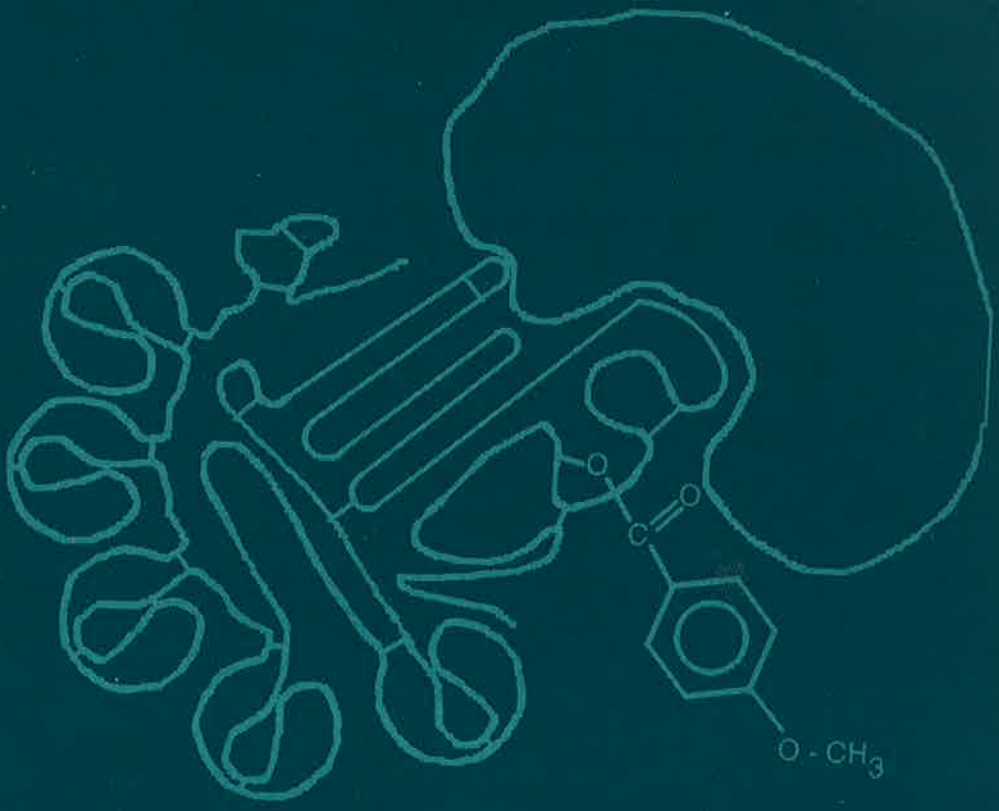
HAEMOSTATIC ASPECTS OF THROMBOLYTIC THERAPY IN ACUTE MYOCARDIAL INFARCTION



J.J.M.L. HOFFMANN

Haemostatic aspects of thrombolytic therapy

in acute myocardial infarction

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Hoffmann, Johannes Jacobus Maria Louis

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STELLINGEN BEHOREND BIJ HET PROEFSCHRIFT "HAEMOSTATIC ASPECTS OF THROMBOLYTIC THERAPY IN ACUTE MYOCARDIAL INFARCTION"

I

Routinematig stollingsonderzoek tijdens kortdurende thrombolytische therapie is niet nuttig.

II

Gezien de verschillen in gevoeligheid voor interferenties, dient altijd gespecificeerd te worden, met welke techniek de concentratie van fibrinogeen gemeten is. Dit geldt vooral, doch niet uitsluitend, tijdens thrombolytische therapie.

III

Het is nog nooit aangetoond, dat antistoffen die reageren met streptokinase, de lysis van coronaire thrombi door streptokinase of anistreplase zouden vertragen of voorkomen.

IV

De storing in de functie van thrombocyten tijdens thrombolytische therapie wordt veroorzaakt door de verhoogde concentratie van afbraakprodukten van fibrinogeen. Mede hierdoor is de incidentie van reocclusie bij niet fibrine-selectieve thrombolytica relatief laag.

V

Thrombolytica, die niet fibrine-selectief zijn, verlagen de viscositeit van bloed en plasma sterker dan de meer selectieve middelen; een mogelijk voordeel hiervan voor de patiënt met een acuut hartinfarct moet echter nog aangetoond worden.

H.R. Arntz et al. Clin Hemorheol 1991; 11: 63-78.

VI

Het aantal waarnemingen, waarop de vermeende voorspellende waarde van de meting van thrombine-antithrombine III complexen in plasma voor het optreden van reocclusie na geslaagde coronaire thrombolyse berust, is te gering om deze bepaling verantwoord toe te passen in de patiëntenzorg.

D.C. Gulba et al. Circulation 1991; 83: 937-944.

VII

De bepaling van de osmotische fragiliteit van erythrocyten met de "acidified glycerol lysis test" is superieur aan de klassieke bepaling met een NaCl concentratie-reeks. Deze laatste bepaling is derhalve obsoleet.

M.J.L. Bucx et al. Eur J Haematol 1988; 40: 227-231
J.J.M.L. Hoffmann et al. Eur J Haematol 1991; 47: 367-370

VIII

Het in de Nederlandse medische spreek- en schrijftaal stelselmatig voorkomende gebruik van het woord "gehalte" wanneer "concentratie" bedoeld wordt, suggereert ten onrechte, dat het menselijk lichaam een mengsel van vaste stoffen is, in plaats van een organisme, dat overwegend uit water bestaat.

IX

Het bepalen van de bloedingstijd bij de voorbereiding van operaties en puncties dient achterwege gelaten te worden, daar aangetoond is dat de bloedingstijd geen betrouwbare voorspelling geeft van het bloedverlies bij zulke ingrepen.

R.P.C. Rodgers & J. Levin. Semin Thromb Hemost 1990; 16: 1-20
S.E. Lind, Blood 1991; 77: 2547-2552

X

Medisch-ethische toetsingscommissies dienen experimenten in patiënten met nieuwe racemische geneesmiddelen af te wijzen, indien de synthese van het werkzame enantiomeer technisch mogelijk is.

E.J. Ariëns. Ned Tijdschr Geneeskd 1990; 134: 2468-2472

XI

In de grote perifere opleidingsziekenhuizen zijn de aard, de omvang en het niveau van gezondheidszorg dermate vergelijkbaar met de academische ziekenhuizen, dat een uniforme wijze van financieren gerechtvaardigd zou zijn.

XII

Gezien de ontwikkelingen op het gebied van middelen met fibrinolytische activiteit, die mogelijk de thrombolytica van de 21e eeuw vormen, doen patiënten met thrombose er verstandig aan, hun afkeer van vampiers, bloedzuigers en teken tijdig te overwinnen.

Dit proefschrift, algemene discussie

XIII

De in sommige sporten heersende gewoonte, dat de winnaar een grote fles champagne leegspuit, degradeert de liefhebber van het drinken van champagne reeds bij voorbaat tot verliezer.

J.J.M.L. Hoffmann
Leiden, 5 november 1992

Haemostatic aspects of thrombolytic therapy

in acute myocardial infarction

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR AAN DE RIJKSUNIVERSITEIT TE LEIDEN,

OP GEZAG VAN DE RECTOR MAGNIFICUS DR. L. LEERTOUWER,

HOOGLERAAR IN DE FACULTEIT DER GODGELEERDHEID,

VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP

DONDERDAG 5 NOVEMBER 1992 TE KLOKKE 14.15 UUR

door

Johannes Jacobus Maria Louis Hoffmann

geboren te Rotterdam in 1952

Promotiecommissie:

Promotor	:	Prof. Dr. P. Brakman
Co-promotor	:	Dr. E.J.P. Brommer, IVVO-TNO Gaubius Laboratorium, Leiden
Referent	:	Prof. Dr. D. Collen, Katholieke Universiteit, Leuven (België)
Overige leden	:	Prof. Dr. R.M. Bertina
		Prof. Dr. A. van der Laarse

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voor Joke

CONTENTS

Introduction

THE FIBRINOLYTIC SYSTEM

The fibrinolytic system is one of the main defense systems, which afford protection against potentially adverse events by dissolving fibrin in blood clots and haemostatic plugs in the circulation and fibrin deposited in extravascular spaces. The fibrinolytic system operates in close concert with other defense systems, of which the coagulation, complement and kinin systems are the most important. All these systems have in common, that under basal, non-disease conditions, their activity remains at a very low level, but they can become very rapidly activated after an appropriate stimulus. Generally, the activation process takes place in multiple steps, which include amplification mechanisms in order to assure quick and sufficient effects. On the other hand, all systems are controlled at various levels by inhibitors, which limit the activation reactions and safeguard against overshoot. So, the eventual result depends on a tedious balance of activators and inhibitors within a particular system and, simultaneously, on a balance between different systems.

For example, the normal circulation of blood is the result of exactly balanced activities of the coagulation and fibrinolytic systems. The coagulation system is necessary to form a fibrin clot when a blood vessel is damaged, in order to prevent blood leaking out of the circulatory system. The fibrinolytic system takes care of dissolving superfluous fibrin whence the vascular damage has been repaired. Insufficient coagulation or too much fibrinolytic activity would result in sustained bleeding from the site of injury, while increased coagulation or defective fibrinolysis would cause pathological clotting (thrombosis) with interruption of normal blood flow and impairment of the viability of tissues, which are dependent of adequate blood supply through the vessel involved.

The activity of the fibrinolytic system is not confined to the blood and fibrin clots. An increasing number of physiological and pathological processes depend on interactions with the fibrinolytic system, for instance inflammation and tumour metastasis, as reviewed recently.[1]

A schematic overview of the major components of the fibrinolytic system is given in Figure 1. The central molecule of the fibrinolytic system is plasminogen, a non-enzymatic plasma protein which can be activated to give the proteolytic enzyme plasmin. Plasmin is capable of dissolving fibrin, the principal constituent of a clot, into fibrin degradation products (FbDP). Various inhibitors regulate the activity of the fibrinolytic system, at the level of plasmin as well as of the plasminogen activators.

Components of the fibrinolytic system[2-4]

Plasminogen

Human plasminogen is a single-chain protein with a low carbohydrate content. Its molecular weight is approximately 90 kD and its concentration in plasma 2 μM. The interactions between plasminogen and other proteins are mediated by specific folded structures, called kringles, of which five copies are present in plasminogen. The kringles contain lysine binding sites, which specifically recognise certain lysine-containing peptides

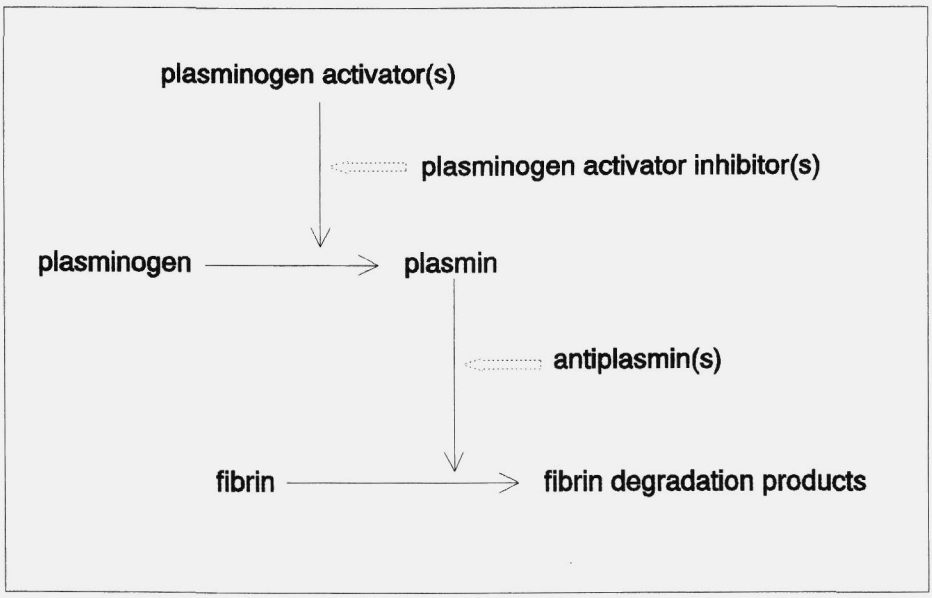

Figure 1 *Schematic representation of the fibrinolytic system. Solid arrows indicate activation and dashed arrows inhibition.*

in fibrin, α_2-antiplasmin, thrombospondin and histidine-rich glycoprotein. The amino terminal of the native protein is a glutamic acid residue ("glu_1-plasminogen"), but limited proteolytic cleavage converts it into a somewhat smaller protein with lysine as the N-terminal amino acid ("lys_{77}-plasminogen"). This conversion does probably not occur under physiological conditions, but may be important during pharmacological fibrinolysis (see below). Lys-plasminogen binds to fibrin with a higher affinity and is more readily activated by plasminogen activators than glu-plasminogen.

The activation of plasminogen to plasmin takes place by cleavage of the arg_{560}-val_{561} peptide bond, resulting in two-chain glu_1-plasmin with its active centre in the light B-chain, followed by autodigestion to lys_{77}-plasmin. The spectrum of proteolytic activity of plasmin is rather broad, but is under physiological circumstances nearly limited to fibrin, due to the specific interactions between fibrin, plasminogen and plasminogen activators on the clot surface and also as a result of plasmin being quickly neutralized by its inhibitors in plasma.

Plasminogen activators

Two types of natural plasminogen activators have been identified, which bring about the activation of plasminogen *in vivo*:

tissue-type plasminogen activator (tPA) is a 68 kD serine protease glycoprotein, with a low plasma concentration (70 pM) under basal conditions. It has a well-defined structure with

different domains, amongst which two kringles with lysine binding sites. It can specifically bind to fibrin and plasminogen and can activate the latter by proteolytic cleavage by means of its serine protease active centre. The activation rate of plasminogen by tPA is normally quite low, but is greatly enhanced by fibrin. In the trimolecular complex of tPA, fibrin and plasminogen the activation rate is about 1000 fold higher than in the absence of fibrin. Thus, in normal circumstances tPA is highly fibrin selective.

tPA is synthesized by vascular endothelial cells and released by a variety of stimuli, for instance ischemia, platelet and fibrin deposition on the vessel wall and certain drugs. The activity of tPA is regulated by a number of plasminogen activator inhibitors (PAI), to be discussed later. Generally, tPA is considered as the most important physiological activator of the fibrinolytic system and is has been extensively used as a pharmacological fibrinolytic agent (see below).

urokinase-type plasminogen activator (uPA) in its native form is a single-chain protein with a molecular weight of 54 kD. Its role in natural fibrinolysis is not completely appreciated. The concentration of single-chain uPA (or pro-urokinase) in plasma is approximately 150 pM. The protein structure of pro-urokinase is highly homologous to that of tPA, but it lacks the specific binding sites for fibrin. It can be transformed by plasmin or kallikrein into enzymatically active two-chain uPA, which is able to directly convert plasminogen into plasmin. Further proteolytic degradation of two-chain uPA leads to the formation of low molecular weight forms of urokinase, which retain their enzymatic activity towards plasminogen. Originally, urokinase was found in and isolated from urine, still the major source of pharmaceutical preparations of urokinase.

The mechanism of action of single-chain uPA is still a matter of controversy. Although it does not bind to fibrin, it has been demonstrated to be reasonably fibrin-selective. Some authors believe that there is a (still hypothetical) inhibitor circulating in plasma, which competes with fibrin for binding to single-chain uPA. Alternatively, single-chain uPA could be activated on the surface of the clot by fibrin-bound plasmin or kallikrein. Both mechanisms would explain the relatively high fibrin selectivity.

The question as to whether single-chain uPA needs conversion into two-chain uPA before it can effectively activate plasminogen is not entirely solved, either. Some investigators have demonstrated that single-chain uPA is a true proenzyme, while others found that it was an enzyme itself, at least under experimental conditions.

factor XII dependent pathway

There is good evidence for a third route of activation of the fibrinolytic system, which is brought about by kallikrein, generated by the contact activation system.[5] The latter system, encompassing factor XII, prekallikrein, high molecular weight kininogen and factor XI, has for long time be considered as the major route of activation of the intrinsic coagulation system. More recently, it was realized that its role in fibrinolysis is probably more important than in coagulation: deficiencies in the contact activation system are associated with thrombophilia rather than with bleeding disorders. Whereas kallikrein can directly

activate plasminogen and also has the potency to activate single-chain uPA, the physiological relevance of the factor XII dependent route is not exactly understood.

Plasminogen activator inhibitors

In the past few years, a number of proteins have been discovered, which inhibit the plasminogen activators described above.[6] The most important one is plasminogen activator inhibitor type 1 (PAI-1), a 52 kD glycoprotein which is derived from endothelial cells. PAI-1 belongs to the large family of serine protease inhibitors, called serpins, and can inactivate single- and two-chain tPA as well as two-chain uPA; single-chain uPA seems not to react with PAI-1. PAI-1 is present in plasma at a concentration of 1 nM, in platelets and in some other cells. In normal plasma, virtually all tPA circulates in a complex with PAI-1; the enzymatic activity of tPA is completely quenched in this complex.

Type 2 plasminogen activator inhibitor (PAI-2) is synthesized under physiological conditions by some blood cells and during pregnancy by the placenta. PAI-2 shares a high degree of homology with other serpins such as antithrombin III, α_2-antiplasmin and PAI-1, but is clearly distinct from the latter. It reacts with tPA, but much less with the different forms of uPA. In normal plasma, there is no or extremely little PAI-2 present, but during pregnancy its level gradually increases with gestation duration and PAI-2 rapidly disappears after delivery. PAI-2 might be of physiological importance in view of the well-known higher incidence of thrombosis during pregnancy.

At least two other types of plasminogen activator inhibitors have been described: PAI-3, which is identical to the inhibitor of activated protein C and protease nexin, which is found in a number of tissue cells, but not in plasma. The physiological significance of both these PAI's is not fully appreciated at present.

α_2-Antiplasmin

The primary inhibitor of plasmin is present in human plasma in a concentration of 1 μM. It is a glycoprotein with a molecular weight of about 70 kD and it is a member of the serpin family, too. Once active plasmin is formed in plasma, it is very rapidly and irreversibly inhibited by α_2-antiplasmin in a 1:1 stoichiometric complex. Further, α_2-antiplasmin can block the incorporation of plasminogen in forming fibrin clots and it can be crosslinked to fibrin itself, thus preventing the lysis of fibrin. Because its plasma concentration is only half of that of plasminogen, α_2-antiplasmin can be completely consumed after extensive activation of plasminogen, as occurs during therapy with fibrinolytic agents. In such circumstances, plasmin can circulate in its free form in blood, because other inhibitors (e.g. α_2-macroglobulin) are less efficient than α_2-antiplasmin. The condition of circulating active plasmin is called the systemic lytic state and is characterized by plasmic degradation of some plasma proteins, notably fibrinogen and coagulation factors V and VIIIC.

Other plasminogen inhibitors

Histidine-rich glycoprotein (HRG) is a plasma protein which can interact with plasminogen via the lysine binding sites. Its plasma concentration is approximately 1.7 μM and based on

equilibrium constants, it is expected that at least half of the plasminogen in plasma is in complex with HRG and is not available for activation by plasminogen activators. The physiological significance of HRG-plasminogen complex formation has not been closely examined.

C1-esterase inhibitor is the main inhibitor of activated components of complement factor C1, but also acts as an inhibitor of kallikrein, activated factors XII and XI_f of plasmin and of tPA. Although there is little, if any evidence from *in vivo* situations, C1-inhibitor probably could play a role in the regulation of fibrinolysis.

Non-physiological plasminogen activators

For therapeutical purposes a number of substances are available, which can activate the fibrinolytic system, but which do not naturally occur in man. The most important of this class of fibrinolytic or thrombolytic drugs is streptokinase (SK), a non-enzymatic protein produced by streptococci. When infused in man, SK complexes with plasminogen and this complex is able to convert plasminogen into plasmin. A preformed and temporarily inactivated complex of SK and human lys_{77}-plasminogen, called anisoylated plasminogen streptokinase activator complex (APSAC), is also available as a thrombolytic drug. The pharmacology of these substances will be discussed later in this chapter.

Since streptococci are ubiquitous micro-organisms, they easily elicit an immune response upon a streptococcal infection. The antibodies formed can react with SK and APSAC and can therefore be regarded as plasminogen activator inhibitors (see chapter 6).

Cellular components

Endothelial cells, platelets, monocytes, tumour cells and many other cells have been found to possess receptors for plasminogen and plasminogen activators.[7,8] These cellular receptors play important roles in the regulation of the fibrinolytic system, especially in inflammatory tissue and in extravascular spaces.

PATHOGENESIS OF ACUTE MYOCARDIAL INFARCTION

Acute myocardial infarction (AMI) is a sudden expression of intra-arterial thrombosis in one or more of the coronary arteries. Arterial thrombosis is associated with atherosclerotic lesions of the arterial wall. The mechanisms leading to atherosclerosis are rather complex and still poorly understood, although thrombus formation and lipid deposition definitely are involved in atherogenesis.[9]

The thrombosis hypothesis

Some 150 years ago, von Rokitansky formulated the hypothesis that atheroma was produced by deposition of fibrin on the arterial wall (cited by Duguid[10]). This theory was challenged by Virchow, who regarded atheroma as an inflammatory process, which caused reactive proliferation of the intimal connective tissue cells.[10] Later, Duguid further developed Rokitansky's hypothesis into the theory of thrombogenic laminations.[10,11] This theory is based on histological examination of the coronary systems of young persons who died of acute heart attack and it considers thrombus formation as the principal event in the development of atherosclerosis. Fibrin becomes attached to the arterial wall surface, either as mural or even occluding thrombi or as thin deposits of fibrin (called encrustations by von Rokitansky) and, if not quickly lysed, this fibrin layer is covered with endothelium. So, a new, smaller vessel lumen is formed and the external thrombus seems to be a part of the vessel wall. Then, phagocytes are attracted, which turn into foam cells at the site of the removed fibrin. The thrombus becomes organized and changes into fibrous tissue and later into hyalin material. During this process of thrombus organization, its volume decreases and finally looks like a thickening of the arterial wall. Duguid stressed that this thickening was not to be regarded as growth of the original intimal tissue with progressive decrease of the lumen of the vessel, like Virchow believed, but as the reverse: a decrease of the thrombus as it becomes incorporated into the vessel wall with a simultaneous increase in the lumen.[10] The newly formed endothelium can become covered with fibrin during the next thrombotic episode and the fresh fibrin is encapsulated again by endothelial cells. Eventually, this gives the classical picture of the "thrombogenic laminations" of Duguid.[10,11]

Based on this thrombosis hypothesis as a cause of AMI, antithrombotic therapy with oral anticoagulant drugs (coumarin derivatives) was a logical step; however, a number of large-scale trials failed to show a significant reduction in mortality and this type of treatment rapidly fell into disfavour, except in the Netherlands where Loeliger and his colleagues had successfully set up a nation-wide network of facilities, which ensured adequate laboratory control and sufficient intensity of oral anticoagulation, and showed successful secondary prevention of thrombosis after AMI (reviewed in reference 12).

Although Duguid knew that lipids were involved in atherosclerosis, he could not fit their significance into his theory. In his view most fatty depositions in atherosclerotic plaques were supposed to result from the degeneration of fibrous tissue and foam cells,

encapsulated by overgrowing endothelium. Opponents of the thrombogenic theory supported a lipogenic cause of atheroma: deposition of initially benign fatty streaks onto the arterial wall would later give rise to atherosclerotic plaques. This theory was substantiated by epidemiologic studies, which showed that hyperlipidaemia was an important risk factor for cardiovascular disease. The prescription of low-fat diets and drugs which lower the concentration of lipids in the blood is based on the lipogenic hypothesis of atherosclerosis.

The "response to injury" theory

Nowadays, both theories are brought together into a more unifying concept of atherogenesis, in which endothelial injury plays a crucial role as the cause of atherosclerosis and arterial thrombosis. Ross called this the response to injury hypothesis.[13] Several critical steps and agents playing a role in this process have been identified.[9,13-16] They are depicted in Figure 2 (the stages mentioned below refer to this

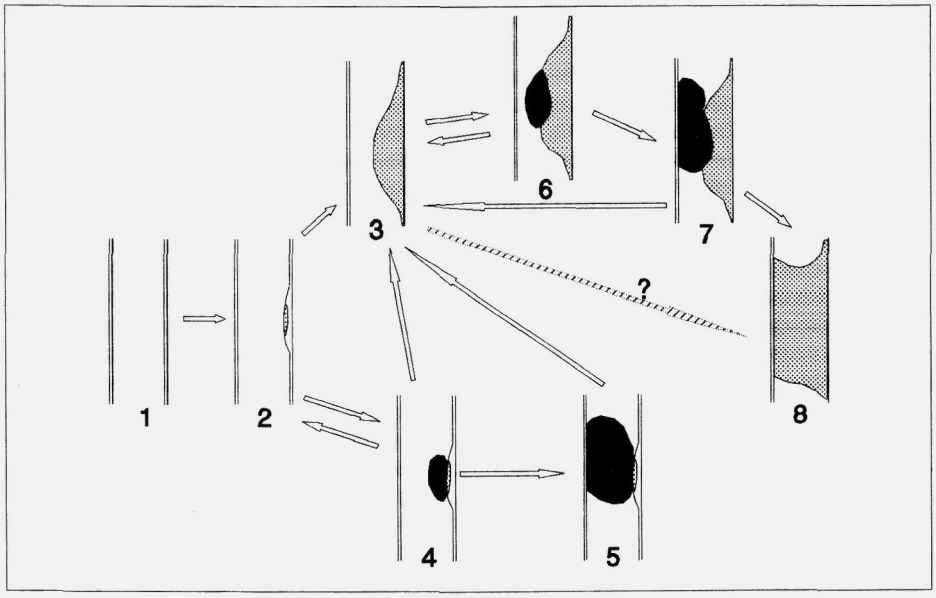

Figure 2 *Schematic illustration of the different stages in the evolution of atherosclerosis (after Fuster et al.[94]). The atherosclerotic plaque is in grey and the thrombus in black.*

figure). The first step in the development of atherosclerosis is a subtle damage of the intimal layer of the vessel located at a fatty streak; this is a flat, lipid-rich lesion consisting of macrophages and smooth muscle cells (stage 1). The origin of these fatty streaks is largely unknown; blood rheology is thought to play a role, because there seems to be a preference for vascular sites with turbulent blood flow, such as branches and bifurcations.

Hyperlipidaemia promotes the emergence of fatty streaks and the accumulation of lipids onto them. The predomination of macrophages in the fatty streaks probably represents a form of inflammatory response. Monocytes are attracted to the site of the lesion, migrate into the subendothelium and deposit their contents. Also platelets can adhere, especially when the subendothelium is exposed to the blood. After activation, they release a number of substances, among others growth factors, which stimulate the smooth muscle cells in the vessel wall to proliferate. The resulting intimal hyperplasia provides the basis for synthesis of connective tissue and for further accumulation of lipids (stage 2). Thus far, the process is asymptomatic.

In the second phase of coronary atherosclerosis, the growth of smooth muscle cells in the intima and deposition of lipids are continuing, especially in the presence of hyperlipidaemia. Then, the fibrous tissue forms into an atherosclerotic plaque (stage 3). As a consequence of either the progression of plaque formation or sudden mural thrombosis (stages 4,5) or both, the lumen of the coronary artery narrows. The blood flow to the cardiac muscle cells distal to the lesion becomes impaired and the patient begins to develop symptoms of angina pectoris. The construction of the plaque is not very stable: it is a relatively soft mass of fats and foam cells covered by a thin layer of fibrous tissue. Then, a variety of triggers (enzymes released from blood cells, shear forces by the blood in the narrowed artery lumen) can cause plaque rupture and exposure of underlying, highly thrombogenic subendothelial collagen.

The next phase is characterized by formation or progression of a mural thrombus on the subendothelium (stage 6) and consolidation of the thrombus. The mural thrombus may grow and eventually occlude the vessel (stage 7), but regression of the thrombus by intrinsic fibrinolysis is also possible (stage 7 to 3). It is important to realise, that the cause of thrombus formation, the coronary lesion, remains present even if the thrombus has disappeared. Thus, rethrombosis can easily occur at the same site. Depending on the size of the thrombus, the speed with which it was formed and the degree of occlusion caused by it, a coronary thrombosis may give rise to deteriorating angina, unstable angina, acute myocardial infarction or sudden cardiac death.[16] Finally, the occlusive thrombus will become organized and the lumen of the vessel may be completely occluded (stage 8).

A sudden thrombotic occlusion of a coronary artery will deprive part of the cardiac muscle cells of oxygen supply and prolonged ischemia leads to irreversible necrosis of these cells and thus to loss of functional capacity. The rationale for thrombolytic therapy is to dissolve coronary thrombi shortly after their formation in order to restore the patency of the infarct-related coronary artery, to reestablish adequate oxygen supply and thus to salvage as much functionally intact myocardial tissue as possible.

THROMBOLYTIC THERAPY IN ACUTE MYOCARDIAL INFARCTION

More than 80 years ago it was already postulated that acute myocardial infarction (AMI) was the consequence of acute thrombosis in one of the coronary arteries.[17] Still in 1974 a group of recognized experts could not reach consensus as to whether an acute thrombosis was the origin or one of the sequelae of AMI.[18] It was only in 1980 that DeWood *et al.* were able to demonstrate with certainty that coronary thrombosis was the cause of AMI.[19] Using coronary angiography, they showed that during the first four hours after AMI, the infarct-related coronary artery was completely occluded in about 90% of the patients, but the prevalence of coronary thrombosis declined with time, to about 50% after 12-24 h. In a high proportion of patients they could remove a clot from the occluded vessel during coronary artery surgery immediately after AMI and thus proved that coronary thrombosis was the cause rather than a consequence of AMI.[19] These findings rendered thrombolysis a rational approach to treat AMI and in 1976 two Russian patients with AMI were the first to receive intracoronary thrombolysis, as reported by Chazov *et al.*[20] Soon thereafter Rentrop and colleagues reported that they could successfully achieve recanalization of occluded coronary arteries by intracoronary application of SK in the majority of patients treated.[21] These observations considerably renewed the interest in the thrombolytic treatment of AMI. Previously, a number of clinical trials had been conducted in which streptokinase or urokinase had been administered to AMI patients intravenously with varying success and, probably therefore, without widespread acceptance.[22,23] Nowadays it is known, that in those trials the timing relative to the onset of AMI was too late, the doses given were generally quite low, the endpoints recorded were not sensitive enough to detect benefits (coronary angiography was not generally available) and probably the size of the patient groups studied was insufficient to detect significant changes in outcome.

In experimental studies it had been demonstrated that timely recanalization of the occluded artery and thus reperfusion of the myocardial tissue at risk could reduce infarct size.[17] The reversible injury of the myocytes in the subendocardium, caused by ischemia of brief duration will normally turn into irreversible damage upon prolonged or severe ischemia and will be followed by a so-called wave front of cell death into the subepicardial tissue. Early reperfusion may prevent the progression of this process and thus salvage part of the viable myocardium at risk. Myocardial necrosis can not be entirely prevented, but can be limited; there exists an inverse relation between the duration of ischemia and the size of salvaged myocardial tissue.[17] Rescued myocardium will limit the loss of left ventricular function, which is the main purpose of thrombolytic therapy in AMI, at least in the short term. Obviously, the long-term purposes are to reduce mortality from AMI and to improve the physical performance status of survivors.

In the early eighties, the results of Rentrop *et al.*[21] were confirmed by various other groups and intracoronary thrombolysis aroused more interest. However, one began to realize that the cardiac catheterization procedure needed for intracoronary thrombolysis was a disadvantage to a potentially effective therapy, because it is available on a limited

scale only and was time-consuming, thus introducing an undesirable delay in treatment. The intravenous route was adopted again, but now using much higher doses than before and maintaining a strict time window of 4, sometimes 6 hours after the onset of chest pain. The results of this new approach were reassuring: survival after AMI could be significantly improved by high-dose, short duration streptokinase therapy.[24-26]

In the meantime, other thrombolytic agents than streptokinase and urokinase were developed. These second generation drugs include recombinant tPA (rtPA), anisoylated plasminogen streptokinase activator complex (APSAC) and recombinant single chain urokinase-type plasminogen activator (rscuPA or pro-urokinase). In comparative studies it was demonstrated that some of these drugs were significantly more effective in attaining early coronary recanalization if given within a few hours after AMI, but these differences did not translate in significant differences in mortality reduction.[27-30] Very recently, two large-scale trials were completed which were aimed at directly comparing the newer thrombolytic drugs: GISSI-2 with its international extension and ISIS-3.[31-33] Both megatrials failed to indicate significant differences in efficacy between rtPA, APSAC and SK.[31-33] However, critical comments have been made concerning the design of these trials, which may have prevented differences between the drug regimens to become clear.[34] There are indications that the heparinization in these studies was not optimal, especially not in the patients treated with tPA.[34,35] In the ISIS-3 trial, there was the additional problem of the effective dose of the tPA preparation used (duteplase), as compared with the alteplase preparation used in many other studies.[34] On the other hand, other experts are convinced of equivalent efficacies for SK and rtPA in terms of salvaging myocardium, survival benefit, safety and side-effects.[36]

The presently available thrombolytic drugs are far from ideal. Each of them fails to dissolve the coronary thrombus in about 25% of the cases and is associated with potentially dangerous side-effects (reocclusion of a previously recanalized vessel, bleeding tendency). The requirements for the ideal thrombolytic drug have been formulated as follows: it should be non-antigenic, inexpensive, thrombus-selective, quickly acting and highly effective.[30,37] The latter authors simultaneously indicated to expect that such an agent probably will never be found, because with our present knowledge it is impossible to imagine how discrimination between dissolving a thrombus or embolus (pathological fibrin) and dissolving a haemostatic plug (benign fibrin) is ever feasible. At the moment there are several lines of development of new thrombolytic agents, which are aimed at improving the selectivity for fibrin; they will be briefly discussed in the general discussion.

Finally, it should be realized that thrombolytic therapy of AMI is not a definitive treatment, but only transiently removes one of the consequences (thrombus formation) of the underlying disease (atherosclerosis and vascular wall damage). It is therefore imperative that thrombolysis be followed by other types of treatment, such as anti-thrombotic and anti-platelet drugs for the prevention of re-thrombosis or anti-lipid agents for reducing the progression of atherosclerosis;[12,15,30] a number of these subjects will be addressed in the general discussion of this thesis.

PHARMACOLOGY OF THROMBOLYTIC DRUGS

Streptokinase

History

The history of thrombolysis goes back to the 1930's when Tillett and Garner discovered that some strains of ß-haemolytic streptococci in culture secreted a substance with a potent fibrinolytic activity, which they called streptococcal fibrinolysin.[38] Studies on this fibrinolysin were mainly aimed at the bacteriological aspects of streptococcal infections and it was soon demonstrated that patients who were recovering from an infection with streptococci had a specific anti-fibrinolysin in their serum, now known to be antibodies to streptococcal proteins. It took nearly one decade before one realized that fibrinolysin required a plasma factor to become enzymatically active; then plasminogen was discovered.[38]

Clinical application of SK was first performed in 1947 by Tillett and Sherry, who demonstrated that partially purified SK could lyse clotted blood in pleural effusion *in vivo* by activating the fibrinolytic system.[39] During the next few years, administration of SK remained confined to extravascular spaces where fibrin and exudate had to be removed. At about the same time, animal models were developed for studying the effects of SK on thrombi and SK was purified on an industrial scale for intravascular applications.[38] Basic research revealed that SK did not directly activate human plasminogen to plasmin, but the SK-plasminogen complex behaves as a plasminogen activator.[40,41]

The concept of a two phase system was developed, which involves that during clot formation plasminogen becomes incorporated into the clot, bound to fibrin (plasminogen in the fibrin or gel phase), while it also remained in the circulation (soluble phase). This concept could explain the local effect of SK (clot dissolution, by activation of fibrin-bound plasminogen) as well as the systemic effects (degradation of fibrinogen and other clotting factors by plasmin in the soluble phase), but also provided insight into the fibrin-selectivity of plasmin during natural fibrinolysis (see earlier).

Pharmacological investigations of SK were performed by Fletcher *et al*,[42,43] who found that a sustained lytic state was necessary to successfully dissolve clots. The first series of AMI patients treated with SK indicated that early thrombolytic treatment probably gave better results in terms of clinical course, cardiac enzyme release and autopsy findings.[43] Initially, dosing of SK was individually determined because it was known that highly variable levels of anti-streptococcal antibodies could occur before treatment, which would neutralize the fibrinolytic capacity of SK.[44] Later, a standard scheme was adopted consisting of a loading dose, which accounted for the neutralizing antibodies in the majority of patients, followed by a sustaining infusion for the thrombolysis.[45] Several large-scale clinical trials were carried out, but as pointed out in the previous section, the results were not generally accepted by the cardiological profession.[22,23] This changed dramatically after the publications by de Wood and Rentrop,[19,21] who paved the way for widespread thrombolytic treatment of patients with AMI.

Mechanism of action

SK is a single chain protein with a molecular weight of approximately 48 kD and possesses no enzymatic activity. It can form a 1:1 stoichiometric complex with human plasminogen, but not with most animal plasminogens. After a conformational change, an active site is exposed in the SK-plasminogen complex, giving it plasminogen activator activity. The active complex converts other plasminogen molecules into plasmin (Fig 3). Alternatively, SK can also form an activator complex with plasmin as soon as the latter has been generated and the plasminogen-SK complex can be degraded into plasmin-SK complex. The SK-plasmin(ogen) complex is essentially not inhibited by α_2-antiplasmin. The reaction between SK and plasminogen is immediate and carries the risk of consuming too much plasminogen for complex formation so that there is insufficient plasminogen left for conversion into active plasmin.[46]

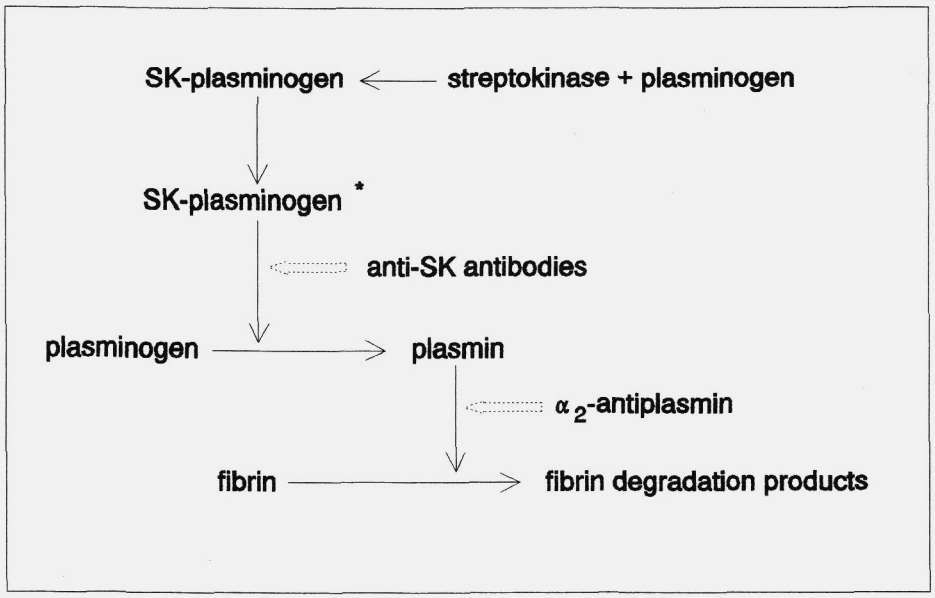

Figure 3 *Mechanism of action of streptokinase. The asterisk indicates enzymatic activity of the SK-plasminogen complex.*

Because SK is a non-human protein, it is antigenic and high titers of anti-SK antibodies may be found after recent infections with streptococci or in patients who had previous therapy with SK or other SK-containing agents (see chapter 6). Such antibodies have been found to inhibit the fibrinolytic action of SK and are therefore generally considered as a limitation of repeated therapy with SK. However, there is no evidence from prospective clinical studies using current dosage schemes to support this hypothesis.

Further, SK is able to generate thrombin activity during thrombolysis; this will be discussed extensively in chapter 8.

Dosage and pharmacokinetics

Due to its relatively early introduction in clinical practice, the pharmacologic properties of SK have not been extensively investigated. Sherry, one of the pioneers in the thrombolytic field, originally defined the dose for clinical applications on the basis of *in vitro* findings in plasma and administered 200,000 to 400,000 U of SK over 30-60 minutes.[38] Similar dosage schemes were later adopted for intracoronary administration.[21] When high-dose intravenous thrombolysis was introduced, initially the SK dose ranged from 0.9 to 3.0 million U,[47] but later a more or less arbitrary amount of 1.5 MU was proposed, infused over 1 hour.[48] The first real dose-finding study was described as late as 1990.[49] These authors established that 750,000 U of SK was the minimal effective dose, while the highest patency rate was obtained after 3 MU. They found no solid arguments for the 1.5 MU dose, which is ubiquitously applied.[49] Despite these findings, this dose appears to be continued as a "standard".

Clearance studies of SK have shown that the disappearance half-life of ^{131}I-labelled SK is about 18 min in immunized patients and 83 min in subjects with low anti-SK titers.[44] Other authors found a comparable half-life for clearance of SK activity, measured in a fibrin lysis system, between 15 and 31 min.[50-52] Estimations of SK clearance using assays with chromogenic substrates indicated a longer half-life, ranging from 59 to 82 min.[52-54] In a mouse model, which resembles the human situation, the plasma half-life of radioactive SK was 15 min and could be increased at least 8 times by conjugating SK to polyethylene glycol.[55] This development might be promising for clinical application, also because the conjugate seems to be non-antigenic.

Fears *et al* showed that the pre-treatment concentration of IgG anti-SK had no influence on the clearance half-life of SK,[52] but anti-SK antibodies play certainly a role in the elimination of SK (chapter 6).

APSAC (anistreplase)

History

APSAC is the most important of the acyl-enzymes, a novel class of thrombolytic agents. These agents were developed around 1980. Since the SK-plasmin(ogen) complex is a highly efficient plasminogen activator, but is rapidly cleared from the circulation (see above), it was conceived that temporary inhibition of the active centre of the plasminogen activator could give a more stable compound, which could be regarded as a pro-drug. Therefore, SK-plasminogen was reacted with a range of acyl groups and the structure-activity relationships examined.[56,57] It was concluded that the anisoyl derivative was most appropriate for clinical use as a thrombolytic drug in patients with AMI.

Mechanism of action

APSAC is the *p*-anisoylated complex of SK and lys_{77}-plasminogen, which gives enhanced fibrin affinity in comparison with native glu_1-plasminogen.[58] After administration, the acyl-

enzyme deacylates by spontaneous hydrolysis so that the active plasminogen activator is released *in situ* with a controlled rate. The deacylation half-life in human plasma *in vitro* is about 105 min and is not influenced by APSAC binding to fibrin or by high concentrations of IgG antibodies to SK.[59] Once the drug is deacylated, it activates plasminogen to plasmin in an identical manner as described above for SK (Fig 4).

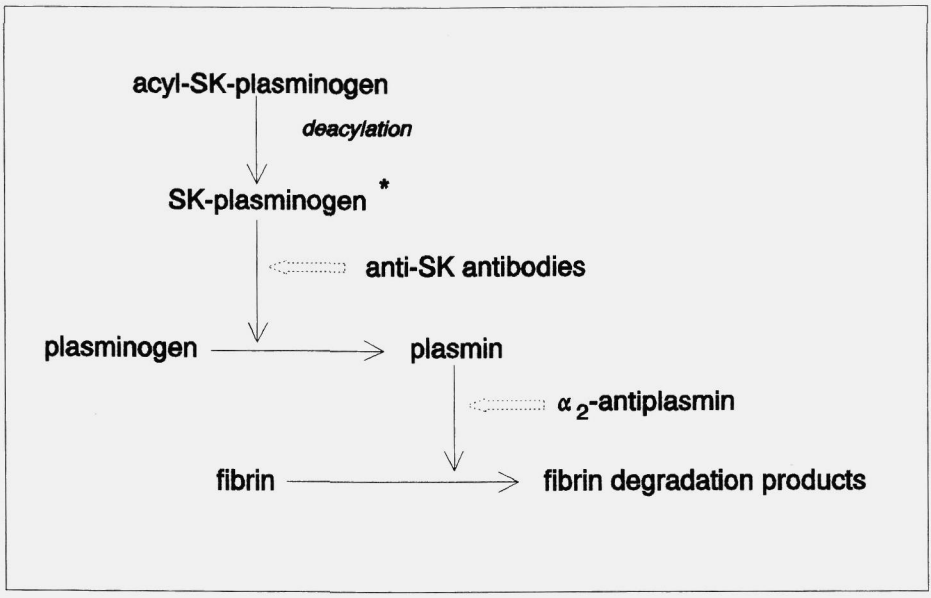

Figure 4 *Mechanism of action of APSAC. The asterisk indicates enzymatic activity of the SK-plasminogen complex.*

Dosage and pharmacokinetics

Early dose ranging studies were performed using the intracoronary route of administration. It appeared that recanalization as assessed by coronary angiography, increased with dosage up to 20 U over up to 1 hour.[60] Soon this route was abandoned in favour of intravenous administration. The dose-response curve indicated that a high recanalization rate was achieved with 30 U of APSAC in 3-5 minutes, and this has become the recommended dose.[60-62]

After intravenous administration as a bolus, the fibrinolytic activity of APSAC, measured on a fibrin plate, disappears with a half-life of approximately 70-90 min.[51,63] This is considerably longer than found for SK (see above), which has been explained by greater stability of APSAC due to acylation and by retarded susceptibility of the SK moiety in APSAC to proteolytic degradation.[58]

The influence of anti-SK antibodies on the clearance rate of APSAC has not been extensively examined. Available data suggest that extremely high levels of anti-SK may occasionally be associated with decreased half-life.[63,64] It has been demonstrated without

any doubt that anti-SK antibodies can inhibit the systemic lytic action of APSAC (chapter 6),[65,66] but it is still uncertain whether its thrombolytic effect is modulated by such antibodies. APSAC induces an anamnestic rise in anti-SK titer, which peaks at 2-3 weeks after administration.[67]

Tissue plasminogen activator (tPA)

History

Whereas the occurrence of one or more plasminogen activators in tissue had been known for a long time, detailed knowledge and therapeutic usage became only accessible after the large-scale isolation and purification of tPA from melanoma cell lines in 1980.[68] Soon thereafter, the tPA gene was cloned and the cDNA coding for intact human tPA expressed in a cell system, yielding recombinant tPA (rtPA) in high quantities.[69] The first clinical application of tPA, the natural material isolated from the melanoma cell line, was reported in 1984: seven patients with AMI were treated with intracoronary and intravenous tPA and successful lysis of the coronary thrombi was achieved in six of them.[70] The recombinant tPA was tested in several large-scale clinical trials and found to be more effective than SK.[71-73]

Mechanism of action

tPA is an effective plasminogen activator only when it is bound to fibrin, in the vicinity of fibrin-bound plasminogen, as pointed out in one of the preceding sections. The plasmin molecules formed there have their lysine binding sites and their active centre occupied, so that they are only slowly inhibited by α_2-antiplasmin. These specific interactions make tPA a reasonably fibrin specific thrombolytic agent.

Because tPA is a natural protein in humans, the risk of antibodies forming after therapy with rtPA is extremely small, if existing anyway. Due to the molar excess of tPA, its major inhibitor, PAI-1, is of no significance in therapeutic thrombolysis, even despite the fact that the PAI-1 concentration in platelet-rich clots may be considerably higher than in blood. In animal studies, high dose PAI-1 is known to limit the blood loss associated with tPA administration,[74] whereas inhibition of PAI-1 by monoclonal antibody accelerates thrombolysis;[75] however, the significance of these findings for therapy in man remains to be defined.

Dosage and pharmacokinetics

In the early years of clinical use of tPA, a great variety of dosage schemes has been investigated, with the aim to establish the optimal equilibrium between efficacy (patency of the infarct-related coronary artery) and side-effects (mainly bleeding tendency). At present the standard dosage amounts to 100 mg of rtPA (equivalent to nearly 60 million U), to be given over 3 hours by continuous intravenous infusion. The continuous infusion is necessary to maintain a sufficiently high plasma level of tPA, in view of the extremely short half-life, which is approximately 4-6 min.[76,77] The steady-state plasma concentrations reached after standard dose tPA are in the order of 1-3 μg/L.[76,77]

At the moment, two pharmaceutical tPA preparations are available. Although both types of tPA are derived from the same cDNA, there appear to be differences in specific activity.[30,34] It is still too early to conclude that one of these preparations would be superior in terms of survival and patient performance. The difference only stresses the need for an adequate reference standard, which allows for comparing different preparations and relating their clinical responses to the dose of active drug administered.

Urokinase plasminogen activator (uPA)

History

Although urokinase (UK) belongs to the first generation of thrombolytic drugs, relatively few clinical trials have been carried out with this drug, because of its limited availability and high cost. The only advantage of UK over SK is, that the former is non-antigenic and therefore can be applied repeatedly in the same patient. When the single-chain inactive precursor of UK had been discovered, more emphasis has been placed on the development of single-chain uPA (or pro-UK) as a thrombolytic drug.[78] Like tPA, pro-UK is nowadays produced on a large scale using recombinant DNA technology. In 1985, pro-UK purified from a human cell line, was used for the first time in clinical thrombolysis, being successful in 5 out of 6 patients with AMI.[79] Recombinant plasminogen activator (rscuPA; a non-glycosylated protein produced in *E.coli*) became soon available for clinical use[80] and the only large-scale clinical trial on rscuPA published thus far indicates that it induced earlier and higher patency than intravenous SK, with less effects on the haemostatic system and less bleeding.[29]

Mechanism of action

The exact mechanism of action of scuPA is not entirely clarified, but there is now accumulating evidence that single chain uPA is able to activate plasminogen without its conversion into two-chain uPA is required. A three-step mechanism has been proposed, in which scuPA first activates plasminogen to plasmin, followed by plasmin-mediated conversion of scuPA to its two-chain form and, finally, two-chain uPA activates plasminogen to plasmin.[81] ScuPA does not bind to fibrin and yet it acts relatively fibrin selective, due to largely unknown mechanisms as discussed previously.[81]

It has been demonstrated that scuPA activates plasminogen which is bound to C-terminal lysine binding sites in fibrin, which become only available after limited digestion of fibrin.[82] This explains the lag phase in scuPA induced lysis of clots in a plasma environment. In contrast, tPA activates plasminogen bound to internal lysine binding sites in fibrin, which are easily accessible and therefore tPA induced fibrinolysis shows no lag phase. As a result of the amplification mechanism by which two-chain UK is generated from scuPA, the rate of fibrinolysis is larger for scuPA than for tPA.[82] The differences in mode of action between scuPA and tPA also provide an explanation for their synergism (see General Discussion).

Dosage and pharmacokinetics

For achieving optimal recanalization or patency of the coronary arteries involved, a total dose of 70-80 mg recombinant scuPA is necessary;[83,84] this corresponds to 12-15 million urokinase units. The clearance half-life of scuPA is rather short, about 8 min, which necessitates sustained administration by continuous infusion for 1 hour.

A recent development is the combination of scuPA with a low dose of urokinase, with the aim to pre-activate scuPA and thus to accelerate its thrombolytic effect.[84,85] The gain in thrombolytic potency is accompanied by increase in haemostatic disturbances.

CLINICAL EFFECTS OF THROMBOLYTIC THERAPY

Patency, reperfusion, infarct size and mortality

A concise overview of the clinical effects of thrombolytic therapy is presented in Table 1. These data are based on two recent reviews of all major clinical trials, which have been conducted during the past decade.[86,87]

Table 1 *Clinical results of therapy with different thrombolytic drugs in acute myocardial infarction.*[86,87]

Drug	Dose	Patency (%)	Reperfusion (%)	Infarct size enzyme (U)	Mortality (%)
SK (ic)	250,000 U	80 - 85	60 - 75	HBDH 770	4 - 8
SK (iv)	1.5 MU	50 - 65	30 - 65	CK-MB 1701	3 - 11
tPA	100 mg	60 - 80	60 - 65	HBDH 698	3 - 9
APSAC	30 U	55 - 70	50 - 85	CK 1588	6
UK	2 - 3 MU	70 - 75	60 - 75	n.a.	4
scuPA	80 mg	70 - 75	n.a.	n.a.	4
placebo		20	8 - 15	HBDH 988 CK-MB 1869 CK 1951	6 - 13

Patency measured after 90 min; reperfusion after 60-120 min; infarct size is median area under the curve up to 72 h after AMI; mortality within 6 weeks from AMI; N.A.: not available.

Adverse effects

Bleeding

Two types of side effect accompany thrombolytic therapy in AMI, irrespective of the drug used: bleeding and reocclusion. Since none of the present thrombolytic agents can discriminate between fibrin in the coronary thrombus and fibrin in haemostatic plugs elsewhere, the risk of haemorrhage is intrinsic to thrombolysis. The most serious complication is intracranial bleeding, which is observed in 0.5-1.0 % of the patients undergoing thrombolytic therapy.[87] Especially in the early, invasive trials one was concerned about increased bleeding, but with growing clinical practice, the incidence of serious bleeding appears to be minimal. In many instances, haemorrhage is not imposed by the thrombolytic drug itself, but rather by repeated invasive procedures, such as coronary

angiography and blood sampling, and by simultaneous therapy with heparin. With proper exclusion criteria it is feasible to routinely treat patients with thrombolytic agents without an increased risk of bleeding complications.[88]

Reocclusion

The eventual benefit of thrombolytic therapy depends not only on rapid recanalization of the infarct-related coronary artery, but also on its sustained patency. As stated previously, the cause of the coronary thrombosis is not eliminated by thrombolysis and this implicates that there may be ongoing thrombosis during and after the thrombolytic treatment. It has even been suggested that the thrombolytic agent, either directly or via plasmin, might generate thrombin, which could promote rethrombosis.[89,90] It remains to be established whether this is a direct effect on the coagulation system or that removal of the thrombus, completely or even partially, could expose thrombogenic material at the original vascular lesion again and thus give rise to activation of prothrombin and rethrombosis (see chapter 8). Because high concentrations of fibrin(ogen) degradation products and low fibrinogen can be regarded as anticoagulants, one might expect that the incidence of reocclusion is higher after therapy using short-acting, fibrin-selective agents (tPA and uPA) than with drugs, which are associated with a prominent systemic lytic state (SK, APSAC). Rates of early reocclusion range from nil to over 30%,[91] and it is striking that among trials reporting the lower reocclusion rates (under 5%), those using the long-acting and non fibrin-selective agent APSAC are relatively common.[92,93] However, reocclusion data from direct, comparative investigations are still lacking. Currently, there are interesting developments in adjunctive therapeutic strategies, which should prevent or minimize the rate of reocclusion after thrombolysis[12,15,30,91,94,95] (see also General Discussion).

Other side effects

Recanalization of coronary arteries may be associated with so-called reperfusion arrhythmias, which are rarely severe. A side-effect restricted to the administration of the non-human proteins SK and APSAC is hypotension and allergic reactions. The ubiquitous antibodies to streptococci, which react with SK and APSAC, are responsible for these effects. In well-documented trials the incidence of mild allergic and hypotensive reactions is generally in the order of 1-2% and severe reactions are infrequent.[62] Occasionally, other adverse reactions have been reported to occur during thrombolytic therapy, which however were not serious in most cases.[96]

HAEMOSTATIC ASSAYS IN THROMBOLYTIC THERAPY

Laboratory tests

Thrombolytic therapy is in essence the pharmacological stimulation of the natural fibrinolytic system of man, since all thrombolytic drugs currently in use act as plasminogen activators. This necessarily implies that thrombolytic therapy is associated with changes in the fibrinolytic system (see above) and in the haemostatic system in general. These changes can be monitored using various haemostatic assays in plasma and the factors measured with these assays can be classified into several groups on the basis of their outcome during thrombolytic therapy.

Activation/conversion

Plasminogen is converted by the thrombolytic drugs to plasmin, especially the fibrin-bound fraction of plasminogen. When using less fibrin-selective drugs, circulating plasminogen is also activated and its plasma concentration may considerably decrease.

The euglobulin clot lysis time (ECLT) is an overall test of fibrinolytic activity. It is performed in the euglobulin precipitate of plasma, so that most fibrinolytic inhibitors no longer interfere in this test. Increased fibrinolytic activity results in a shortened lysis time for the ECLT.

Degradation

The desired effect of thrombolytic therapy is the dissolution of the thrombus. Breakdown of fibrin in the thrombus leads to an increase in fibrin degradation products (FbDP) in plasma.

As a result of systemic plasmin formation, fibrinogen may be degraded as well. Apart from a sometimes dramatic decrease in plasma fibrinogen, this will give an increase in the concentration of fibrinogen degradation products (FgDP). These derivatives now can be distinguished from FbDP, by virtue of specific monoclonal antibodies.[97] Previously, methods were used to estimate the concentration of all degradation products, irrespective of their fibrin or fibrinogen origin (FDP/fdp).

Free plasmin in the circulation will degrade other proteins of the haemostatic system as well, notably factor V, factor VIIIC, von Willebrand factor and histidine-rich glycoprotein.[98-101]

Consumption of inhibitors

As discussed in one of the above sections, α_2-antiplasmin is the most important inhibitor of plasmin. When plasmin is generated in the circulation, it is rapidly inhibited by α_2-antiplasmin, more specifically by its plasminogen-binding form.[102] However, the normal level of α_2-antiplasmin becomes exhausted as soon as about half of the available plasminogen has been activated to plasmin. Depletion of α_2-antiplasmin is one of the earliest events in the emergence of the systemic lytic state.

In addition to α_2-antiplasmin, the protease inhibitor α_2-macroglobulin is also consumed during the systemic lytic state, but to a more limited extent because its normal concentration is higher than that of plasminogen.[102]

Antibodies to streptokinase can be regarded as inhibitors, too; changes in the plasma level of these antibodies after treatment with SK and APSAC will be examined in chapter 6. Similarly, the plasminogen activator inhibitor PAI-1 is completely neutralized during therapy with tPA, at least in plasma.[102]

Formation of enzyme-inhibitor complexes

The reaction of α_2-antiplasmin with plasmin involves a rapid complex formation. Assay of these plasmin-α_2-antiplasmin complexes in plasma is a very sensitive method for detecting activation of the fibrinolytic system in pathological or therapeutical situations.[103]

Other haemostatic effects

As a result of the low fibrinogen concentration and the increased FgDP, which act as anticoagulants, the clotting times observed in global coagulation tests will be prolonged: the activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) and the reptilase time (RT). However, one should realize that the three former ones can also be prolonged by heparin, which is often given concomitantly during thrombolytic therapy. Since the RT is affected by FgDP, but is insensitive to heparin, this test may be used to discriminate between these two anticoagulant effects.[104]

The effects of thrombolytic agents on blood viscosity and on platelet function are the subjects of later sections of this thesis (see chapters 9-12).

Laboratory monitoring of thrombolytic therapy

There are three major goals of monitoring thrombolytic therapy using haemostatic assays: to assess the therapeutic success of the treatment and to predict the two important complications of thrombolytic therapy, namely bleeding and reocclusion/rethrombosis.

Control of therapeutic efficacy

Several studies have shown that improved clot dissolution may be associated with lower levels of residual fibrinogen after SK, tPA and APSAC in groups of patients, but low fibrinogen is not predictive of successful clot lysis in an individual patient.[105-107] The same holds true for other parameters of the fibrinolytic and coagulation systems. Even an increase in fibrin-derived degradation products does not necessarily reflect lysis of the coronary thrombus.[108-110] The median FbDP concentration after thrombolysis corresponded to complete lysis of a clot, composed of about 5 mL blood, whereas coronary thrombi are generally not larger than 0.2 mL.[102,111] Therefore, the major source of increased FgDP and other fragments of crosslinked fibrin is most probably not the coronary thrombus, but rather soluble fibrin in plasma and fibrin in haemostatic plugs or vascular intima deposits at non-coronary sites in the circulation.[108-111]

Prediction of bleeding

Clinical experience from countless investigations has revealed that there is no single laboratory test which can predict bleeding during thrombolysis.[106,107,112] The incidence and severity of bleeding during thrombolytic treatment show little, if any, correlation with the degree of systemic lysis.[112] Of course, it is not surprising that any bleeding occurring during thrombolytic treatment would be more severe in the presence of disturbances of the haemostatic system, but the coagulation defect can not be regarded as the primary cause for bleeding. Other factors, such as concomitant heparin administration and the integrity of the vascular system are probably more important reasons for haemorrhage.

Prediction of reocclusion/reinfarction

Two factors can be implicated in the occurrence of reocclusion: activation of platelets and continued or renewed clotting at the site of the original thrombus. The contribution of platelets in reocclusion has not been fully defined at the moment; moreover, the laboratory assays in this field are not well suited for routine applications (see also Part 3).

Thrombolysis has been shown to be accompanied by generation of thrombin activity. Although these mechanisms involved are not appreciated yet, there are indications that measuring parameters of thrombin activity, for instance thrombin-antithrombin III complex (TAT), might be useful in predicting reinfarction.[89] This subject will be discussed in detail in chapter 8.

As for the monitoring of thrombolytic therapy in non-investigational circumstances, the state of the art can not be summarized more meaningful than by quoting Victor Marder, who wrote: "the sole reason for performing a laboratory test is to prove that the activator has been effectively delivered to the patient and that is has overcome the inhibitory effect of natural or acquired plasma inhibitors".[105]

AIM OF THE INVESTIGATIONS

At the time of initiating the studies described in this thesis, the situation in the field of thrombolytic therapy was quite different from the one in 1992. For instance, the first clinical trials with the new drugs rtPA and APSAC had just been reported, the favourable outcome of thrombolysis in AMI, *i.e.* the reduced mortality, was still unknown and haemostatic assays based on the monoclonal antibody technology were not widely available. The two major side-effects of coronary thrombolytic therapy, bleeding and reocclusion, were of course well-known, but the mechanisms leading to these adverse effects were still unidentified. It was also not clear why administration of thrombolytic drugs failed to induce lysis of coronary thrombi in such a high percentage of patients with AMI. Major research efforts were being given to assessing the clinical efficacy of new dosage schemes, minimizing side-effects and searching for additional strategies in order to consolidate the rapid recanalization achieved with thrombolytic drugs. When our laboratory became involved in the early application of new thrombolytic agents in a peripheral cardiologic clinic, the opportunity was used for studying those aspects of thrombolysis which are at the interface of laboratory and clinical practice.

Part 1: (pre-)analytical aspects of haemostatic assays

As stated before, some thrombolytic agents will activate plasminogen not only when it is bound to fibrin, but also free plasminogen in plasma. During the early phase of thrombolytic therapy, substantial amounts of thrombolytic drug may circulate in blood and continue to activate plasminogen *in vitro* after blood collection for laboratory studies. Chapter 1 describes the results of a study on the addition of several substances for inhibiting ongoing plasminogen activation or plasmin activity after blood sampling, in order to reflect the *in vivo* situation at the moment of blood sampling as closely as possible.

In chapter 2 a new technique is evaluated for measuring the fibrinogen concentration in plasma. It is based on nephelometric monitoring of clotting plasma in the PT assay, using a fully automated coagulation analyzer. Special consideration was given to the accuracy of this method when measuring fibrinogen in plasma samples from patients undergoing thrombolytic therapy.

A comparative study on the specificity of four different methods for determining fibrinogen in plasma is reported in chapter 3. Two established techniques, based on rapid and slow thrombin-induced clotting of plasma, respectively, were compared with two novel methods: one was the nephelometric method described in chapter 2 and the other was an immunological assay using two monoclonal antibodies, which allow recognition of fibrinogen molecules with at least one intact Aα-chain.

Part 2: haemostatic assays in thrombolytic therapy of AMI

Chapter 4 describes the clinical results of one of the earlier studies comparing the new thrombolytic agent APSAC, administered intravenously, with intracoronary SK, the then

standard approach for thrombolytic treatment of AMI. The design of the study included a pre-treatment coronary angiography, which allowed for assessing the recanalization of the infarct-related artery rather than its patency. The results of haemostatic determinations performed in this study are presented in chapter 5.

Because APSAC is a SK-containing compound, it might react with and become neutralized by anti-SK antibodies, formed after an infection with streptococci or in response to previous treatment with SK or APSAC. Chapter 6 describes a study on anti-SK antibodies in plasma of patients undergoing thrombolytic therapy with APSAC or SK, addressing the early course of the anti-SK level after thrombolysis and potential correlations between pretreatment anti-SK antibodies and clinical parameters as therapeutic efficacy and side-effects.

Chapter 7 reports an investigation of the relevance of histidine-rich glycoprotein for thrombolytic therapy of AMI. HRG is believed to regulate the availability of plasminogen for activation by plasminogen activators and therefore, HRG could in theory represent a factor contributing to the therapeutic effect of thrombolysis.

Chapter 8 deals with the role markers of thrombin activity in the development of reinfarction after thrombolysis. The patients investigated were selected from a large clinical trial, comparing the efficacy of recombinant pro-urokinase (saruplase) and urokinase in nearly 550 subjects with AMI.

An explorative study on the rheology of blood during thrombolytic therapy is presented in chapter 9. It was chosen to investigate this item in patients receiving APSAC, a thrombolytic drug known to create a significant systemic lytic state. Since fibrinogen is an important determinant of plasma viscosity, this type of treatment represents a useful model for examining the role of haemorheology in thrombolysis.

Part 3: thrombolytic therapy and platelet function

The role of platelets during and after thrombolytic therapy appears to be much more important than supposed until recently. There is growing evidence that platelets are involved in the bleeding complications associated with thrombolysis and in the emergence of reocclusion. The study described in chapter 10 was designed to document the effects of treatment with APSAC on platelets in AMI patients, who did not use other medication affecting platelet function.

Chapter 11 presents the results of a flow cytometric study on the immunological integrity of platelet surface glycoproteins, after treating the platelets *in vitro* with thrombolytic agents and with plasmin. Some of these glycoproteins serve as receptors for platelet-vessel wall and platelet-platelet interactions and are necessary for adequate platelet function.

The effects of plasmic fibrinogen digests and purified fibrinogen degradation products on the aggregation of platelets is reported in chapter 12. This *in vitro* model was used to verify the hypothesis that fibrinogen fragments generated during thrombolytic therapy, are competitive inhibitors of fibrinogen-dependent platelet function and might be causally related to the bleeding tendency associated with this therapy.

In the General Discussion section, some unsolved problems and controversial issues related to thrombolytic therapy are discussed. In addition, a perspective is provided on current and future developments in the field of thrombolysis, which are expected to enter clinical practice within the next few years.

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PART 1

(PRE-)ANALYTICAL ASPECTS OF HAEMOSTATIC ASSAYS

Chapter 1

Prevention of in vitro fibrinogenolysis during laboratory monitoring of thrombolytic therapy with streptokinase or APSAC

JJML Hoffmann and M Vijgen

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SUMMARY

In the present study, we systematically investigated aprotinin, epsilon-aminocaproic (EACA) and tranexamic acid as inhibitors of fibrinogen breakdown and of the generation of fibrinogen degradation products (FgDP). The experimental setting very closely imitated the conditions in practice when collecting blood from patients receiving thrombolytic therapy with streptokinase or APSAC. The minimal concentration of aprotinin required to completely inhibit fibrinogen breakdown and FgDP generation was 200 KIU/mL blood. This was sufficient even at the highest concentrations of streptokinase and APSAC expected to occur in patients (300 U/mL and 46 nM, respectively). However, 200 KIU/mL aprotinin heavily interfered in the determinations of plasminogen and α_2-antiplasmin. Relatively low concentrations of EACA (200 mM) and tranexamic acid (35 mM) were sufficient to prevent FgDP generation, but they interfered in the Clauss assay of fibrinogen. A non-interfering concentration of EACA (7 mM) allowed the inhibition of lower concentrations of APSAC (20 nM) and streptokinase. We conclude that at least 200 KIU aprotinin per mL blood is necessary to effectively inhibit in vitro fibrinogenolysis under circumstances likely to be met in clinical practice during thrombolytic therapy.

INTRODUCTION

Thrombolytic therapy for acute myocardial infarction is rapidly gaining widespread application now it has been convincingly demonstrated that early thrombolysis significantly reduces the mortality in patients with this disease.[1-3] Therefore, laboratory monitoring of thrombolytic therapy is likely to become routine practice, although the need for laboratory control is still under debate.[4,5]

Thrombolytic drugs are plasminogen activators with a highly variable selectivity for fibrin-bound plasminogen. None of the drugs presently in clinical use is sufficiently selective to avoid activating free, circulating plasminogen and prevent fibrinogen breakdown.[5] Especially in the early phase of therapy this poses a problem for laboratory monitoring since plasminogen activation and plasmin activity will proceed *in vitro* after blood collection. Unless adequate precautions are taken, spuriously low concentrations of fibrinogen, α_2-antiplasmin and plasminogen and falsely elevated concentrations of fibrinogen degradation products (FgDP) will be measured.[6]

Several methods for preventing *in vitro* fibrinogenolysis have been proposed. During therapy with tissue-type plasminogen activator (tPA), aprotinin, specific polyclonal or monoclonal anti-tPA antibodies and a synthetic peptide were found to efficiently inhibit *in vitro* plasminogen activation.[6-9] For other thrombolytic drugs like streptokinase (SK) or anisoylated plasminogen-streptokinase activator complex (APSAC), such specific inhibitors are not available and agents with broad specificity are to be used: aprotinin and epsilon-aminocaproic acid (EACA).[10-12] However, the concentrations of inhibitors recommended vary substantially between reports [4,10-12] and EACA is even considered ineffective by some workers.[10] Moreover, in a number of studies the conclusions were based on experiments with plasma[9,10] and these authors disregard the variations in haematocrit occurring in patients, especially those with acute myocardial infarction.

The aim of the present study was to systematically investigate three inhibitors (aprotinin, EACA and tranexamic acid) in order to determine the minimum concentrations required for effectively preventing *in vitro* fibrinogenolysis and FgDP generation during monitoring of thrombolytic therapy with SK or APSAC. Special attention was given to rigorously mimic the conditions occurring in routine practice in a clinical laboratory serving a cardiology department involved in the thrombolytic therapy of acute myocardial infarction.

MATERIALS AND METHODS

Materials

Aprotinin (Trasylol®; 10,000 KIU/mL) was purchased from Bayer AG, Leverkusen, FRG. Tranexamic acid (TA; *trans*-4-aminomethylcyclohexane carboxylic acid) and EACA (6-aminohexanoic acid) were from Sigma, St. Louis, MO, USA and streptokinase

(Kabikinase®, clinical grade) from KabiVitrum, Amsterdam, the Netherlands. APSAC (Eminase®, clinical grade) was a gift from Beecham Research Laboratories, Amstelveen, the Netherlands. Bovine thrombin (Topostasin®) was from Hoffmann-LaRoche, Basel, Switzerland and fibrinogen standard, a calibrated human plasma derivative, from Dade (Baxter, Utrecht, the Netherlands). Reagents for the determination of FgDP in plasma (Fibrinostika-FgDP) were purchased from Organon Teknika, Boxtel, the Netherlands.

Blood collection

Blood was drawn from healthy volunteers through a clean puncture of an antecubital vein after short, minimal stasis; the first few millilitres were discarded. It was collected into plastic syringes containing 0.1 volume of 0.1 M trisodium citrate and, depending on the experiment (see below), was kept at 37 °C until use.

Inhibition of fibrinogenolysis

The effect of varying concentrations of the inhibitors was studied with blood, prewarmed to 37 °C. Appropriate dilutions of inhibitor in 0.15 M NaCl or control saline were added in a constant volume (0.05 mL) to 0.45 mL prewarmed blood, quickly followed by 0.015 mL of SK or APSAC solution; APSAC and SK were dissolved in water, immediately (< 2 min) before addition to plasma. The samples were either transferred into melting ice (0 °C) or kept at ambient temperature (22 °C), both for 30 min. They were then centrifuged (15 min, 2500 *g* at 4 °C), the plasma was pipetted off and stored at 0 °C for subsequent fibrinogen assay or snap-frozen and stored at -70 °C for FgDP determination.

The inhibiting capacity of fixed concentrations of the three inhibitors was studied similarly by adding 0.015 mL of appropriate dilutions of APSAC or SK solutions to 0.45 mL prewarmed blood supplemented with 0.05 mL of inhibitor solution. All samples were then incubated for 30 min at 0 °C prior to processing as described above.

The effects of the inhibitors on other coagulation and fibrinolytic parameters were determined in plasma from citrated blood, to which 0.1 volume of inhibitor solution had been added; an equal volume of saline served as control.

Assays

Plasma fibrinogen was determined using the Clauss thrombin clotting time method.[13] All tests were performed in quadruplicate in a Schnitger and Gross coagulometer (H. Amelung, Lemgo-Brake, FRG) using bovine thrombin; a calibration curve was constructed using five different dilutions of fibrinogen standard. FgDP in plasma was determined using a commercial ELISA kit, which is based on two specific monoclonal antibodies.[14] Other assays were carried out according to standard procedures and using commercially available reagents.

Statistics

For statistical evaluations we used student's t-test at a significance level of 0.05.

RESULTS

The effect of a range of aprotinin concentrations on fibrinogenolysis in blood containing APSAC is shown in Figure 1. The minimal aprotinin concentration required to conserve fibrinogen and to prevent FgDP generation was 100 KIU/mL blood if the sample was cooled to 0 °C immediately after addition of the thrombolytic agent and inhibitor. When the blood was kept at 22 °C, at least 200 KIU/mL of aprotinin was necessary to keep fibrinogen and FgDP statistically indiscernible from control (Fig 1). Experiments with SK as a thrombolytic agent (final concentration 300 U/mL blood) yielded essentially identical results: depending on the storage temperature, 100 or 200 KIU aprotinin per mL blood were necessary to completely inhibit fibrinogen degradation (not shown).

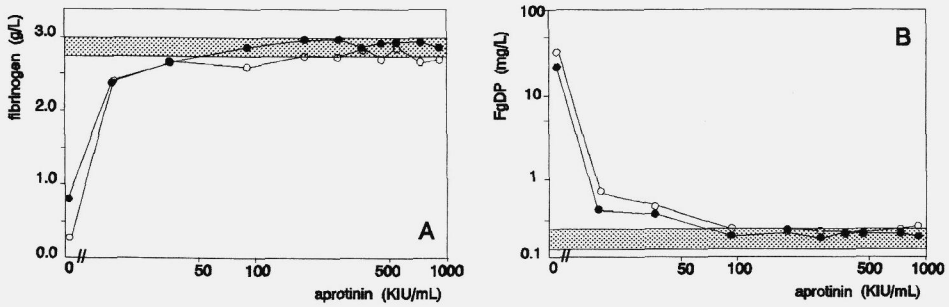

Figure 1 *Effect of a range of aprotinin concentrations on fibrinogen (A) and FgDP (B) in blood, supplemented with APSAC (46 nM; about 70 nM in plasma); means of four determinations. Samples were kept at 0 °C (●) and 22 °C (○) prior to processing. Shaded area is mean ± SD of control sample without thrombolytic or aprotinin.*

Aprotinin, 250 KIU/mL blood (equivalent to about 400 KIU/mL plasma), added to a series of samples from patients not receiving thrombolytic treatment, had no measurable effect on fibrinogen, FgDP, prothrombin time and thrombin time, but caused highly significant increases in APTT and α_2-antiplasmin activity and a decrease in plasminogen activity (Table 1).

When EACA and TA were tested for their inhibiting capacity, it appeared that neither could prevent a decrease in plasma fibrinogen, relative to the control, not even at the highest concentrations attainable (Fig 2). However, 180 mM EACA and 35 mM TA were sufficient to completely inhibit FgDP generation at 0 °C (Fig 2). This apparent discrepancy was caused by a direct, negative interference of EACA and TA in the fibrinogen assay: EACA progressively decreased fibrinogen of control plasma by about 10% at 100 mM and over 20% at 400 mM. The experiments with APSAC at 22 °C and with SK (300 U/mL blood), both at 22 °C and 0 °C, gave very similar results. Complete inhibition of FgDP generation was achieved with final EACA concentrations of 200 mM or higher or TA concentrations over 30 mM, but fibrinogen always remained significantly below its control value (not shown).

Table 1 *Effect of aprotinin (250 KIU/mL blood) on several coagulation and fibrinolytic parameters (mean ± SD; n = 22).*

parameter (unit)	control	aprotinin
PT (sec)	12.9 ± 2.3	13.0 ± 2.3
APTT (sec)	30.7 ± 3.3	69.9 ± 8.6*
thrombin time (sec)	13.0 ± 1.9	13.0 ± 1.8
fibrinogen (g/L)	2.52 ± 0.82	2.51 ± 0.79
plasminogen (U/mL)	0.87 ± 0.17	0.15 ± 0.07*
α_2-antiplasmin (U/mL)	1.01 ± 0.09	1.43 ± 0.05*
antithrombin III (U/mL)	0.96 ± 0.14	0.97 ± 0.13

* $P < 0.001$

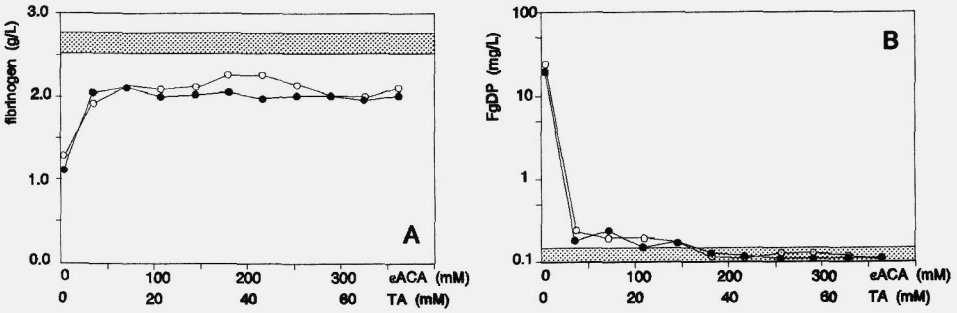

Figure 2 *Effect of EACA (○) and TA (●) on fibrinogen (A) and FgDP (B) in blood, supplemented with APSAC (46 nM; about 70 nM in plasma); means of four determinations. Samples were stored at 0 °C for 30 min prior to processing. Shaded area indicates mean ± SD of control sample not containing inhibitor or thrombolytic agent.*

In order to investigate whether fibrinogen concentrations could effectively be kept constant when lower than maximal concentrations of APSAC and SK were used, we studied the inhibiting capacity of a number of fixed amounts of inhibitors; inhibitor concentrations identical with or close to ones previously published were chosen. Figure 3 shows that, over the entire range of APSAC and SK concentrations, only aprotinin was able to prevent breakdown of fibrinogen. There was no significant difference between the commonly used concentration of 250 KIU/mL blood and 1000 KIU/mL. EACA at 400 mM consistently resulted in fibrinogen concentrations far below control, but at 7 mM, EACA effectively inhibited low concentrations of APSAC (to about 20 nM). TA at 71 mM gave similar results as 7 mM EACA. It was also found that in blood without any inhibitor, fibrinogen remained constant only at very low concentrations of thrombolytic agents, at least under the circumstances used here (saline control, Fig 3).

Additional testing indicated that aprotinin (250 KIU/mL blood) prevented FgDP generation and fibrinogen breakdown for at least 90 min, provided the samples were kept at 0 °C after addition of thrombolytic and inhibitor; once plasma had been obtained, freezing and storage at -70 °C equally did not influence FgDP or fibrinogen values at this aprotinin concentration (not shown).

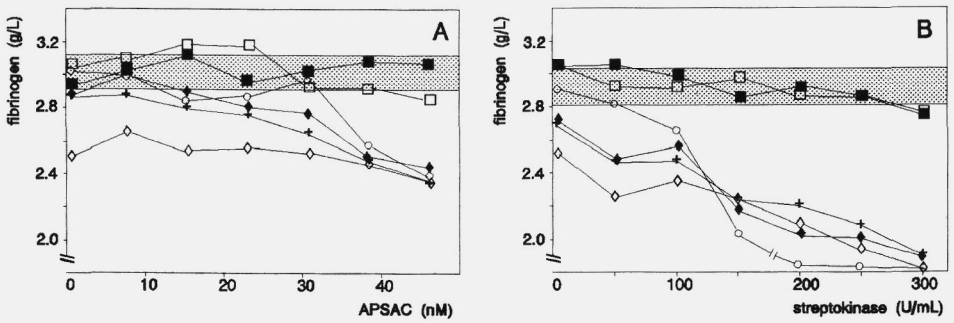

Figure 3 *Inhibiting capacity of aprotinin 250 KIU/mL (■), aprotinin 1000 KIU/mL (□), EACA 7 mM (♦), EACA 400 mM (◇), and TA 71 mM (+) as a function of the concentration of APSAC (A) and SK (B). Saline control (○) and control without inhibitor (shaded area; mean ± SD). All points represent mean of four assays; blood stored at 0 °C prior to processing.*

DISCUSSION

Despite recognition of the precautions required when collecting blood samples for coagulation studies during thrombolytic therapy, surprisingly few systematic studies on this topic have been reported. Most of these deal with prevention of *in vitro* fibrinogenolysis during treatment with tPA[6-9] and we know of only a single study focusing on APSAC.[10] In addition, the recommendations arising from these studies are far from uniform. Our study was aimed at preventing fibrinogenolysis by SK and APSAC, two agents currently used in clinical thrombolysis, for which no specific inhibitors are available, in contrast to tPA.[6-9] Furthermore, we tried to closely imitate the circumstances which probably occur in a clinical setting; this item was insufficiently addressed in the previously mentioned study.[10] For instance, Fears *et al.*[10] added the thrombolytic agent to plasma at 25 °C or 0 °C; such a procedure ignores the temperature of blood at collection from a patient and may seriously underestimate fibrinogenolysis at temperatures between 37 and 0 °C. We also deliberately chose to add the plasminogen activators to blood after the inhibitors, because *in vitro* fibrinolytic activity of APSAC or SK is so rapid at 37 °C that fibrinogen cannot be kept at its original concentration when inhibitor is added after the thrombolytic; in the latter way, the fibrinogen concentration will remain constant, but at an unpredictable level, which is invariably lower than its control value (results not shown).

Like others, we have used the Clauss method to estimate fibrinogen because this technique is very sensitive for interference by FgDP.[15] Unlike others, however, we decided to also include determination of FgDP using a sensitive and specific enzyme immunoassay[14] in order to improve the detection of fibrinogen degradation.

The experiments depicted in Figures 1 and 2 were carried out at APSAC and SK concentrations which represent the theoretically highest possible values, calculated from a blood volume of 5 L and dosages currently in clinical use; 30 mg of APSAC and 1.5 x 10^6 U of SK, respectively. The conversion of the proenzyme APSAC to active SK-plasmin(ogen) occurs by deacylation. The deacylation half-life of APSAC in plasma *in vitro*, is similar to the *in vivo* clearance half-life of the total fibrinolytic activity of APSAC,[17,18] suggesting that the deacylation rate is the prime determinant of clearance in most patients.[17] The clearance of active SK-plasmin(ogen) is much faster[16] than the deacylation of APSAC. Thus, *in vivo* there will not be any accumulation of SK-plasmin(ogen), but *in vitro* there will, although this complex is not very stable.[19] Given the fact that under the circumstances mentioned, at least 200 KIU of aprotinin per mL plasma will efficiently inhibit FgDP generation and fibrinogen breakdown *in vitro* for at least 90 min, we are convinced that 200 KIU/mL of aprotinin is sufficient to prevent *in vitro* fibrinogen degradation also when the highest therapeutic doses are used. Further increasing the concentration of aprotinin to 1000 KIU/mL blood had no significant effect on the stability of fibrinogen or FgDP. Although the aprotinin preparation used by us did not allow testing at 3000 KIU/mL, as recommended by Fears *et al.*,[10] it is difficult to imagine that such a high concentration of aprotinin would be necessary at all, in view of the results is Figures 1 and 3.

It appeared that relatively low concentrations of EACA and TA were already sufficient to prevent formation of FgDP (Fig 2), but they caused simultaneous interference in the Clauss assay of fibrinogen to such a degree that fibrinogen was consistently lower than in control samples. These results limit the usefulness of EACA and TA as inhibitors of APSAC and SK to those circumstances in which parameters can be measured using non-functional techniques, e.g. immunoassays, or when samples are collected containing much less APSAC or SK than the maximal concentrations. If, for instance, samples are taken from patients some hours after dosing, the amount of plasminogen activator will be decreased to such an extent,[16,17] that it can effectively be inhibited by low concentrations of EACA, which no longer cause interference in the Clauss assay (Fig 3). In this view, the low concentration of EACA applied in one of our previous studies[12] might have been suboptimal for the first samples after dosing, but if any relevant overestimation of fibrinogenolysis *in vitro* occurred, it was certainly not present in the later samples.

Although aprotinin was shown to be very effective in preventing fibrinogenolysis, the results in Table 1 show that the functional determinations of plasminogen and α_2-antiplasmin are grossly disturbed by aprotinin. Collection of citrated blood without inhibitor is necessary for these substances; rapid cooling and fast processing of the blood samples will reduce but probably not eliminate *in vitro* consumption of these fibrinolytic proteins.

When comparing fibrinogen concentrations in fresh and frozen (-70 °C) plasma containing aprotinin, no significant difference was observed in our study. This is in contrast with the results of Holvoet *et al.*,[7] who found extensive fibrinogen breakdown in plasma containing tPA which was kept at -20 °C. Important differences in storage temperature and in binding properties of the respective thrombolytics used can account for this discrepancy.

In conclusion, our results underline the absolute necessity for adding a plasmin inhibitor to blood collected during thrombolytic therapy.[4,6-10] We recommend that blood should be collected on to citrate with aprotinin (final concentration at least 200 KIU/mL blood), rapidly cooled to 0 °C, in order to inhibit *in vitro* fibrin-ogenolysis, and plasma stored at -70 °C if not used immediately. Depending on the parameters to be measured, EACA and TA may also be suitable substances for preventing fibrinogen degradation in samples from patients receiving thrombolytic therapy with SK or APSAC.

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Chapter 2

Automated nephelometry of fibrinogen: analytical performance and observations during thrombolytic therapy

JJML Hoffmann and MAL Verhappen

Clinical Chemistry 1988; **34**: 2135-2140

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SUMMARY

We evaluated the performance of an automated nephelometric determination of fibrinogen, which is an integral part of the prothrombin time assay, in a new coagulation analyzer, the ACL-810 (Instrumentation Laboratory). Results were compared with those by a total clottable protein assay and with the thrombin clotting time assay for fibrinogen. In normal and slightly abnormal plasma, the performance of the ACL method was quite satisfactory (CV 3-10%). However, in abnormal plasma (prolonged prothrombin times because of heparin or oral anticoagulants) the accuracy of the ACL method was poor. Nor couls the instrument determine fibrinogen in clearly lipaemic plasma. In plasma containing high concentrations of fibrin(ogen) degradation products (FDP), collected during thrombolytic therapy with streptokinase-containing drugs, the ACL method gave spuriously high values for fibrinogen concentration. We determined that this was mainly because of interference by intermediate FDP (fragment Y). Finally, we demonstrated that early FDP (fragment X) increased the ACL results for fibrinogen to the same extent as in the total clottable protein method and that late FDP (fragments D and E) affected the thrombin clotting time method, but not the ACL fibrinogen determination.

INTRODUCTION

Because fibrinogen plays a central role in the haemostatic system, fibrinogen determinations are frequently requested in the management of ill patients. Ideally, fibrinogen assays in the routine coagulation laboratory should be sensitive, specific and fast. The numerous published methods for determining fibrinogen in plasma are based on different principles: heat or salt precipitation, thrombin clotting time,[1] total clottable protein,[2,3] turbidimetry of clotting plasma,[4] and immunological techniques. In general, the more nearly accurate assays are too laborious for routine use and the simple and fast methods lack specificity or have limitations with regard to automation.

Recently, a centrifugal analyzer fully dedicated to coagulation assays was introduced: the ACL-810, which exploits a new concept for measuring fibrinogen. As an integral part of the prothrombin time (PT) determination, light scattering by the clotted plasma is measured by laser nephelometry at 632 nm and the fibrinogen concentration is calculated from the net light-scattering signal. At least in normal plasma, the light scattering by the clot in the PT assay and fibrinogen concentration seem to be linearly related.[5] The new analyzer has been evaluated in a few, mostly preliminary, reports,[5-7] but the performance of the unique fibrinogen determination has not been studied in detail. Here we present results of our evaluation of the ACL fibrinogen determination, with special reference to its behavior in plasma samples from patients receiving thrombolytic treatment. We compared the ACL method with a total clottable protein (TCP) method[2] and with the widely used thrombin clotting time (TCT) method developed by von Clauss.[1]

MATERIALS AND METHODS

Materials

The PT/fibrinogen determination in the ACL (Automated Coagulation Laboratory, ACL-810®; Instrumentation Laboratory, Milan, Italy) was performed with the Thromborel-S® reagent (Hoechst-Behring, Marburg, FRG), a sensitive thromboplastin preparation from human placenta. Bovine thrombin was from Hoffmann-LaRoche, Basel, Switzerland and aprotinin (Trasylol®) from Bayer, Leverkusen, FRG. Streptokinase (SK), purified human fibrinogen and Intralipid® - a 10% fat emulsion containing soya oil, lecithin, and glycerol - were all purchased from KabiVitrum, Stockholm, Sweden.

Fibrin/fibrinogen degradation products (FDP) were measured semiquantitatively in serum by latex agglutination (Thrombo-Wellcotest®; Wellcome Diagnostics, London, UK), and cross-linked fibrin degradation products (XDP) in plasma by the monoclonal antibody-based Dimertest® reagent (Ortho Diagnostics, Beerse, Belgium). Normal pooled plasma was prepared from fresh blood donated by 46 healthy volunteers. Aliquots of it were snap-frozen in liquid nitrogen, stored at -70 °C and thawed just before use at 37 °C.

Methods

The ACL-810 was operated and calibrated as indicated by the manufacturer. For the calibration of the fibrinogen assay we used our own pooled specimen of normal plasma, the fibrinogen concentration of which had been determined from 10 replicate assays with the TCP method (see below).

For the thrombin clotting time, measured according to von Clauss,[1] we used bovine thrombin (100 kU/L) and electric detection of the fibrin strand in a Schnitger & Gross coagulometer (H. Amelung, Lemgo-Brake, FRG). It was calibrated with either commercial calibration plasma or normal pooled plasma. For comparison, we also determined fibrinogen by the total clottable protein method, according to Blombäck.[2] In brief, we diluted 0.3 mL of plasma with 0.75 mL of buffer (0.2 M phosphate, pH 5.9) and 195 μL of a 10 g/L solution of ϵ-aminocaproic acid, and clotted it by adding 7.5 U of bovine thrombin. After 2 h at ambient temperature, the clot was collected onto a plastic stick, squeezed out, and washed three times in a 0.15 M solution of NaCl. We dissolved the fibrin in 2.5 mL of alkaline urea solution (6.7 M urea in 0.2 M NaOH), let this stand for 2 h and then measured the absorbance at 282 nm. The fibrinogen concentration in the plasma sample was calculated by the absorptivity value of 1.65 cm^2/mg.[2]

Fibrinogen degradation products (FDP) were prepared by digesting purified, plasminogen-rich fibrinogen with SK in the absence of calcium.[8] This plasmin-mediated digestion was stopped by adding aprotinin (final concentration 250 KIU/mL) after 3, 20 and 60 min for early, intermediate and late FDPs, respectively. We verified the composition of the digests by electrophoresis on agarose and SDS-polyacrylamide gel.[8]

Artificial lipaemia of plasma was created by adding to plasma small volumes of various dilutions of Intralipid in saline.

Patients

Plasma samples were collected from patients who were undergoing thrombolytic therapy for acute myocardial infarction as a part of a clinical trial comparing SK with a new drug, APSAC.[9] Further samples were from healthy volunteers and from the routine workload of our laboratory.

Statistics

We used orthogonal regression analysis[10] to compare the methods. In addition, standard statistical methods were applied as indicated below. Significance was considered at P values < 0.05.

RESULTS

Precision and accuracy

We assessed the analytical precision of the ACL-810 in determining fibrinogen at different concentrations, both within and between runs. The coefficients of variation ranged between 3.3 and 7.0% and between 4.9 and 10.4%, respectively.

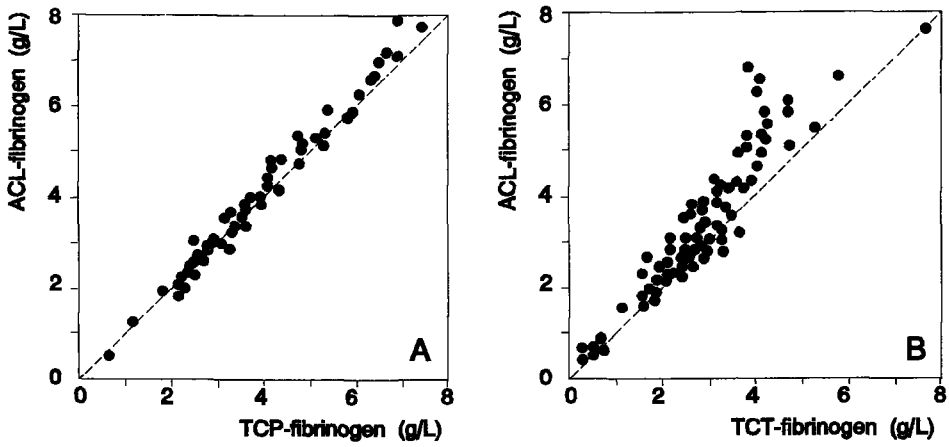

Figure 1 *Correlations between ACL and TCP fibrinogen (A) and ACL and TCT fibrinogen (B) in non-thrombolytic plasma samples. Regression equations by orthogonal regression analysis: (A) y = 1.07x - 0.17 (r = 0.996, n = 60) and (B) y = 1.25x - 0.28 (r = 0.977; n = 81). Dashed lines represent y = x.*

The accuracy of fibrinogen measurements with the ACL-810 was assessed by comparing it with results by the TCP method (Fig 1A) and the TCT method (Fig 1B), all in plasma from patients who were not receiving thrombolytic therapy. Dilution experiments indicated that the ACL-810 method maintained its linearity from about 0.4 to at least 5.0 g/L (not shown). The detection limit was found to be 0.3 g/L, as judged from determinations in plasma samples from patients receiving therapy with asparaginase (EC 3.5.1.1) for acute leukaemia, who have severe hypofibrinogenaemia because of diminished synthesis.

However, when hypofibrinogenaemia was the result of thrombolytic therapy with SK or APSAC,[9] we found gross discrepancies between the three fibrinogen methods. Figure 2 shows results of fibrinogen measurements in plasma from a patient who received 1.5 million U of SK intravenously for acute myocardial infarction: in the early post-treatment period there were significant differences among the three methods, while the results were more comparable longer after the SK therapy.

Figure 3 shows the correlation of the three fibrinogen methods in samples from patients who received SK or APSAC within the previous 12 hours. In these samples the ACL measured significantly higher values than the TCP method (means ± SD, 1.71 ± 0.62 g/L and 1.02 ± 0.78 g/L, respectively; $P < 0.001$ by Student t-test; Fig 3A), whereas with the TCT method the results were significantly lower than with the TCP method (mean 0.57 ± 0.63 g/L; $P < 0.001$ by Student t-test; not shown). As a consequence, we found a highly significant difference between the ACL and the TCT method in these samples (Fig 3B). All these samples contained increased concentrations of FDP, thus a relationship was sought. We detected a statistically significant correlation (r = 0.66; $P <$

0.001 by Spearman rank test) between the overestimation in fibrinogen by the ACL-810 (ACL fibrinogen minus TCP fibrinogen) and the FDP value, as illustrated in Figure 4.

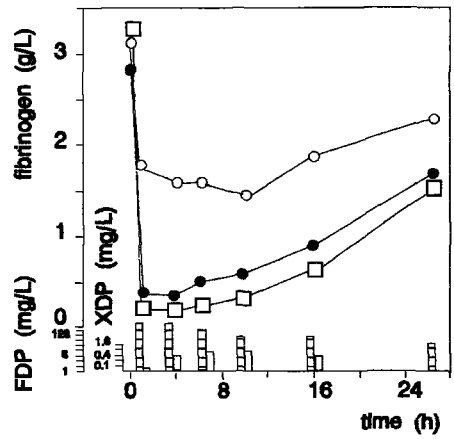

Figure 2 *Course of plasma fibrinogen in a patient treated with intravenous streptokinase (1.5 MU in 60 min), as measured with measured with the TCP method (●), the ACL assay (○) and the TCT assay (□). Hatched and white bars: FDP and XDP concentrations, respectively.*

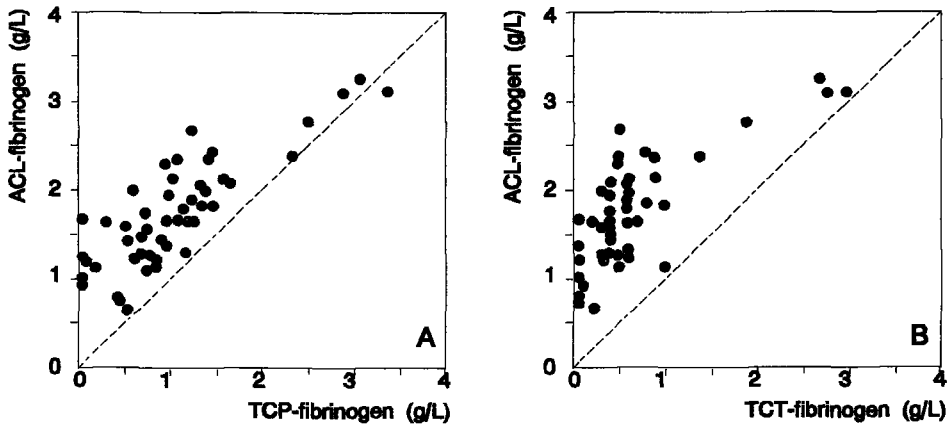

Figure 3 *Correlations between ACL and TCP fibrinogen (A) and ACL and TCT fibrinogen (B) in plasma samples collected during thrombolytic therapy. Dashed lines are y = x.*

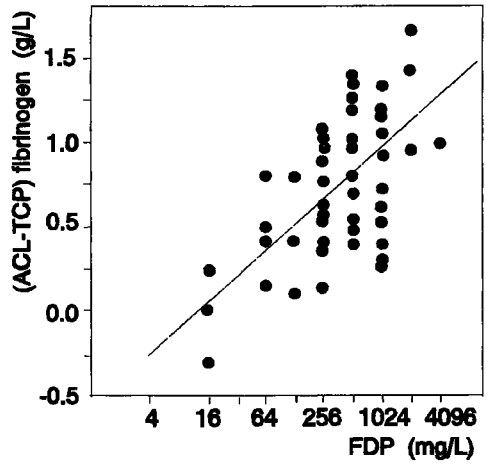

Figure 4 *Relationship between the difference [ACL minus TCP fibrinogen] and FDP concentration. r = 0.61 (linear regression analysis) n = 53.*

There was no such relationship between the fibrinogen difference and the XDP concentrations.

Effects of FDP on fibrinogen

Different FDP solutions were prepared by digesting purified fibrinogen, and the digests were analysed by electrophoresis. After a 3-min digestion (early FDP), the preparation mainly contained fragment X, but no intact fibrinogen. The 20-min digest (intermediate FDP) largely consisted of fragments Y and D, whereas in the 60-min FDP preparation (late FDP) essentially only fragments D and E and some smaller peptides were present (cf. reference 8). When added in increasing concentrations to pooled normal plasma, the early FDP caused an increase in fibrinogen in all three methods (Fig 5A), with no significant differences among them. However, the intermediate FDP preparation caused a substantial increase in fibrinogen as measured in the ACL-810 analyzer, did not affect the TCP fibrinogen, and decreased the fibrinogen concentration as measured with the TCT method (Fig 5B). The inter-method differences were statistically highly significant at FDP concentrations > 0.9 g/L ($P < 0.001$ by Student t-test). Finally, the late FDPs did not influence the TCP method, they only slightly affected the ACL fibrinogen assay, and they substantially decreased plasma fibrinogen as measured with the TCT technique (Fig 5C). The differences between TCP and ACL-810 methods were not significant ($P < 0.1$; by Student t-test), but they were when the TCT method was compared with either TCP or ACL-810 methods, starting at the lowest FDP concentration added ($P < 0.005$ by Student t-test).

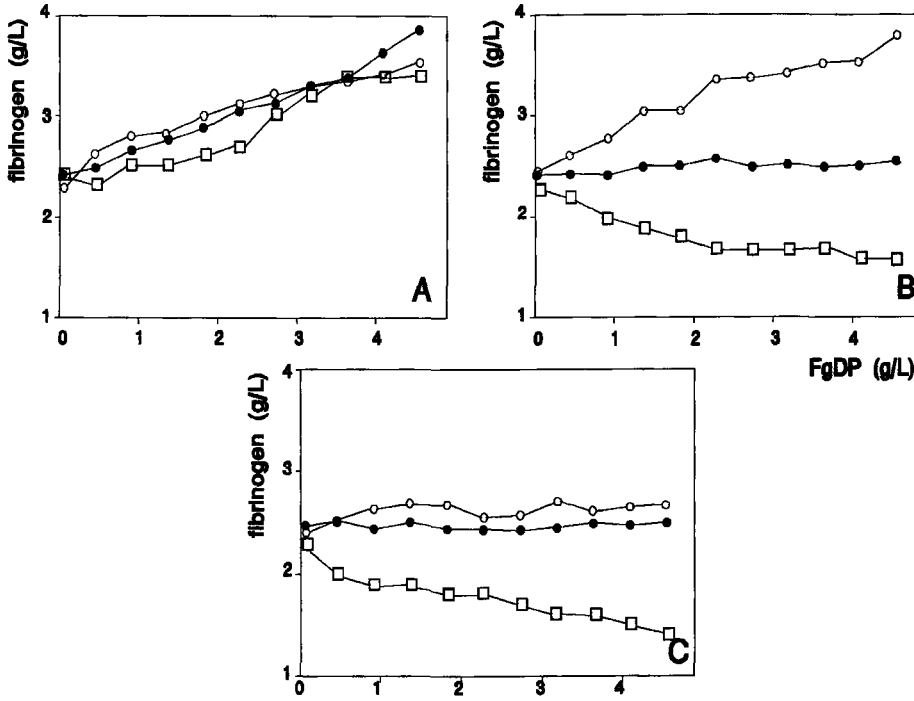

Figure 5 *Influence of increasing concentrations of different FDP preparations on fibrinogen concentration of normal plasma, determined with the TCP method (●), the ACL method (○) and the TCT method (□). Early (A), intermediate (B) and late (C) FDP; shown are means of at least duplicate determinations. * smallest significant difference (P < 0.02).*

Other interferences

Because fibrinogen determinations with the ACL-810 rely on the PT test, we investigated the effects of heparin and oral anticoagulants on the results for fibrinogen. The effect of heparin is shown in Figure 6. In all plasma samples, fibrinogen remained constant up to about 1.0 U of heparin per mL plasma. At higher heparin concentrations fibrinogen appeared to increase by an average of 30% and at even higher concentrations there was a sharp drop in values for fibrinogen below the baseline value. In most samples, the PT times now were excessively long and the ACL-810 indicated a warning message together with the fibrinogen and PT results. As for oral anticoagulant therapy, the fibrinogen result was spuriously low (not shown) with prolonged PT times (activity > 3.5 INR).

Although lipaemia is generally not a problem in clotting assays, it is of course in nephelometry. Accordingly, we investigated the effect of lipaemia -mimicked by adding a milky fat emulsion to plasma- on fibrinogen determinations with the ACL-810. The ACL-

810 no longer accurately determined fibrinogen when the samples were visually turbid (triglycerides exceeding ~5.0 mmol/L; data not shown).

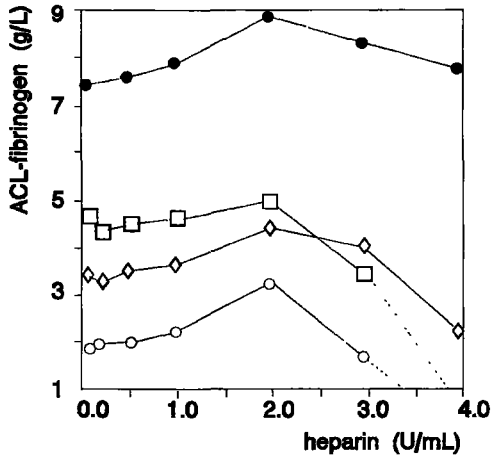

Figure 6 *Effect of heparin added in vitro on ACL fibrinogen for four different plasma samples.*

DISCUSSION

The technique for measuring fibrinogen in plasma with the ACL-810 constitutes a novel approach for determining this important coagulation protein. At the endpoint of the PT test the right-angle light scattering of the clotted sample is measured, corrected for the light scattering of the unclotted reaction mixture, and read from a calibration curve to give the fibrinogen concentration. The precision and accuracy of this method were satisfactory over a broad range of concentrations, in agreement with other reports.[5-7]

However, when we compared fibrinogen results with the ACL-810 to the two other methods, using plasma obtained during thrombolytic therapy, we observed very poor correlations (Figs 2 and 3). This lack of agreement between the ACL-810 and the TCT method was described earlier.[6] Because we suspected the increased FDP concentrations in such plasma samples[9] to be the principal explanation for these discrepancies (Fig 4), we carried out some experiments with different *in vitro* FDP preparations. Figure 5 shows that early FDPs (mainly fragment X) increase the fibrinogen concentrations to a similar degree in all three methods, probably because fragment X is still clottable by thrombin.[8] We could confirm the well-known underestimation of fibrinogen in the TCT method resulting from the anticoagulant properties of the fragments Y, D and E[8,11-13] in our study (Figs 5B and 5C). Further, we showed that the TCP method is insensitive to interference by the intermediate and late FDP fragments Y, D and E. The difference in behaviour of the TCT

method and the TCP method with respect to intermediate and late FDP fragments can be explained by the difference in reaction time, a few seconds in the former and 2 h in the latter method.

In contrast to the TCP and TCT methods, where the intermediate FDPs had a similar effect on fibrinogen as the late fragments, we noted a remarkable difference with the ACL-810 method: intermediate FDPs increased the apparent fibrinogen concentration considerably, but late FDPs had hardly any such effect (Figs 5B and 5C). We consider the analogy with the turbidimetric method, used in the DuPont *aca* instrument,[13] as the most probable explanation for this phenomenon. Recently it was demonstrated that purified fragment D added to normal plasma did not alter the light absorption properties of fibrin protofibrils and oligomers, but completely inhibited the polymerization of fibrin in a clotting-rate method.[12,14] Our results, depicted in Figure 5C (fragments D and E), support this finding. Thus, the apparent increase in fibrinogen with the ACL-810 in the presence of intermediate fragments (Fig 5B) might be explained by the fact that fragment Y would contribute substantially to the light scattering, but would act as a strong anticoagulant or polymerization inhibitor in the clotting-rate method.[8] This assumption remains to be proven in experiments with purified fragment Y. In contrast to the findings of Hoffman and Greenberg,[14] who state that the DuPont *aca* assay more nearly reflects the true amount of fibrinogen in plasma in the presence of FDP, we find that the ACL-810 grossly overestimates plasma fibrinogen in samples with increased FDP, especially when intermediate FDPs (fragment Y) are present (Figs 2-5).

The inadequate performance of the ACL-810 assay in plasma samples with very high heparin concentrations and in plasma from patients on high-intensity oral anticoagulant therapy most probably results from the fact that under these conditions the maximal measuring time of the PT test (120 sec) is too short for a fully stablilized fibrin polymer to form and thus too low a light scattering signal is used for the calculation of the fibrinogen concentration. We cannot present a simple explanation for the phenomenon of apparent increases in fibrinogen at heparin concentrations of 2.0 U/mL. Perhaps heparin in these concentrations interferes in the polymerization process such that the light scattering of the non-polymerized or partly polymerized fibrin is enhanced in comparison with completely polymerized fibrin.

In conclusion, fibrinogen determination by the ACL-810 coagulation analyzer performs adequately for most samples which constitute the workload of a routine haemostasis laboratory. However, in a number of well-described circumstances, which the laboratory should have knowledge of (thrombolytic therapy) or could easily be detected (prolonged PT because of heparin or oral anticoagulation; overt lipaemia), the characteristics of the fibrinogen determination with the ACL-810 will be inadequate for clinical use. An alternative method for determining fibrinogen remains necessary as a back-up under these conditions.

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Chapter 3

Comparison of the specificity of four fibrinogen assays during thrombolytic therapy

JJML Hoffmann, M Vijgen and W Nieuwenhuizen[1]

[1] Gaubius Institute-TNO, Leiden

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SUMMARY

Three functional assays for fibrinogen were compared with an ELISA method which, as a result of the use of two monoclonal antibodies, one reacting with N- and the other with C-terminal domains of the Aα chain, only detects fibrinogen molecules with at least one intact Aα chain. In normal plasma and plasma from patients not receiving thrombolytic therapy, all four methods showed excellent correlations. In the presence of high concentrations of fibrinogen degradation products (FgDP), however, there was very poor agreement between the methods. Two clotting assays (Clauss and Blombäck methods) were negatively interfered by FgDP and the ACL nephelometric method positively. Therefore, the degradation of fibrinogen during thrombolytic therapy with (pro-)urokinase proved much less extensive than expected from previous studies. The well-documented fibrinogen rebound 48-72 hours after thrombolysis was not observed using the ELISA for intact fibrinogen.

INTRODUCTION

Since none of the thrombolytic drugs presently in clinical use is entirely fibrin-specific, increased plasma concentrations of fibrinogen degradation products (FgDP) can occur during thrombolytic therapy. Some of these FgDP can inhibit the activity of thrombin and fibrin polymerization.[1] Because most fibrinogen assays are based upon thrombin-induced clotting, FgDP can interfere in the determination of fibrinogen. Both positive (increase in apparent fibrinogen concentration) and negative (spuriously low fibrinogen) interferences have been reported for different fibrinogen assays.[2-5]

Recently, a quantitative enzyme immunoassay for fibrinogen has been described which, as a result of the use of two specific monoclonal antibodies, only reacts with fibrinogen molecules with at least one intact Aα chain.[6] Since the latter assay is not interfered by FgDP, we compared it with three other fibrinogen assays (the clotting-rate method according to Clauss, the clottable protein method of Blombäck and an automated nephelometric determination of fibrin polymerization in the prothrombin time assay),[7-9] using plasma samples with high FgDP concentrations. They were obtained during thrombolytic therapy or by addition of exogenous FgDP.

MATERIALS AND METHODS

Plasma

Blood was collected from patients with acute myocardial infarction during their thrombolytic treatment with urokinase or pro-urokinase. Blood was drawn into a mixture of citrate and aprotinin (final concentration 250 KIU/mL blood), it was centrifuged immediately at 4 °C, plasma was snap-frozen and stored at -70 °C until analysis. Further samples, not containing aprotinin, were from 77 patients for whom routine haemostatic determinations were requested; 31 of them were on oral anticoagulant therapy.

Methods

The Clauss method was performed manually in a Schnitger & Gross coagulometer (Amelung) and the Blombäck method was carried out as previously described.[5,8] The nephelometric fibrinogen determination was performed in an ACL-300 coagulation analyzer (Instrumentation Laboratory SpA, Milan, Italy) using human placenta thromboplastin (Thromborel-S®; Behringwerke). The ELISA for "intact" fibrinogen was carried out exactly as described.[6] All four fibrinogen assays were calibrated with frozen aliquots of pooled normal plasma with an assigned fibrinogen concentration of 2.71 g/L (from 10 replicate determinations with the Blombäck assay). The plasma concentration of FgDP was determined using an ELISA technique and commercially available reagents (Fibrinostika®-FgDP; Organon Teknika, Boxtel, the Netherlands).[10]

FgDP were prepared by digesting purified, plasminogen-rich fibrinogen (Kabi) with streptokinase in the absence of calcium and stopping the digestion with aprotinin (final concentration 250 KIU/mL) after 3, 20 and 60 min to obtain early, intermediate and late FgDP, respectively.[1,5]

RESULTS

In plasma from 77 patients not receiving thrombolytic treatment, we found excellent correlations between the different methods: Blombäck and Clauss (r = 0.98), ACL and Clauss (r = 0.96), ELISA and Clauss (r = 0.96), ELISA and Blombäck (r = 0.95) and ACL and Blombäck (r = 0.96). The correlation between ELISA and ACL methods was somewhat less (r = 0.87). At fibrinogen concentrations >5 g/L, the ACL gave significantly higher results than the Clauss and Blombäck methods. The results in the ELISA were consistently lower than those of the other methods.

In 53 plasma samples from patients receiving (pro-)urokinase, the correlations between the fibrinogen assays were rather poor, at least during the first day of treatment when there was significant fibrinogenolysis. Figure 1 shows the mean percentage residual fibrinogen as measured with the four assays, along with the FgDP concentrations.

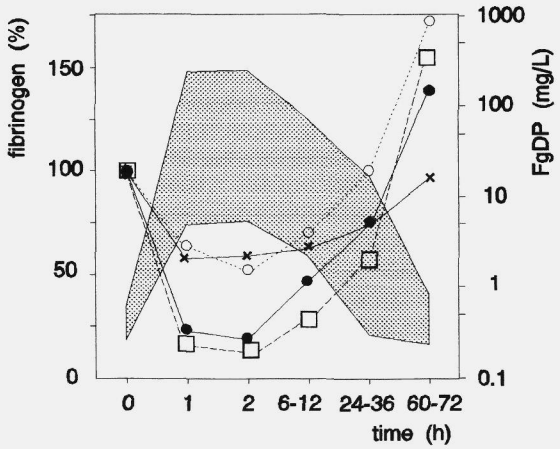

Figure 1 *Mean residual fibrinogen (% of pre-treatment) during three days after infusion of (pro-)urokinase in 9 patients with acute myocardial infarction. Determined using the methods of Blombäck (●), ACL (○) Clauss (□) and ELISA (x). Shaded area represents median ± SD of FgDP concentrations.*

Further, we added fibrinogen digested to various extent to pooled normal plasma (final concentration equivalent to 5.0 g/L of fibrinogen). The 3-min digest, which mainly consisted of FgDP fragment X as determined by SDS-PAGE, significantly increased apparent fibrinogen in the Clauss method (+ 1.5 g/L), the Blombäck method (+ 1.4 g/L) and the ACL method (+ 1.0 g/L), but not appreciably in the ELISA (+ 0.2 g/L). Intermediate digests (20 min; FgDP-Y and D) still increased ACL fibrinogen (+ 1.2 g/L) and had only a small effect on the other methods: Blombäck (+ 0.5 g/L), Clauss (+ 0.2 g/L) and ELISA (- 0.2 g/L). Finally, the late digest (60 min; FgDP-D and E) significantly decreased Clauss fibrinogen (- 0.7 g/L), increased ACL (+ 1.2 g/L) and had no measurable influence on either Blombäck or ELISA methods (both < 0.1 g/L).

DISCUSSION

The ELISA used in this study is highly specific for "intact" fibrinogen. It does not crossreact with the early FgDP fragments X or Y nor with SK-digested plasma.[6] We confirmed these findings by adding SK digests of purified fibrinogen to normal plasma. The three investigated functional assays for fibrinogen performed equally well in plasma from patients not receiving thrombolytic drugs. However, in plasma with high FgDP concentrations (Fig 1), it was confirmed that the Clauss method is sensitive to negative interference by FgDP.[1-3] Also the Blombäck method is interfered with to a considerable extent by high concentrations of FgDP, although clearly less than the Clauss technique. FgDP interference in the ACL fibrinogen determination can be explained by the fact that some FgDP fragments contribute to the light scattering of polymerizing fibrin without supporting the clotting of fibrin.[4,5]

The degradation of fibrinogen during treatment with (pro-)urokinase as assessed by the ELISA appeared to be less extensive than expected from the PRIMI-trial where mean residual fibrinogen (Clauss) was only 14% at 2 hours.[11] Our study indicates a mean of 60% residual intact fibrinogen at 2 hours (Fig 1). These findings are qualitatively similar to those of Seifried *et al*, who described 81% residual intact fibrinogen (ELISA) 3 hours after lytic dosages of tPA and only 51% as assessed by the Clauss method.[12] Apparent overestimation of fibrinogen breakdown can explain that after tPA infusion, only part of the degraded fibrinogen could be recovered as FgDP.[13]

The lack of fibrinogen rebound as assessed with the ELISA method is not easy to explain. It might be suggested that the rebound phenomenon seen with functional assays is a consequence of circulating, slightly proteolyzed fibrinogen or of soluble fibrin which readily clots but remains undetected in the ELISA. This will need further investigation.

In conclusion, the specificity of three functional assays for fibrinogen (Clauss, Blombäck and ACL methods) in plasma samples containing elevated concentrations of FgDP, is poor in comparison with a double-antibody ELISA which only detects "intact" fibrinogen molecules.

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PART 2

HAEMOSTATIC ASSAYS IN THROMBOLYTIC THERAPY

Chapter 4

Comparison of intravenous anisoylated plasminogen streptokinase activator complex and intracoronary streptokinase in acute myocardial infarction

JJRM Bonnier[1], RF Visser[2], HC Klomps[3], JJML Hoffmann, and the Dutch Invasive Reperfusion Study Group

[1] Department of Cardiology, Catharina Hospital, Eindhoven

[2] Department of Cardiology, St.Joseph Hospital, Eindhoven

[3] Department of Cardiology, St. Jans Hospital, Weert

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SUMMARY

Coronary angiography was used to compare the efficacy of anisoylated plasminogen streptokinase activator complex (APSAC) administered intravenously and streptokinase given by intracoronary infusion in inducing reperfusion in patients with a proven acute myocardial infarction. Forty-two patients received 30 U of APSAC intravenously over 5 minutes and 43 patients received 250,000 IU of streptokinase given via intracoronary infusion over 90 minutes, after occlusion of the infarct-related vessel was demonstrated by angiography. Reperfusion was achieved in 23 (64%) of 36 patients (mean time to reperfusion 46 minutes) treated with APSAC and 25 (67%) of 35 patients (mean time to reperfusion 45 minutes) treated with intracoronary streptokinase, who were angiographically evaluated 90 minutes after the start of treatment. Twenty-four hours after treatment, reocclusion had occurred in 1 (5%) of 22 patients in the APSAC group and 3 (13%) of 23 patients in the streptokinase group. No major bleeding was observed in either treatment group despite a similar systemic lytic state that lasted for up to 48 hours. Two patients treated with APSAC died after severe left ventricular failure unrelated to therapy. The results indicate that APSAC given intravenously is as effective as streptokinase given intracoronary in producing thrombolysis in acute myocardial infarction. The major advantages of APSAC are its rapid and convenient administration by a single intravenous injection, the low rate of arterial reocclusion and good patient tolerance.

INTRODUCTION

The role of coronary thrombosis as an initiator of acute myocardial infarction (AMI) has been well documented and is generally accepted.[1] The beneficial effect of intravenous thrombolytic therapy with streptokinase (SK) was demonstrated in a large European multicentre trial,[2] which showed that for successful lysis of coronary artery thrombi patients should receive lytic therapy within 2 to 6 hours after the onset of symptoms of an AMI.

The development of coronary catheterization and angiographic techniques, and the realization that standard intravenous infusion rates of SK may have been insufficient to produce thrombolysis and hence salvage of myocardial tissue, led Rentrop *et al.*[3,4] to treat early AMI with direct intracoronary infusions of SK. These investigators were able to demonstrate rapid arterial reperfusion in more than 70% of the patients and their results have been reviewed in detail by Udall.[5] Recently, Vermeer[6] compared intravenous and intracoronary SK therapy in AMI and concluded that the intracoronary route gave better patency rates than the intravenous route. The principal disadvantage of SK is that it has to be administered over 30 to 60 minutes[7] because of a significant incidence of hypotension and because systemic administration of SK is followed by rapid nonspecific degradation by inactivators, thus reducing the delivery of active material to the site of the thrombus. Also, while the intracoronary route is the more effective,[6] the technique is more complicated and may further delay the start of treatment. Research has been directed to the development of better intravenous thrombolytic agents, with properties that would facilitate early treatment of AMI. The properties of the anisoylated plasminogen streptokinase activator complex (APSAC), with its longer fibrinolytic activity and greater resistance to early inactivation, make it particularly suitable for rapid intravenous injection in patients with AMI.[8]

We performed this open, multicentre randomized trial to compare the reperfusion rates produced by intravenous APSAC with those of intracoronary SK at 90 minutes after dosing, and to assess in both groups the reocclusion rates at 24 hours after dosing, side effects and coagulation and fibrinolytic parameters.

PATIENTS AND METHODS

Patients

Eighty-five patients (71 men and 14 women) ranging in age from 36 to 75 years (mean 55) participated in the study. All patients had been referred to emergency departments within 4 hours of the onset of chest pain caused by AMI. Participating patients had chest pain of at least 30-minute duration and ST-segment elevation of at least 0.1 mV in one or more of the standard leads or at least 0.2 mV in one or more of the V leads in a 12-lead electrocardiogram. The symptoms were not relieved by sublingual glyceryl trinitrate. If a

patient had no contraindications to thrombolytic therapy and informed consent was obtained, a coronary angiography was performed. In case of an occluded infarct-related vessel (TIMI grade 0 or 1), the patients were randomly assigned to treatment with either 30 U of intravenous APSAC or 250,000 IU of intracoronary SK; 42 patients received APSAC and 43 received SK on an open basis. The two groups were similar in sex ratio, age, site of infarction and cardiovascular characteristics (Table 1). The mean time between onset of pain and start of lytic therapy was slightly shorter in the SK group (2.3 hours) compared with the APSAC group (2.5 hours).

Table 1 *Clinical variables on admission.*

therapy	APSAC (n=42)	SK (n=43)	total (n=85)
male / female	35 / 7	36 / 7	71 / 14
age (yrs) mean (range)	55 (37-70)	55 (36-75)	55 (36-75)
anterior infarct	18	21	39
inferior infarct	24	22	46
initial perfusion grade at angiography			
TIMI 0	36	32	68
TIMI 1	6	11	17
time from onset of pain to therapy			
mean (hrs)	2.5	2.3	2.4
≤ 2.5 hrs	19	26	45
> 2.5 hrs	23	17	40

Treatment

APSAC is formulated in a mixture of clinical grade human albumin, D-mannitol and L-lysine (Eminase®, Beecham). It was supplied in vials containing 30 units of APSAC as a sterile off-white powder. Each 30 U dose of APSAC was dissolved in 5 mL of water for injection or physiologic saline and administered intravenously over 5 min. SK was supplied as lyophilized powder in vials containing 250,000 IU of purified SK (Kabikinase®, KabiVitrum). It was administered by intracoronary infusion in physiologic saline at rates

between 2,000 and 5,000 IU/min. Ten patients were given intravenous bolus injections of 10,000 IU after each angiographic procedure. The total doses ranged from 64,000 IU in 45 min (1 patient) to 500,000 IU in 90 min (1 patient). The mean SK dosage was 275,000 IU and the mean time of administration 67 min. All patients received 5,000 IU of heparin before catheterization. No additional heparin was given during the thrombolytic therapy. Anticoagulation therapy with heparin was started 4 h after dosing.

Angiographic evaluation

Angiography of the occluded infarct-related coronary artery was carried out by the Judkins technique before the patients were randomized. After the start of lytic therapy, angiography was performed at 15-min intervals for up to 90 min to assess coronary artery reperfusion. The catheter was removed after 90 min, but the sheath was left in the peripheral artery for 48 h to minimize bleeding from the puncture site. Approximately 24 h after dosing, additional angiograms were performed to assess the degree of reperfusion or to discover if reocclusion had occurred. The degree of perfusion at each angiographic assessment was classified according to the criteria used in the TIMI trial[9] (Table 2). The angiograms were read by an independent cardiologist. In the postdosing assessments, patients with grades 0 or 1 were considered to show no reperfusion of the infarct-related vessels and those with grades 2 or 3 to show reperfusion.

Table 2 *Definitions of perfusion.*[9]

grade 0 (no perfusion)	No antegrade flow beyond the point of occlusion
grade 1 (penetration without perfusion)	Contrast material passes beyond the area of obstruction but "hangs up" and fails to opacify the entire coronary bed distal to the obstruction
grade 2 (partial perfusion)	Contrast material passes across the obstruction and opacifies the coronary bed distal to the obstruction. However, the rate of entry of contrast material into the vessel or its rate of clearance from the distal bed, or both, are perceptibly slower than its entry into or clearance from comparable areas not perfused by the previously occluded vessel
grade 3 (complete reperfusion)	Antegrade flow into the bed distal to the obstruction occurs as promptly as antegrade flow into the bed proximal to the obstruction. Clearance of contrast material from the involved bed is as rapid as clearance from an uninvolved bed in the same vessel or the opposite artery

Coagulation parameters and fibrinolytic assays

Blood samples for analysis in the central coagulation laboratory were taken before lytic therapy and at 90 min, 12, 24 and 48 h after therapy to assess fibrinogen concentration (clotting-rate method of Clauss), euglobulin clot lysis time (ECLT), SK resistance titre and

other parameters of fibrinolysis and coagulation. Blood for the fibrinogen determination was collected in tubes with a citrate anticoagulant, to which 0.7 M ϵ-aminocaproic acid had been added as an inhibitor of *in vitro* fibrinogenolysis. All samples were processed immediately after collection and plasma was frozen at -70 °C for future analysis.

Adverse effects

Two deaths occurred, both in the APSAC group. One patient, a 58-year-old man, died of severe left ventricular failure during catheterization. This patient had had an AMI 5 y earlier. The second, a 61-year-old woman, also died of left ventricular failure 2 days after treatment. This patient had shown reperfusion of an anterior infarct-related vessel after 90 min and early reocclusion had not occurred. There was no evidence that there was a relation between these deaths and the patients' APSAC therapy. Otherwise, only minor adverse events occurred, with two patients developing haematoma at the puncture site after APSAC therapy and another one showing hypotension of 5-min duration that resolved after treatment. None of the patients required blood transfusions.

One patient in the SK group had angiography stopped because of vomiting. Another developed hypertension during infusion (infusion was stopped after 45 min and a total dose of 96,000 IU of SK). The hypotension disappeared after treatment.

Statistical analysis

A two-tailed χ^2 test was used in analyzing the reperfusion data, with $\alpha = 0.05$ and df = 1. The Fisher exact test was used to test for possible differences between the group of patients treated within 2.5 h and those treated 2.5 to 4 h. Values obtained from the Fisher exact test were two-tailed, tested at $\alpha = 0.05$. One-factor analysis of variance was performed on coagulation parameters and the results of fibrinolytic assays.

RESULTS

Angiographic findings

The findings 90 min after the start of lytic therapy were taken as the point for determining whether or not a patient had gained satisfactory reperfusion of the infarct-related vessel, with the angiographic examination 24 h after therapy carried out to determine if the infarct-related vessel was still patent or if rethrombosis had occurred (Tables 3 and 4).

Intravenous APSAC

Thirty-six of the patients who received i.v. APSAC had coronary angiography performed 90 min after treatment. Twenty three (64%) of these patients showed reperfusion; nine (60%) from the group with anterior infarcts and 14 (67%) from the group with inferior infarcts. The mean time to reperfusion was 46 min (Table 3). All 13 patients who did not show reperfusion at 90 min had shown no reperfusion (TIMI grade 0) at the pretreatment angiography. Six (46%) of these patients had anterior infarcts and seven (54%) inferior infarcts. Of the 13 evaluated patients who received APSAC within 2.5 h of the onset of

Table 3 *Results of coronary angiography 90 min after start of treatment.*

therapy		intravenous APSAC (n=42)				intracoronary SK (n=43)			
evaluated		36				37			
not evaluated		6				6			
	site of infarct	reperfusion n	(%)	no reperfusion n	(%)	reperfusion n	(%)	no reperfusion n	(%)
all evaluated patients	anterior	9	(60)	6	(40)	13	(72)	5	(28)
	inferior	14	(67)	7	(33)	12	(63)	7	(37)
	total	23	(64)	13	(36)	25	(68)	12	(32)
initial perfusion grade 0	anterior	7	(54)	6	(46)	8	(62)	5	(38)
	inferior	13	(65)	7	(35)	10	(67)	5	(33)
	total	20	(61)	13	(39)	18	(64)	10	(36)
initial perfusion grade 1	anterior	2	(100)	-		5	(100)	-	
	inferior	1	(100)	-		2	(50)	2	(50)
	total	3	(100)	-		7	(78)	2	(22)

Table 4 *Results of coronary angiography by time of treatment after onset of symptoms.*

therapy	intravenous APSAC (n=42)				intracoronary SK (n=43)			
evaluated	36				37			
not evaluated	6				6			
time to treatment after onset of symptoms	reperfusion n	(%)	no reperfusion n	(%)	reperfusion n	(%)	no reperfusion n	(%)
< 2.5 hours	10	(77)	3	(23)	15	(65)	8	(35)
≥ 2.5 hours	13	(57)	10	(43)	10	(71)	4	(29)
total	23	(64)	13	(36)	25	(68)	12	(32)

symptoms, reperfusion was achieved in ten (77%) patients, compared with 13 (57%) of the 23 patients who were treated between 2.5 and 4 h after the onset of symptoms (difference not significant) (Table 4). One of the six patients not assessed at 90 min had died of severe left ventricular failure during catheterization. The other five patients had shown reperfusion, but coronary angiography was discontinued before 90 min. In two of these

patients a percutaneous transluminal coronary angioplasty was performed at 60 and 75 min after treatment, respectively; in the other three angiography was stopped for various reasons at 45, 60 and 75 min after treatment, respectively.

Intracoronary SK

Coronary angiography was performed 90 min after the start of infusion of SK in 37 of the 43 patients. Twenty-five (68%) patients showed reperfusion of the infarct-related vessels: 13 (72%) from the group with anterior infarcts and 12 (63%) from the group with inferior infarcts (Table 3). The mean time to reperfusion was 45 min. Five (42%) of the 12 patients who did not show reperfusion had anterior infarcts and seven (58%) had inferior infarcts. Ten of these 12 had shown no reperfusion (TIMI grade 0) at pretreatment coronary angiography. Fifteen (65%) of the 23 evaluated patients who received SK within 2.5 h of the onset of symptoms showed reperfusion, compared with ten (71%) of the 14 patients who were treated between 2.5 and 4 h after the onset of symptoms (not significant) (Table 4). Six patients could not be evaluated at 90 min. Three of them showed reperfusion at 60 min but angiography was not continued; one of these patients had angiography discontinued because of vomiting while in the other two it was discontinued because of other reasons. Two of the patients who were not examined at 90 min did not show reperfusion at 60 min and 75 min, respectively, requiring mechanical clearance by guidewire perforation of the infarct-related vessel followed by percutaneous transluminal coronary angioplasty. One patient was withdrawn from the trial as his age (75 y) was outside the range specified in the protocol ($\leq$ 70 years).

Reocclusion

Of the 23 patients in the APSAC group, 22 patients with reperfusion at 90 min were reassessed by coronary arteriography after 24 h. One patient was not reassessed because the procedure failed. Only one (5%) patient with an inferior infarct showed reocclusion (Table 5). Coronary angiography was performed at 24 h in 23 of the 25 patients treated with SK who had shown reperfusion at 90 min. One of the two patients not assessed had undergone percutaneous transluminal coronary angioplasty and the other coronary artery bypass grafting. Three (13%) patients showed reocclusion, two of whom had anterior infarcts and one an inferior infarct (Table 5).

Coagulation and fibrinolytic assays

The changes in the most important parameters, plasma fibrinogen concentration and ECLT, are given in Figure 1. Fibrinogen rapidly decreased in both groups after therapy, reaching minimal concentrations 90 min after the start of therapy. In the APSAC treated patients the mean fibrinogen concentration at this time (0.33 $\pm$ 0.31 g/L) was lower than that in the patients treated with SK (0.50 $\pm$ 0.34 g/L) (analysis of variance not significant). Then, fibrinogen concentrations increased and returned towards pretreatment values between 24 and 48 h after treatment.

The ECLT sharply decreased (*i.e.* total fibrinolytic activity in plasma increased), reaching a minimum 90 min after dosing and thereafter gradually increased in both groups.

The ECLT was significantly lower among patients treated with APSAC, both at 24 hours ($P < 0.02$, analysis of variance) and at 48 hours ($P < 0.005$, analysis of variance) after lytic therapy, reflecting the sustained fibrinolytic activity of APSAC.

Table 5 *Results of coronary angiography 24 h after the start of treatment in patients with reperfusion after 90 min after the start of treatment.*

therapy		intravenous APSAC (n=23)				intracoronary SK (n=25)			
evaluated		22				23			
not evaluated		1				2			
	site of infarct	reocclusion n	(%)	no reocclusion n	(%)	reocclusion n	(%)	no reocclusion n	(%)
all evaluated patients	anterior	-		8	(100)	2	(17)	10	(83)
	inferior	1	(7)	13	(93)	1	(9)	10	(91)
	total	1	(5)	21	(95)	3	(13)	20	(87)

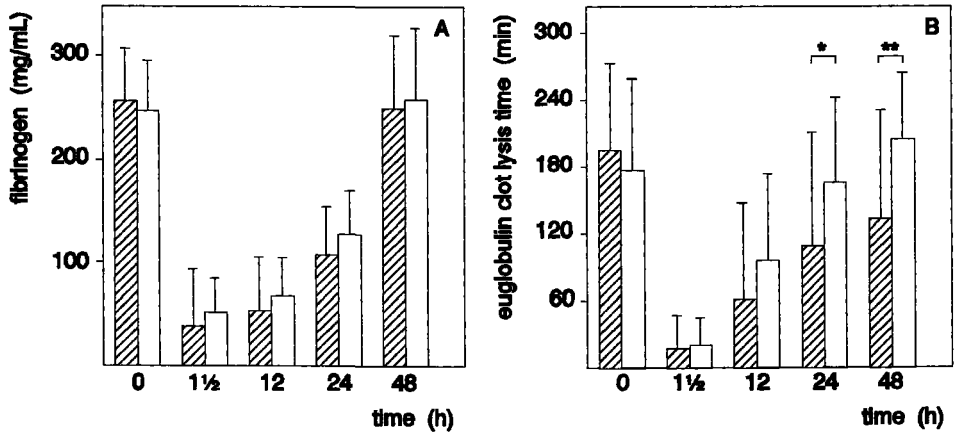

Figure 1 *Course of fibrinogen (A) and euglobulin clot lysis time (B) (mean ± SD) in patients treated with intravenous APSAC (hatched bars) and intracoronary SK (clear bars). * $P < 0.02$; ** $P < 0.005$ by analysis of variance.*

The SK-inhibiting capacity of plasma, measured as SK resistance titre in pretreatment plasma, was identical in both groups (Fig 2): mean 148 U/mL (range 20 to 1,000) in the

APSAC treated patients and mean 148 U/mL (range 13 to 1,000) in the patients who received SK. There was no correlation between pretreatment SK resistance titer and occurrence of reperfusion in either group, as indicated in Figure 2. More detailed results of coagulation and fibrinolysis studies in these patients have been published elsewhere.[10]

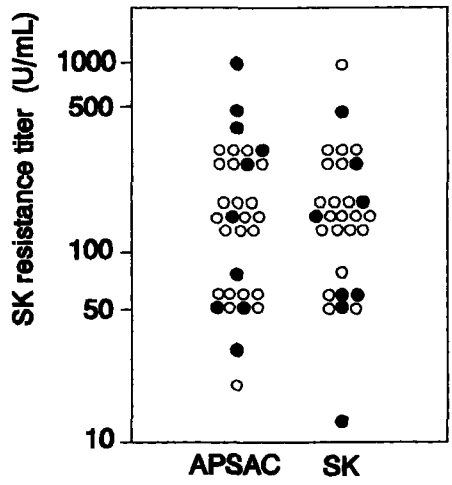

Figure 2 *SK resistance titre in both treatment groups. Note logarithmic ordinate. ○: reperfused; ●: not reperfused (at 90 min).*

DISCUSSION

In nearly 90% of patients, coronary arterial occlusion is likely to be present in the first 4 h after the onset of symptoms of AMI.[1] However, in an individual patient it is not possible to be certain that occlusion and subsequent reperfusion have occurred unless coronary angiography has been performed before and after lytic therapy.

The importance of thrombolytic therapy in the treatment of AMI has been confirmed by recent studies carried out in Europe and the US using intracoronary SK. In the Western Washington Trial[11,12] a reperfusion rate of 69% was achieved after thrombolytic therapy, although there was no significant difference in patient survival 1 y after therapy between these patients and a control group who received non-thrombolytic therapy. These results are comparable with those of the Interuniversity Cardiology Institute of the Netherlands Study Group[6], which found an 85% patency rate in infarct-related vessels after thrombolytic therapy and a significantly higher survival rate 1 y after therapy among the patients who received thrombolytic treatment (90 *vs* 84% in the control group). Simoons *et al.*[13] have noted a similar decrease in mortality rate in-hospital after thrombolytic therapy. Vermeer[6] suggested that the differences in long-term mortality rates

between the Interuniversity Cardiology Institute of the Netherlands Study Group findings and those in the Western Washington Trial are partly attributable to the greater delay between onset of symptoms and start of therapy in the latter trial. The benefits of early thrombolytic therapy have been demonstrated in the GISSI trial[14] (1.5 x 10^6 U SK by intravenous infusion *vs* placebo), when a marked reduction in hospital mortality was seen in patients who received thrombolytic therapy within 4 h of the onset of symptoms, compared with those whose treatment was more delayed. The largest decrease in mortality was seen among patients treated within 1 h of the onset of symptoms.

The present study shows that 30 U of APSAC by intravenous injection is as effective as 250,000 IU of SK by intracoronary infusion in producing reperfusion in occluded coronary arteries within 90 min of treatment. Furthermore, the administration of APSAC as a single intravenous injection in 5 min is easier and quicker than intracoronary infusion of SK in patients with AMI because of the delay caused by catheterization of the patient for the intracoronary procedure.

After SK therapy, fibrinogen decreased to about 22% of pretreatment values, which was close to the values found in other studies on intracoronary SK.[15,16] In the APSAC treated patients there was a marked reduction in fibrinogen to 15% of pretreatment values, a result identical to our earlier findings.[17]

When given in equally effective doses, both drugs systemically activate the fibrinolytic system in most patients (93%) to similar degrees, without causing significant bleeding. The differences in ECLT between the treatment groups indicate that APSAC has a more sustained fibrinolytic activity than SK. In the present study, reocclusion occurred in more patients treated with SK (3 patients, 13%) than in the APSAC group (1 patient, 5%). However, the small number of patients in this trial should be noted.

We believe that APSAC is well suited for rapid thrombolysis of relatively fresh clots, as evidenced by the 77% of the patients in this study treated within 2.5 h of the onset of symptoms of AMI who showed reperfusion 90 min after dosing, as compared to 56% of patients whose treatment was more delayed. In contrast, the difference in reperfusion rate between patients who were treated early and those treated late with SK was far less marked.

APSAC can be given successfully and safely as a single intravenous injection. These are advantages that the other known plasminogen activators, which are either hypotensive at high doses or are cleared too rapidly, are not likely to have.

APSAC apparently reduced the incidence of early reocclusion observed by most investigators after thrombolytic therapy with SK in AMI. This can be explained by APSAC's prolonged fibrinolytic activity, resistance to autodegradation and strong fibrin affinity. However, further trials are needed to confirm that the incidence is, indeed, lower.

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APPENDIX

The members of the Dutch Invasive Reperfusion study group were: JJRM Bonnier, MF El Deeb, MIH El Gamal, HR Michels, JBRM de Swart, JJML Hoffmann, C Werter (Catharina Hospital, Eindhoven); LC Slegers, AH Bosma, MC Huige, RF Visser (St. Joseph Hospital, Eindhoven); HC Klomps, HJAM Penn (St. Jans Hospital, Weert).

Chapter 5

Systemic effects of thrombolytic drugs in acute myocardial infarction: comparison of intravenous APSAC (BRL 26921) and intracoronary streptokinase

JJML Hoffmann, JJRM Bonnier[1] and JBRM de Swart[1]

[1] Department of Cardiology, Catharina Hospital, Eindhoven

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SUMMARY

The systemic effects of intravenous anisoylated plasminogen-streptokinase activator complex (APSAC; BRL 26921; 30 U) and intracoronary streptokinase (250,000 U) were compared in 70 patients with acute myocardial infarction. In 5 patients no signs of a systemic lytic state were observed. In all other patients, significant consumption of coagulation and fibrinolytic factors occurred; fibrinogen concentrations decreased by 85% in the APSAC group and 78% in the streptokinase-treated patients. For plasminogen the decreases were 68% and 64% and for α_2-antiplasmin activity > 95% and 87% (APSAC and streptokinase, respectively). Fibrin(ogen) degradation products were generated to mean concentrations of 739 mg/L and 355 mg/L, respectively. Although there was a trend for the lytic state to be more profound in the APSAC-treated patients, no difference with the streptokinase group was observed with regard to bleeding complications or therapeutic efficacy, which was 64% and 68% respectively for APSAC and streptokinase. The total fibrinolytic activity, measured as euglobulin clot lysis time, sustained longer in the APSAC group, which might be the reason for the low reocclusion rate (4.5%) in comparison with the SK group (13%).

INTRODUCTION

Acyl-enzymes with plasminogen activator potency constitute a new approach in the field of clinical fibrinolysis.[1,2] Their principle of action is that the acyl form of the enzyme, which is catalytically inactive, will be hydrolysed under physiological conditions according to first-order kinetics. By this spontaneous deacylation an active plasminogen activator is generated in the circulation with a controlled rate, so that a long-acting fibrinolytic activity will be obtained.

Of the acyl-enzymes available, the anisoylated plasminogen-streptokinase activator complex (APSAC; formerly BRL 26921) has been best investigated. From prior investigations in limited numbers of patients with acute myocardial infarction (AMI) it appears that APSAC is an effective thrombolytic agent.[3-6] Unfortunately, its relative fibrin-selectivity present in animal systems[1,2,7] has not be found in humans, probably because of interspecies differences in plasminogen activation kinetics[2] or in binding to fibrin. Because of its efficacy and its favourable kinetic properties, which allow i.v. bolus administration, APSAC is considered as a promising thrombolytic drug. Several clinical trials in patients with AMI are at present in progress in order to assess its value in clinical practice.

Up to now there are no studies published in which APSAC was compared with the standard drug for thrombolytic treatment, namely streptokinase (SK). Therefore we studied 85 patients with AMI in an open, randomised multicentre trial comparing SK (250,000 U by intracoronary infusion) and APSAC (30 U by rapid intravenous injection); this dosis of APSAC contains approximately 1 million units of SK, complexed to plasminogen and inactivated by active-site acylation. The aim of this study was to compare both drugs with respect to their effect on major components of the coagulation and fibrinolytic systems and to investigate possible relations between the systemic lytic state and clinical items as reperfusion, reocclusion and bleeding complications.

PATIENTS AND METHODS

Patients

Eighty five patients with AMI entered the study. There were 14 females and 71 males, 39 with anterior and 46 with inferior AMI. The age of the patients ranged from 36 to 70 y, mean age 54.8 y. All patients gave their informed consent for participating in the study, which had been approved by the local ethical committee.

Study Protocol

In the patients fulfilling the inclusion criteria, angiography was performed to document occlusion of one of more coronary vessels. Then, the patients were randomised to either of two treatment regimens: APSAC (30 U; equivalent on a molar basis to about 1 million U of SK) by single intravenous injection during 2-4 min, or SK (250,000 U) by intracoronary infusion for 60 min. Thrombolytic therapy was always started within 4 h from onset of

chest pain. The administration of heparin was allowed as a catheter flush (5000 U by bolus injection) at the insertion of catheters and heparin was mandatory after thrombolytic therapy (1000 U/h as a continuous infusion): in the APSAC-treated patients it started 4 h after starting thrombolytic therapy and in the SK-group as soon as the thrombin time decreased below twice the upper normal limit (usually 3-5 h after starting SK). In all cases heparin infusion was maintained up to 24 h after starting thrombolysis; thereafter oral anticoagulants were given if necessary. Repeat coronary angiograms were made at 15 min intervals during the first 90 min and 24 h after starting therapy for assessing coronary reperfusion and reocclusion, respectively. The study was carried out by members of the Dutch IRS group (see Appendix) in three hospitals in the south-eastern part of the Netherlands.

Methods

Blood for coagulation and fibrinolysis studies was collected in plastic syringes containing 1/10 volume of 0.1 M sodium citrate (for α_2-antiplasmin, euglobulin clot lysis time and streptokinase resistance titer determinations) and 0.1 M sodium citrate plus 0.07 M ϵ-aminocaproic acid for all other determinations. Blood was centrifuged immediately at 4 °C and plasma was snap-frozen and stored at -70 °C until testing. All analyses were carried out in the central coagulation laboratory. From 15 patients no or insufficient plasma was available for testing.

Fibrinogen concentration was measured with the clotting-rate method (Clauss); prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and reptilase time (RT) according to standard methods using commercial reagents. APTTs were measured without and with addition of Polybrene for heparin neutralization.[8] Serum fibrin/fibrinogen degradation products (FDP/fdp) were estimated semi-quantitatively using a latex agglutination assay (Thrombo-Wellcotest®; Wellcome Diagnostics). Plasminogen and α_2-antiplasmin activities were determined using the chromogenic substrate S-2251 (Kabi) and euglobulin clot lysis time (ECLT) according to Nilsson *et al.*[9] Streptokinase resistance titers in plasma were measured using the technique of Deutsch.[10]

A systemic lytic state was defined as a decrease in fibrinogen concentration after thrombolytic therapy by at least 10% of the pretreatment value.[11]

Statistics

For statistical evaluation we used analysis of variance, if indicated after logarithmic transformation of the data. P values < 0.05 were considered significant.

RESULTS

The patient characteristics and clinical findings are summarised in Table 1. The composition of the two treatment groups was closely comparable. The efficacy of APSAC, expressed as reperfusion rate at 90 min was 64%, not significantly different from 68% for SK. The incidence of reocclusion of initially reperfused coronary arteries was 4.5 and 13%,

respectively. Minor bleeding from puncture sites occurred frequently in both groups, but major bleeding did not occur in this study and no patient required blood transfusion.

Table 1 *Patient characteristics and outcome of thrombolytic therapy. Non-evaluable patients have been omitted.*

	APSAC	SK
number of patients	36	34
sex (M : F)	30 : 6	27 : 7
age (mean ± SD) (y)	54.6 ± 8.1	54.8 ± 10.1
systemic lytic state	33/36 (91.7%)	32/34 (94.1%)
reperfusion	23/33	21/32
no reperfusion	10/33	11/32
no systemic lytic state	3/36 (8.1%)	2/34 (5.9%)
reperfusion	0/3	2/2
no reperfusion	3/3	0/2
reperfusion at 90 min	23/36 (63.9%)	23/34 (67.7%)
major bleeding	none	none
reocclusion at 24 h	1/22 (4.5%)	3/23 (13.0%)

In 5 out of the 70 patients of whom adequate samples were available for testing, no signs of a systemic lytic state could be observed (3 APSAC and 2 SK). In both SK-treated patients, successful reperfusion was achieved, in the 3 others, treated with APSAC, it was not. The data of these 5 patients have been excluded from the statistical analysis. The course of the coagulation parameters of the remaining patients is shown in Figure 1. The pretreatment values were identical in both groups and only slight deviations from the normal range were seen in some patients. After therapy, however, considerable changes became apparent: fibrinogen concentration fell to very low values, while the Polybrene-APTT, PT (not shown), thrombin time and reptilase time were strongly prolonged during the early post-treatment period.

The nadir of fibrinogen was approximately 15% (APSAC) and 22% (SK) of the respective pretreatment values, and it was normal again at 48 h; the differences between both groups were never statistically significant. The course of the other coagulation parameters was also comparable between the two groups, except significant differences in PT and reptilase time at 90 min and 12 h post-treatment.

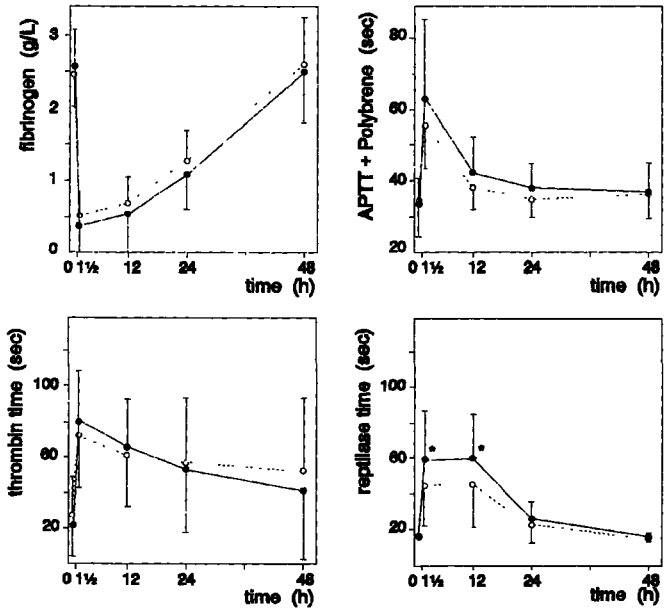

Figure 1 *Course of coagulation parameters during thrombolytic therapy with APSAC (●) and SK (○). Mean ± SD is shown.* * $P < 0.05$.

The course of the fibrinolytic parameters is shown in Figure 2. In the APSAC group, significantly more FDP/fdp were generated during therapy than in the SK group. Plasminogen was consumed to about one third its original concentration in both groups. α_2-Antiplasmin activity became undetectably low in all but one APSAC-treated patients, but in only a part of the patients in the SK-group. The difference in α_2-antiplasmin at 90 min was significant ($P < 0.005$) between both groups. The total fibrinolytic activity in euglobulin fractions sharply increased (*i.e.* ECLT decreased) after therapy and then slowly decreased. The differences in ECLT between both groups at 24 and 48 h were statistically significant ($P < 0.02$ and < 0.005, respectively), indicating that APSAC has a more sustained fibrinolytic effect than SK. The streptokinase-inhibiting activity in plasma, measured as SK-resistance titer (SKRT), was identical in both groups: mean 148 (range 15-1000) U/mL in the APSAC group and mean 148 (range 13-1000) in the SK group. The degree of fibrinogenolysis as a function of pretreatment SKRT is shown in Figure 3: the correlation coefficient (by linear regression) was 0.173 (not significant).

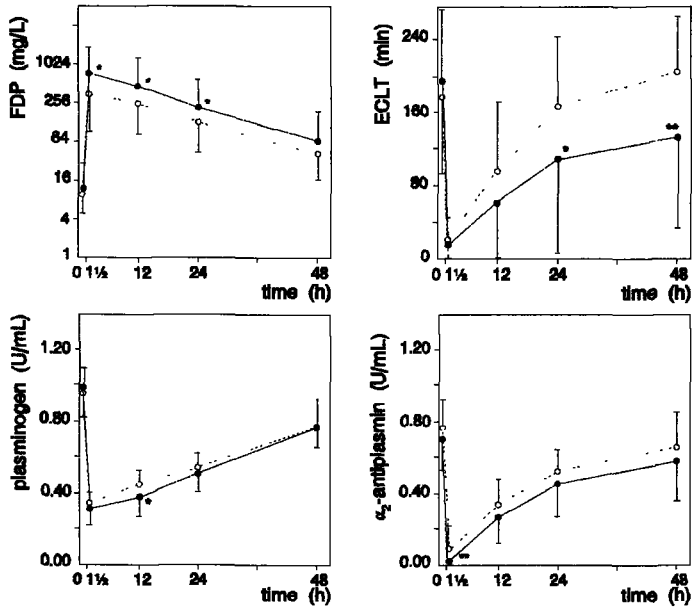

Figure 2 *Course of fibrinolytic parameters during thrombolytic therapy with APSAC (●) and SK (○). Mean ± SD is given. * $P < 0.05$; ** $P < 0.005$.*

DISCUSSION

Although APSAC is much less fibrin-selective in humans[3-6] than in animals,[2,7,12] it might be better suited for clinical usage than SK, due to the possibility of rapid intravenous administration. Then, APSAC should not cause more side effects, mainly systemic lysis, than SK does. These systemic effects of APSAC were the subject of this comparative study with intracoronary SK, which is considered as the standard for thrombolytic treatment of AMI.[13]

A major problem in assessing the coagulation system during thrombolytic therapy is that the fibrinolytic activity will continue *in vitro* after blood collection, thereby giving falsely low results for fibrinogen and other factors and falsely increased values for FDP/fdp.[14] In order to prevent in vitro fibrinogenolysis to go on, we have collected blood in anticoagulant containing ϵ-aminocaproic acid for those determinations, in which this inhibitor had been shown not to interfere (unpublished results). Only α_2-antiplasmin, euglobulin clot lysis time and streptokinase resistance had to be determined in plain anticoagulated plasma.

After SK therapy, fibrinogen decreased to about 22% of pre-treatment values in our patients (Fig 1), a value close to that of other studies on intracoronary SK.[15,16] In the

APSAC group the decrease in fibrinogen was marked, to 15% of baseline values, which is identical with our earlier observations,[5] but clearly lower than in two recent studies on intravenous APSAC.[17,18] This discrepancy is probably caused by the different methods used: we used the Clauss technique, which reflects rapidly coagulable fibrinogen and can be negatively influenced by higher concentrations of FDP. The methods used by the other authors measure total clottable protein and may include some slowly-coagulable fibrinogen fragments. In any case, all available studies with SK and APSAC, administered locally or systemically, indicate that after administration of therapeutically effective doses, fibrinogen will decrease to low concentrations, often below 0.5 g/L, but rapidly increases to normal values within 48 h (Fig 1).[5,6,14-18]

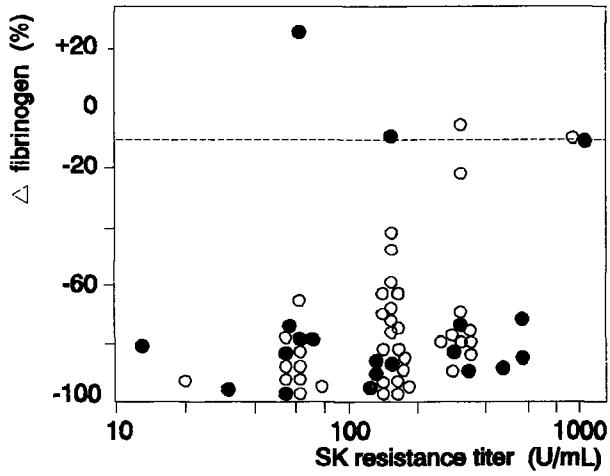

Figure 3 *Change in fibrinogen concentration relative to pre-treatment value, as a function of SK resistance titre (log scale). Dotted line represents definition of systemic lytic state. Reperfusion (○) and no reperfusion (●) is indicated.*

From Figure 1 it is concluded that global coagulation tests provide little, if any additional information during thrombolytic therapy. In order to appreciate the effect of thrombolytic therapy on these global assays without the effect of heparin interfering, we included two heparin-insensitive coagulation assays, the Polybrene-APTT[8] and the reptilase time. Even with these tests the information obtained is limited. As Figure 1 shows, the reptilase time is probably the best global test for monitoring thrombolytic therapy,[6] although fibrinogen concentration alone appears to be sufficient for this purpose.

As a consequence of fibrino(geno)lysis FDP/fdp increased to very high values in both treatment groups (Fig 2). The peak concentration of FDP/fdp in the APSAC group (739

mg/L) corresponds to about 30% of the pretreatment fibrinogen concentration, a value identical with that found in high-dose (1.5 million U) intravenous SK therapy.[14]

In line with expectations, the ECLT decreased extremely after therapy in both groups. In most patients, the clot in the euglobulin preparation dissolved immediately, as has been described by others.[17,18] In the early post-therapy period we found no difference in ECLT between APSAC and SK, but the fibrinolytic activity in the SK group was approximately normal at 24 and 48 h, while in the APSAC group it was still elevated (Fig 2). This sustained fibrinolytic activity might be speculated to be a factor of importance in preventing early reocclusion. A similar suggestion on a relation between the lytic state and reocclusion has been made by others.[4,17]

Plasminogen activities were identical in both groups throughout therapy; the lowest activity, about 30% of the pretreatment value, is in reasonable agreement with data from studies on APSAC[4,18] and SK,[14] but the minimum was not as low as in our earlier study;[5] the reason for this remains unexplained.

Functional α_2-antiplasmin decreased to undetectable concentration in the majority of patients, both in APSAC and SK (Fig 2). This is fully in keeping with the concept that fibrinogenolysis only occurs once the plasmin generated has completely exhausted circulating free α_2-antiplasmin. This process is much better reflected by measurement of α_2-antiplasmin activity than by an immunological assay.[5] Data on α_2-antiplasmin during thrombolytic therapy are rather scarce: Walker *et al.*[3] observed a mean decrease to 28% of the baseline value after APSAC in varying doses, but there was no systemic lysis in all of their patients and if it was, not as profound as in our study (mean decrease in fibrinogen: 44%). The results on α_2-antiplasmin reported by Collen *et al.*[14] in patients treated with high-dose intravenous SK are not easily explained: fibrinogen decreased below 10% of its initial concentration, but α_2-antiplasmin had decreased to only 24% of its pretreatment activity, while one would expect extremely low values with such a degree of fibrinogenolysis. The results shown in Figures 1 and 2 indicate only a few statistically significant, but clinically hardly relevant differences between the systemic effects of APSAC and SK, whereas their clinical efficacy and safety was identical (Table 1). The degree of systemic lysis appeared to be somewhat more profound in the APSAC group, but one should realise that 30 U of APSAC is equivalent to approximately 1 million U of SK.

Table 1 shows a correlation between reperfusion and the occurrence of a systemic lytic state, especially in the case of APSAC, while a truly local effect of SK was observed in two patients who had reperfusion without a systemic lytic state. However, the small number of patients does not allow to draw a firm conclusion on this subject. In agreement with others we found no correlation between presence of systemic lysis and major bleeding complications.[6,18]

The significance of anti-SK antibodies has up to present not been investigated in detail and certainly not so in APSAC therapy. The effect of SK-resistance on systemic lysis is difficult to interpret from the results of Walker *et al.*[3] because of the low number of patients and the wide range of doses applied. Figure 3 indicates that the effects of APSAC may be modulated by anti-SK antibodies: high SK-resistance titers sometimes prevent a

systemic lytic state to occur, but there is no significant correlation between these parameters. Reperfusion appears not to be related at all with SK resistance titers, irrespective whether APSAC or SK was the thrombolytic drug administered.

In conclusion, the results of our present investigation can be summarised as follows: when administered in equally effective doses, intravenous APSAC and intracoronary SK both give rise to systemic activation of the fibrinolytic system in the majority (93%) of the patients, to a closely comparable degree, but without the systemic lysis causing significant bleeding. Since early thrombolytic treatment can substantially reduce early mortality from AMI[19] and time-consuming cardiac catheterisation is obviated, intravenous APSAC seems one of the better thrombolytic drugs currently available.

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APPENDIX

The members of the Dutch Invasive Reperfusion Study (IRS) Group are: JJRM Bonnier (study coordinator), MF El Deeb, MIH El Gamal, HR Michels, JBRM de Swart (Catharina Hospital, Eindhoven); AH Bosma, MC Huige, P Slegers, RF Visser (St. Joseph Hospital, Eindhoven); HC Klomps and HJAM Penn (St. Jans Hospital, Weert); JJML Hoffmann (central coagulation laboratory) and DALG Aelvoet (organisation).

Chapter 6

Significance of antibodies to streptokinase in coronary thrombolytic therapy with streptokinase or APSAC

JJML Hoffmann, R Fears[1], JJRM Bonnier[2], R Standring[1], H Ferres[1] and JBRM de Swart[2]

[1] Beecham Pharmaceuticals, Research Division, Great Burgh, Epsom, England

[2] Department of Cardiology, Catharina Hospital, Eindhoven

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SUMMARY

In 61 patients with acute myocardial infarction, who received thrombolytic therapy with intracoronary streptokinase (250,000 U) or intravenous anisoylated plasminogen-streptokinase complex APSAC (30 U), the concentration of IgG antibodies to streptokinase (IgG anti-SK) was measured using a recently developed micro-radioimmunoassay technique. The distribution of IgG anti-SK in the patients was approximately log-normal with a geometric mean of 0.84 μg/mL. The correlation with the streptokinase resistance titre was statistically significant, but there was a wide scatter of individual results.

In both drug regimens a significant correlation was found ($r = 0.51$ and 0.67) between degree of systemic lytic state, expressed as residual fibrinogen 90 min after dosing, and pre-treatment IgG anti-SK, as well as between post-treatment euglobulin clot lysis time and pre-treatment IgG anti-SK ($P < 0.025$). Comparable, angiographically-defined reperfusion rates were obtained for intravenous APSAC (66%) and intracoronary SK (69%). For both APSAC and SK there was no significant difference in initial IgG anti-SK between the patients who achieved reperfusion and those who did not. However, the rarity of patients with very high antibody concentrations in this study precludes a definitive conclusion on reperfusion success in such patients. There was no correlation between time at which sustained reperfusion was achieved (mean interval 45 min for APSAC and 47 min for streptokinase) and baseline IgG anti-SK concentrations. The incidence of side effects of APSAC and SK was too low to examine in relation with antibody concentrations.

The time course of changes in IgG anti-SK during the first 48 h after therapy was independent of drug regimen and pre-treatment antibody concentration. The maximum decrease in IgG anti-SK concentration after dosing was approximately 1.0 μg/mL. This is less than the amount expected by stoichiometic binding of APSAC with antibody and indicates that APSAC might be poorly bound by antibodies to streptokinase.

Although concurrent studies in vitro demonstrate that IgG anti-SK can bind and at high concentrations even cause some loss of activity for a streptokinase-containing thrombolytic agent, the present clinical results show that the reperfusion response in our population of patients was not significantly affected by IgG anti-SK at therapeutic doses of intravenous APSAC or intracoronary SK, while the systemic lytic state was significantly influenced by anti-SK antibodies.

INTRODUCTION

More than 40 years after the discovery of the fibrinolytic properties of streptokinase (SK), renewed interest in thrombolytic agents has arisen after the demonstration that SK can effectively restore the blood flow in occluded coronary arteries of patients with acute myocardial infarction (AMI).[1] It recently became evident that early thrombolytic treatment with SK significantly reduces the mortality in such patients,[2,3] but reperfusion trials indicate that 20-30% of the patients fail to respond to thrombolytic treatment.[1] The reasons for this failure are largely unknown.

Some authors have explained at least part of the therapeutic failures of SK by high titres of antibodies to SK in those patients.[4,5] It is known that following infections with streptococci and after administration of SK, antibodies to streptococcal proteins are formed,[6,7] which are capable of reacting with SK and neutralizing its potency to activate the fibrinolytic system.[4,8] However, absence of systemic fibrinolytic activity is not necessarily identical with failure to lyse coronary thrombi, although there may be a relationship in some patients.[9-11] So, adequate evidence from clinical studies that antibodies to SK can prevent successful dissolution of thrombi is currently not available in the literature, certainly not concerning thrombolytic therapy in AMI.

Most authors who reported measurements of anti-SK activity in blood or plasma have relied upon the SK resistance test (SKRT) or some modification of it.[6,7,12] However, this functional assay of total SK inhibiting capacity is not specific for antibodies to SK, since it also measures other inhibitors, such as antiplasmin.[7] Moreover, the SKRT cannot be carried out in samples containing SK; therefore, studies on antibodies to SK during thrombolytic therapy have been impossible. Recently a sensitive radioimmunoassay has been described which does not have these disadvantages.[13]

We now present results obtained using this assay in a recent randomized comparative study on intravenously administered anisoylated plasminogen-SK activator complex (APSAC; BRL 26921), a novel thrombolytic agent,[14] and intracoronary SK in AMI.[15,16] Overall, these drug regimens achieved comparable reperfusion rates (64% for APSAC and 68% for SK) and the aim of the present study was to investigate the significance of anti-SK antibodies for the clinical response (reperfusion and haemostatic changes). In addition, data are presented on the course of IgG anti-SK concentrations during the first 48 h after thrombolytic treatment with APSAC or SK. Thus, the study was intended to assess the effects of antibodies on short term events as reperfusion, reocclusion and haemostatic changes and the depletion of antibodies in response to drug, but not to examine the induction of an anamnestic response and other long term effects.

PATIENTS AND METHODS

Patients

The Dutch Invasive Reperfusion Study[15] was an open, randomized, multicentre trial comparing i.v. APSAC and i.c. SK in patients with AMI. Eighty-five patients were recruited, but the present analysis is confined to 61 patients from whom sufficient plasma for measurement of SKRT and IgG anti-SK was available. These 61 patients were in no respect different from the entire study group. There were 50 male and 11 female patients with a mean age of 54.8 y (range 37-70 y). Thirty-two of them were treated with APSAC and 29 with SK. Each patient had given his or her informed consent prior to entering the study, which had been approved by our ethical committee.

Study protocol

After angiographical demonstration of coronary artery occlusion, the patients were randomized to thrombolytic treatment with either APSAC (30 U by an intravenous injection of 5 min) or SK (250,000 U over 60 min by intracoronary infusion). Administration of the thrombolytic drugs was always started within 4 h (mean 2.3 h) from the onset of chest pain. Heparin was given, when necessary, as a catheter flush (5000 U i.v.) and then as a continuous i.v. infusion (1000 U/h), from about 4 to 24 h after initiating thrombolytic therapy.

Vascular patency was assessed by coronary angiography every 15 min for 90 min. All angiograms were read independently by two investigators in a 'blind' fashion and classified on a 4-class scale, according to the definitions used in the TIMI trial.[17] Reperfusion was assumed if at 90 min a sustained change from class 0 or 1 to class 2 or 3 had occurred. Reocclusion was assessed by coronary angiography at 24 h (range 18-30 h) after starting thrombolytic therapy.

Methods

Blood for coagulation studies was collected before and 90 min, 12, 24 and 48 h after therapy.[16] In all patients pre-treatment plasma was used for measuring SKRT and IgG anti-SK; moreover, in 22 patients IgG anti-SK was determined also in post-treatment plasma samples. SKRT was performed as described by Deutsch and Fischer.[7] The assay of IgG anti-SK was carried out according to Moran *et al.*[13] Briefly, 25 μL of appropriately diluted patient plasma was pipetted into the wells of a microtitre plate. Then 25 μL of ^{125}I-labelled SK dilution (approximately 100 ng SK/mL) was added to each well and after 3 h incubation at ambient temperature, the IgG anti-SK/^{125}I-SK immunocomplexes were precipitated by adding 50 μL of a Protein A-Sepharose suspension (50 g/L) in buffer. The plate was continuously agitated for 1 h and centrifuged (10 min, 3000 *g*). The supernatant was removed and the precipitate washed six times. Finally, each well was cut loose from the plate and counted for radioactivity. The results were expressed in μg SK bound per mL plasma (μg/mL).

Inhibition of fibrinolytic activity *in vitro* in a model system was measured using a semi-purified IgG preparation. Sera containing high anti-SK binding titres were obtained from

individuals previously treated with APSAC and IgG-enriched serum fractions were prepared by salt-fractionation. The fibrinolytic activity of a range of concentrations of SK and APSAC was measured by the fibrin plate assay using a range of anti-SK antibody concentrations. The inhibition (%) was calculated by measuring the amount of activator required to produce an equivalent lysis area in the presence of antibody as that produced in the absence. Thus, the inhibition by a range of antibody concentrations over a range of activator concentrations was quantified and a plot made of relative inhibition versus fold excess of antibody (that is, ratio of antibody to antigen).

Statistics

Standard methods were used for the statistical evaluation of the results, as indicated in the results section. In case of non-normal distributions, the data were transformed prior to testing. Significance was assumed at P values < 0.05.

RESULTS

Distribution of IgG anti-SK in patients

The distribution of pre-treatment IgG anti-SK concentrations in our population of patients with AMI is shown in Figure 1. The arithmetic mean was 1.39 μg/mL and the geometric mean, after logarithmic transformation of the data, 0.84 μg/mL. The tenth and ninetieth

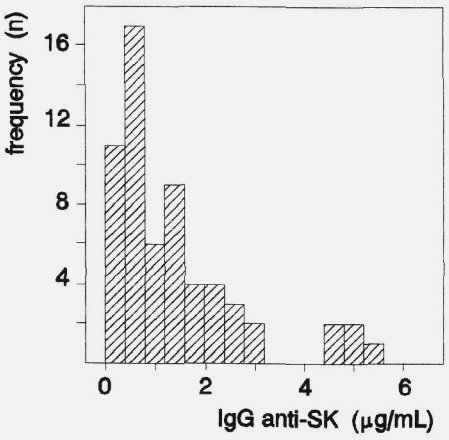

Figure 1 *Histogram of the distribution of IgG anti-SK concentrations in a population of patients with AMI (total n = 61).*

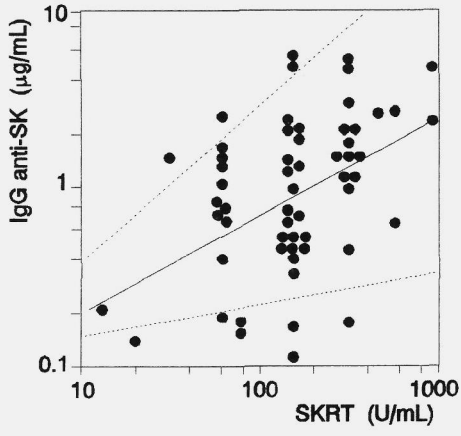

Figure 2 *Relationship between IgG anti-SK and SKRT in pre-treatment plasma. Correlation coefficient r = 0.43 (P < 0.001). Dotted lines represent 95% confidence interval of regression. Note logarithmic scale of both axes.*

percentile values were 0.21 and 2.67 μg/mL, respectively. There was no difference in IgG anti-SK between the patients who were treated with i.v. APSAC and i.c. SK ($P > 0.5$; student t-test).

The concentration of IgG anti-SK appeared to be dependent on age; younger patients had significantly higher concentrations than older ones ($P < 0.02$; linear regression). The highest values of IgG anti-SK (4.7 - 5.5 μg/mL) were found in 5 patients whose mean age was only 44.4 y as compared to a mean age of 55.7 y in the other patients, whose IgG anti-SK concentration was below 3.0 μg/mL ($P < 0.005$; Student t-test). There was no sex-dependent difference apparent in our population.

The relation between IgG anti-SK and SKRT, both in pre-treatment plasma, is demonstrated in Figure 2. When calculated from log-log regression analysis, the coefficient of correlation was statistically significant ($r = 0.43$; $P < 0.001$; 95% confidence limits of r: 0.18-0.61). However, a very wide scatter of data in individual patients was obvious.

Inhibition of fibrinolytic activity in vitro

Results from the fibrin plate assay, comparing the inhibition of fibrinolytic activity of SK and APSAC by partially purified preparations of IgG anti-SK, are shown in Figure 3. At an antibody : antigen ratio of 1.0 (equivalence), SK was rather more inhibited than was APSAC. At excess antibody concentrations, the inhibition of SK and APSAC became similar, but for both agents a large excess of antibody was required to produce an appreciable inhibition of fibrinolytic activity in the fibrin plate.

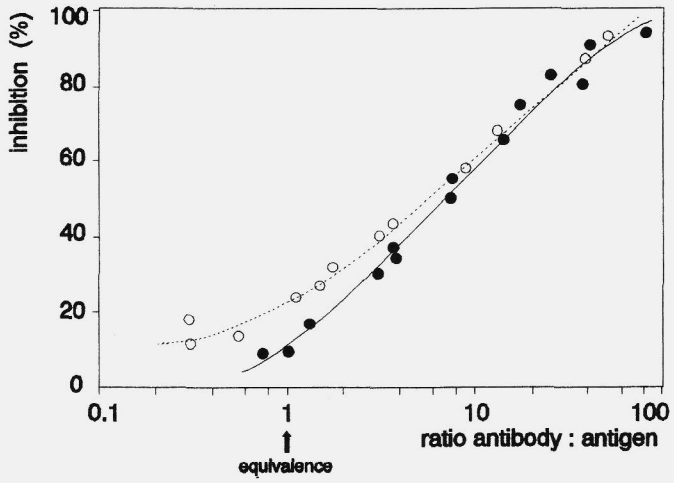

Figure 3 *Effect of IgG antibodies to SK on in vitro fibrinolytic activity of APSAC (●) and SK (○) in a fibrin plate assay.*

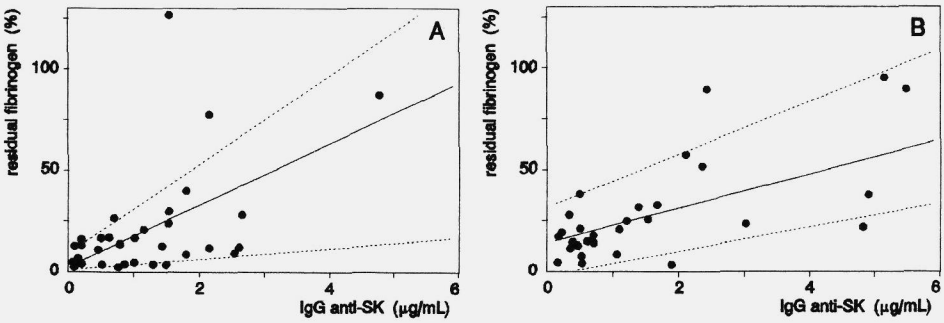

Figure 4 *Degree of systemic lysis, expressed as residual fibrinogen at 90 min after dosing, as a function of pre-treatment IgG anti-SK in patients treated with i.v. APSAC (A) or i.c. SK (B). Coefficients of correlation 0.51 (A) and 0.61 (B), respectively (both P < 0.005). The 95% confidence limits are represented by dotted lines.*

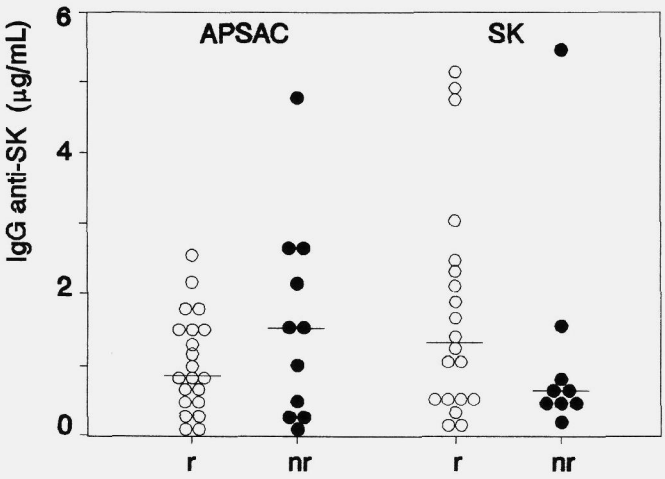

Figure 5 *Distribution of pre-treatment IgG anti-SK concentrations in both patients groups relative to reperfusion success; r: reperfused (○), nr: not reperfused (●) at 90 min. Median values are indicated by horizontal lines.*

IgG anti-SK and the systemic lytic state

Figure 4 shows the degree of systemic lysis, expressed as residual fibrinogen at 90 min, as a function of pre-treatment IgG anti-SK. In both groups the best fit of correlation was described by a linear regression function. The coefficient of correlation was 0.51 ($P < 0.005$; 95% confidence limits: $0.19 < r < 0.73$) in the APSAC group (Fig 4A) and

0.67 ($P < 0.001$; 95% confidence limits: $0.41 < r < 0.83$) in the SK group (Fig 4B), respectively. On the contrary, the relation between SKRT and residual fibrinogen was not significant, neither in the patients treated with i.v. APSAC ($r = 0.10$; n.s.), nor in those treated with i.c. SK ($r = 0.19$; n.s.).

The relationship between pre-treatment IgG anti-SK and euglobulin clot lysis time (ECLT) at 90 min after initiation of therapy, was also assessed. In both therapy regimens, the post-dosing ECLT became very short (10-20 min) in the majority of patients. More prolonged ECLTs were nearly exclusively found amongst those patients with a relatively high pre-treatment antibody concentration (Table 1), although of those five patients with the highest pre-treatment IgG anti-SK, only two (both receiving SK) demonstrated a substantially prolonged ECLT at 90 min.

Table 1 *Euglobulin clot lysis time (ECLT) at 90 min in patients with low and high pre-treatment IgG anti-SK concentrations.*

treatment	n	baseline IgG-anti SK (μg/mL)	ECLT (min) mean ± SEM	P
i.v. APSAC	23	≤ 2.0	10.4 ± 0.4	< 0.025
	5	> 2.0	57.2 ± 35.3	
i.c. SK	20	≤ 2.0	16.0 ± 3.9	< 0.005
	8	> 2.0	82.5 ± 32.2	

* by Wilcoxon signed rank test

IgG anti-SK and reperfusion

The distribution of pre-treatment IgG anti-SK according to whether successful reperfusion was achieved or not, is shown in Figure 5. For both APSAC and SK there was no significant difference (Mann-Whitney U-test) in pre-treatment IgG anti-SK between the groups of patients who reperfused and those who did not. Also, there was no statistically significant correlation between pre-treatment IgG anti-SK concentration and time to sustained reperfusion for those patients who reperfused within 90 min: $r = 0.20$ (n.s.; linear regression analysis) for APSAC and $r = 0.17$ (n.s.) for SK. We also found no significant difference in reperfusion rate between patients with high and low SKRT, irrespective of the type of drug given (data not shown).

In the entire group of patients studied, reocclusion of an initially reperfused coronary artery at 24 h was documented in only four cases; one patient had been treated with i.v. APSAC and three with i.c. SK. In three of these four patients plasma was available for measuring pre-treatment IgG anti-SK; the concentrations were 1.54 μg/mL (APSAC) and 1.10 and 1.39 μg/mL (SK) respectively. Adverse side-effects were mild and of low

incidence:[15] hypotension, vomiting and haematomas and so it was impossible to investigate a relationship with anti-SK concentrations.

IgG anti-SK after therapy

The course of IgG anti-SK during the first two days after treatment was determined in 22 patients, 11 of whom had received APSAC and 11 SK. There was no difference between these two groups with respect to change in IgG anti-SK concentration: in all patients it decreased significantly ($P < 0.001$; paired t-test) immediately after therapy, remained more or less constant up to 24 h and began to rise slowly on the second day. However, within both groups, two entirely different patterns emerged, when the proportional decrease in IgG anti-SK was calculated (Fig 6): in 14 patients (8 APSAC, 6 SK) there was a considerable decrease to only 17% of baseline on the average, while in the remaining patients (3 APSAC, 5 SK) the mean residual IgG anti-SK concentration was still 70% of baseline. This significant difference was closely related to the patients' pre-treatment antibody concentration: the former pattern was confined to patients with IgG anti-SK concentrations < 2.0 μg/mL, while seven of the eight patients exhibiting the latter pattern had IgG anti-SK concentrations above this value.

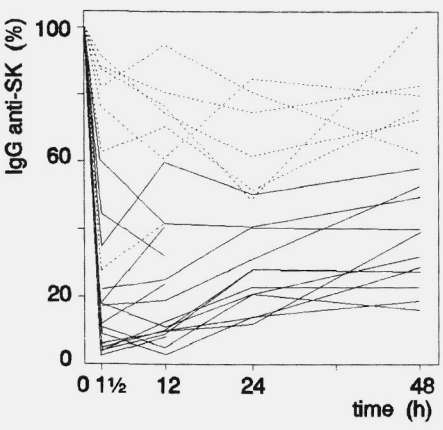

Figure 6 *Course of IgG anti-SK concentrations after thrombolytic therapy in patients with pre-treatment concentrations > 2.0 (dashed line) and ≤ 2.0 μg/mL (solid line). Differences between both groups were significant ($P < 0.001$) at all post-treatment moments.*

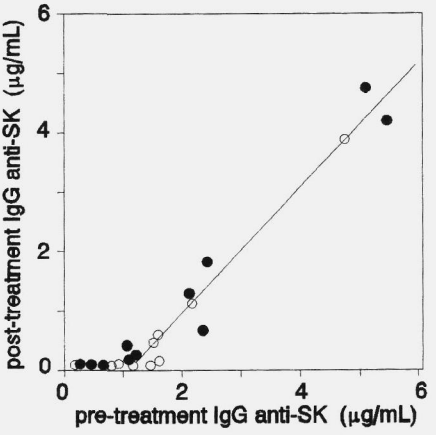

Figure 7 *Post-treatment IgG anti-SK as a function of pre-treatment concentration in patients treated with i.v. APSAC (●) or i.c. SK (○). Slope of regression line 1.03 and intercept on x-axis 1.06 μg/mL ($r = 0.98$).*

Upon plotting the post-treatment IgG anti-SK value as a function of the pre-treatment concentration, as shown in Figure 7, it became clear that up to a baseline value of approximately 1.0 μg/mL nearly all IgG anti-SK had disappeared after therapy, but in patients with initial concentrations above this value, the depletion in IgG anti-SK was

independent of pre-treatment concentration and amounted to approximately 1.0 μg/mL. This was observed for the APSAC-treated patients as well as for those who had received i.c. SK (Fig 7).

DISCUSSION

High reperfusion rates can often be achieved with current regimens of thrombolytic agents, but reasons for failure in approximately one in four patients are not usually clear.[1] Resistance to lysis may be related to location, age and composition of the thrombus, but also, perhaps, to variations in the circulating amounts of substrate and inhibitors. One of the theoretical explanations for the failure of SK-containing thrombolytic drugs is the inactivation by antibodies to streptococcal proteins. Reports on the role of anti-SK in thrombolytic therapy are scarce and are predominantly confined to older studies in patients with deep venous thrombosis or thrombophlebitis, who were treated with intravenous SK.[4,5,8] Moreover, in the few patients with AMI described, the effect of anti-SK on systemic lysis rather than on reperfusion was reported.[4] It is highly questionable whether such results can be extrapolated to intracoronary SK therapy or to reperfusion in AMI. Only two reports on anti-SK in AMI thrombolysis are available. The study by Rothbard and colleagues, describing 15 patients receiving i.c. SK, showed no correlation between anti-SK, measured as SKRT, and presence of the lytic state or between SKRT and reperfusion; however, the patient with the highest SKRT in their study did not show systemic lysis or reperfusion.[11] Lew *et al.* described a single patient who failed to develop a systemic lytic state and did not reperfuse after high dose i.v. SK;[18] this patient had an extremely high anti-SK antibody titre, measured using a semi-quantitative electrophoresis method. Thus, there is no convincing evidence that anti-SK antibodies can prevent coronary reperfusion within a normal population.

In the present report we describe the relevance of antibodies to SK, measured using a new and specific assay for IgG anti-SK, in patients with AMI who were treated with intravenous APSAC or intracoronary SK. Only short-term effects, *e.g.* on reperfusion success and the systemic lytic state, were examined in this study; we did not investigate the longer term effects such as the anamnestic rise in antibodies[6] and the consequences thereof for possible re-treatment.

The distribution of IgG anti-SK concentrations in our patient population was most probably log-normal (Fig 1), which is in agreement with that in healthy volunteers.[13] Since the distribution of SKRT is also known to be log-normal,[8] the statistical correlation of IgG anti-SK and SKRT is not surprising. However, the inconsistencies amongst individuals described by Moran *et al.*[13] were also present in our patients (Fig 2). Hence there is a poor association between SKRT and IgG anti-SK concentration for the individual patient.

The mean concentration of IgG anti-SK in our patients (1.4 μg/mL) is comparable with a mean of 0.9 μg/mL, found in a small group of patients in Scotland,[19] but much lower than in English volunteers, being approximately 3.3 μg/mL.[13] Similar geographical differences have been reported for SKRT and are postulated as resulting from differences

in exposure to streptococcal infections.[4,5] The age-dependence in IgG anti-SK (higher concentrations in younger subjects) has not been reported previously, but might also account for the difference between the mean values in Dutch and Scottish patients compared to English volunteers. In the present study none of the five younger patients exhibiting higher concentrations had a history of recent infection, but one had been treated with intracoronary SK two years previously.

We evaluated the potential influence of IgG anti-SK in a model system *in vitro*. Although the fibrin plate method provides an insight into the effect of antibodies on fibrinolytic activity, it requires an incubation time of 24 h and it is possible that antibody exchange could occur, leading to overestimation of the inhibition. Our results *in vitro* suggest that for the antibody : antigen ratio expected in a normal population of patients receiving thrombolytic therapy for AMI, the inhibition of SK might be rather greater than that of APSAC, but for both drugs the net inhibition is small (Fig 3). For example, for APSAC there might be 10% inhibition of the dose at an IgG anti-SK concentration of 3.3 μg/mL, *i.e.* above the ninetieth percentile in the current study. Although *in vitro* methods can only provide a partial estimate of what might happen *in vivo*, our use of a specific assay for IgG anti-SK allows a more precise prediction of the role of antibody in the response to SK-containing thrombolytic agents than has been possible previously.[20]

When the clinical response to i.v. APSAC and i.c. SK therapy was examined, there was a statistically significant relationship found between pre-treatment IgG anti-SK concentration and residual fibrinogen (at 90 min post-dosing) in both groups (Fig 4). This is consistent with the correlations noted previously between SKRT and systemic lysis in patients receiving intravenous SK,[4,5,8,20] but not after intracoronary SK.[11] It is most likely that increasing concentrations of antibody bind and inactivate increasing proportions of the SK-containing plasminogen activators administered and thus modulate the circulating fibrinolytic activity and the development of the systemic lytic state. These *in vivo* results are comparable with the *in vitro* results described above (Fig 3) and those described by Matsuo *et al.*[21]

It is also interesting to note the correlation between pre-treatment IgG anti-SK and post-dosing ECLT (Table 1). Since post-treatment ECLT is mainly determined by the residual concentrations of fibrinogen and plasminogen as well as by the circulating amount of plasminogen activator, where present, the relationship between IgG anti-SK and ECLT presumably reflects the same process as the correlation between IgG anti-SK and residual fibrinogen. However, it is also conceivable that because APSAC is cleared much slower from the circulation than SK-plasminogen, the contribution of APSAC to the ECLT at 90 min is still considerable and therefore that in the APSAC group the ECLT is less dependent on pre-treatment IgG anti-SK than in the SK group. In addition we feel that caution must be advised in interpreting this correlation because under the acidification conditions involved in measuring ECLT, we cannot exclude the possibility that not all complex is precipitated or the possibility that the complexes may dissociate.

With regard to thrombolytic efficacy, when the patients' response to APSAC and SK was examined there was found to be no significant correlation between pre-treatment anti-SK concentration and reperfusion success. Because patients with high IgG anti-SK were

rare in our population, any possible effect of high antibody concentrations on reperfusion remains to be established using larger groups of patients.

In the patients in whom successful recanalization was achieved there was no correlation found between pre-treatment IgG anti-SK concentration and time at which sustained reperfusion was achieved, neither for SK (mean interval 47 min), nor for APSAC (mean interval 45 min). Furthermore, we found no statistically significant correlation between residual fibrinogen at 90 min and time interval to sustained reperfusion. This finding contrasts with a recent study[22] using 750,000 U of SK i.v., where both high residual fibrinogen concentration and delayed reperfusion were attributed to a high pre-treatment antibody concentration. The difference between our results and this conclusion[22] might be explained by an augmented delivery of the thrombolytic drug to the thrombus or to a suboptimal dose of SK used in the previous study. Furthermore, Lew *et al.* employed only a semi-quantitative estimate of anti-SK concentration and recognised reperfusion by non-angiographic criteria.[22]

The very small number of patients who reoccluded within 24 h does not permit any conclusions to be drawn. However, it would be improbable that IgG anti-SK would affect reocclusion; the three reoccluded patients had only averaged IgG anti-SK concentrations, even in comparison with those who reperfused successfully without reocclusion. Reocclusion beyond 24 h after dosing would be very unlikely, since there will be no longer SK or APSAC in the circulation by that time, in view of the half-life of the drugs.

The course of IgG anti-SK after administration of the thrombolytic drug is compatible with binding of antibody to SK or APSAC and clearance of the complexes, as has been suggested by others.[4,8,18] Somewhat surprisingly, it was found that the clearance of antibody-SK and antibody-APSAC complexes apparently became saturated at approximately 1.0 μg/mL IgG anti-SK (Fig 7) and thus gave rise to two different patterns of antibody depletion (Fig 6), depending on the pre-treatment IgG anti-SK concentration. In theory, the dose of SK applied in the present study (250,000 U) should deplete IgG anti-SK by about 0.8 μg/mL, assuming a normal plasma volume.[13] Thus, in the SK-treated patients, the results are compatible with the theoretical calculation. On the contrary, the quantity of SK complexed in the dose of APSAC applied, would theoretically be expected to deplete IgG anti-SK by more than 3.0 μg/mL, since 30 U of APSAC is equivalent to approximately 1 million U of SK on a molecular basis. In fact, APSAC depleted IgG anti-SK to the same extent as occurred in the SK group, thus to only 25% of the theoretical value. These data on decrease in IgG anti-SK after APSAC are similar to those from the only other report available on this subject.[19] In that study, six patients were treated with i.v. APSAC (30 U) and from the data an average depletion in IgG anti-SK of 0.80 μg/mL can be derived, similar to the mean in our APSAC group (0.90 $\pm$ 0.42 μg/mL). Only partial disappearance of IgG anti-SK after APSAC therapy might be explained by circulation of antigen-antibody complexes after dosing if the clearance process was saturated. However, we searched for such complexes in plasma taken 90 min after dosing, using an immunoblotting technique, but without success (R. Standring, unpublished result). The steric conformation of the APSAC molecule may also be important. For example the

plasminogen moiety or the anisoyl-group might render the SK part less accessible for antibody binding than in the unmodified SK molecule.

In conclusion, although the degree of systemic lysis (residual fibrinogen) was shown to correlate with pre-treatment IgG anti-SK, most patients could still be defined as exhibiting a systemic lytic state after treatment with SK or APSAC. Whether or not APSAC is relatively protected from binding to and inhibition by IgG anti-SK, we conclude from the present results that such antibodies, at least in the range studied here, have little effect on the reperfusion success in a normal population of patients with AMI who are treated with therapeutic doses of intravenous APSAC or intracoronary SK.

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Chapter 7

Histidine-rich glycoprotein in thrombolytic therapy: has it clinical relevance ?

JJML Hoffmann and JJRM Bonnier[1]

[1] Department of Cardiology, Catharina Hospital, Eindhoven

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ABSTRACT

The plasma concentrations of histidine-rich glycoprotein and plasminogen were measured and those of free plasminogen calculated in 34 patients with acute myocardial infarction treated with thrombolytic drugs. We investigated whether the plasma concentrations of histidine-rich glycoprotein and free plasminogen were associated with clinical parameters such as the occurrence and time of successful reperfusion, the incidence of reocclusion and the presence and extent of the systemic lytic state. The mean (± SD) pre-treatment concentration of histidine-rich glycoprotein was 1.08 ± 0.24 U/mL, which is almost identical with the normal reference values. After thrombolysis, the mean concentration decreased slightly. We found no support for a role of these proteins in achieving therapeutic success and only a faint suggestion that histidine-rich glycoprotein might contribute to reocclusion. Surprisingly, the non- or only limited occurrence of a systemic lytic state appeared to be associated with a low concentration of histidine-rich glycoprotein, instead of the high concentration expected on theoretical grounds. It is therefore unlikely that histidine-rich glycoprotein is of clinical relevance for thrombolytic therapy of acute myocardial infarction.

INTRODUCTION

Histidine-rich glycoprotein (HRG) is an α_2-globulin which has been shown to bind to plasminogen,[1,2] heparin,[3,4] fibrinogen and fibrin[5], at least when purified components are investigated *in vitro*. The biological significance of these binding properties is not yet known. In theory, the reversible binding of HRG to plasminogen in plasma might have important consequences, since it diminishes the availability of free plasminogen (fPlg) and thus inhibits clot lysis.[1] A number of patients with thrombophilia have been described with a reduction in functional plasminogen concentration (types I or II plasminogen deficiency), although the association between plasminogen deficiency and thrombosis is far from complete.[6] In addition, several cases of thrombophilia associated with a familial elevation of plasma HRG concentration have been described,[7,8] but a causal relationship could not be proven.[8] Thus, there are clinical indications that HRG acts as an inhibitor of natural fibrinolysis.

In therapeutic fibrinolysis plasminogen also plays a key role, since the desired effect of thrombolytic therapy, namely the dissolution of occlusive thrombi, depends on the activation of fibrin-bound and perhaps also of fPlg by the thrombolytic drug. Analogous to the defective endogenous plasminogen activation occurring in some types of thrombophilia,[6-8] it is quite possible that HRG is a determinant of the clinical result of thrombolytic therapy. Firstly, in the presence of high HRG concentrations there might be so little fPlg left for incorporation into the thrombus that drug-induced lysis could be retarded or even prevented. Secondly, HRG might limit the amount of systemic plasminogen available to activation once the thrombolytic drug has been administered. The scanty literature on HRG and thrombolytic therapy[9,10] does not address its possible clinical relevance. We therefore designed the present study to investigate whether the occurrence and time of reperfusion, the incidence of reocclusion and the extent of systemic fibrinogenolysis were correlated with pre-treatment concentrations of HRG and fPlg. Our study was part of a multicentre trial comparing intracoronary streptokinase (SK) and intravenous anisoylated plasminogen streptokinase activator complex (APSAC) in patients with acute myocardial infarctions (AMI).[11,12]

PATIENTS AND METHODS

Patients

We studied 34 patients with AMI, who had angiographically proven occlusion of their infarct-related coronary arteries and were treated with thrombolytic therapy within the framework of the Dutch Invasive Reperfusion Study.[11] This was an open, randomized, multicentre trial comparing intravenous APSAC (30 U by a 5-min injection) with intracoronary SK (250,000 U over 60 min as an infusion). Of these 34 patients, 17 (four

females, 13 males; mean age 52.3 y) received APSAC and the other 17 (three females, 14 males; mean age 55.5 y) were treated with SK.

Coronary angiography was used to assess coronary patency every 15 min for 90 min after starting the lytic therapy and the degree of perfusion was graded according to the TIMI-criteria.[13] A sustained grade 2 or 3 perfusion at 90 min was considered as successful reperfusion. After 24 ± 6 hours, reocclusion was assessed also by coronary angiography.

The study, including blood collection for laboratory analysis,[12] had been approved by the ethical committees of the participating hospitals.

Methods

Blood was collected into 1/10 volume of 0.1 M trisodium citrate as described previously,[12] before and 90 min, 12, 24 and 48 h after the start of thrombolytic treatment. Plasma was stored at -70 °C until analysis.

HRG and plasminogen were measured in duplicate using rocket immuno-electrophoresis according to Laurell,[14] with specific rabbit antisera to human HRG and plasminogen (Hoechst-Behringwerke, Marburg, FRG). Concentrations were expressed relative to a pooled plasma obtained from 40 healthy individuals, to which a potency of 1.00 U/mL was arbitrarily assigned. The inter-assay precision (coefficient of variation) of these assays was about 6%. Plasminogen activity (chromogenic substrate assay) and fibrinogen (clotting-rate method) were determined as described elsewhere.[12]

The concentration of free, non-HRG bound, plasminogen (fPlg) was calculated from the antigen concentrations of HRG and plasminogen, using a dissociation constant of the HRG-plasminogen complex of 0.55 μM[15] and regarding 1.00 U/mL HRG as being equal to 1.7 μM and 1.00 U/mL plasminogen to 1.8 μM.[1,16]

Standard statistical methods (Student t-test, analysis of variance, Wilcoxon test and rank correlation test) were used as indicated below. P values < 0.05 were considered significant.

RESULTS

HRG and plasminogen in patients

The patients who were given intracoronary SK were not significantly different from those treated with APSAC with respect to age, sex, duration of chest pain, location of infarction and laboratory parameters, including HRG, plasminogen and fibrinogen. Therefore, the results of both groups were pooled for the present study.

The mean pre-treatment concentration of HRG (± SD) was 1.08 ± 0.24 U/mL (range 0.68 -1.52 U/mL), which is identical with the reference values for HRG in our laboratory: 1.07 ± 0.25 U/mL (n = 55).

After thrombolytic therapy, the mean HRG concentration remained unchanged for 24 h and then slightly, but not significantly, decreased to approximately 87% of pre-treatment after 48 h (Fig 1). Again there was no difference between the SK and APSAC groups (Student t-test).

Plasminogen antigen before treatment was 0.99 ± 0.11 U/mL (range 0.80-1.29 U/mL) and the calculated mean fPlg concentration 0.33 ± 0.08 (range 0.21-0.51) U/mL. Thus, on average, 66% of total plasminogen antigen was HRG-bound and 34% was free. Plasminogen activity was 0.97 ± 0.13 U/mL before treatment and correlated closely with the antigen concentration (r = 0.94); after treatment the correlation became very poor. There was no correlation between plasminogen activity and calculated fPlg (r = 0.03; linear regression analysis) either before or after therapy.

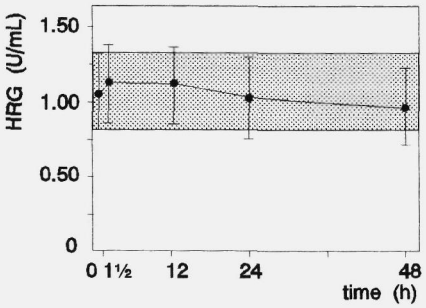

Figure 1 *HRG concentrations before and at several times after thrombolytic therapy. Mean ± SD is given. Shaded area indicates reference range.*

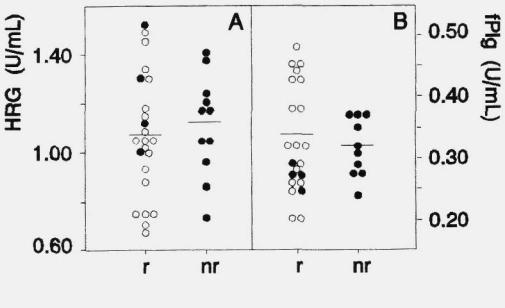

Figure 2 *Distribution of HRG (A) and fPlg (B) in patients who successfully reperfused (r) and who did not (nr). Sustained reperfusion at 24h angiography (○); no reperfusion at 90 min or reocclusion after 24h (●).*

HRG and reperfusion

Pre-treatment concentrations of HRG and fPlg were compared between the patients who achieved angiographically proven reperfusion within 90 min (n = 23) and those in whom no reperfusion was seen (n = 11). Using analysis of variance, no significant differences were found in HRG, fPlg, plasminogen antigen and fibrinogen ($P > 0.8$), as shown in Figure 2. In addition, the moment at which reperfusion was documented was not correlated with HRG, fPlg or fibrinogen (rank correlation test, $r < 0.32$, $P > 0.2$).

HRG and reocclusion

There were four patients in the study in whom reocclusion of an initially reperfused coronary artery was demonstrated after 24 h; one had been treated with APSAC and the three others with SK.[11] The mean pre-treatment HRG in these four patients was higher than in those in whom no reocclusion occurred (1.24 ± 0.23 *vs* 1.03 ± 0.25 U/mL; Fig 2) but this difference did not reach statistical significance ($0.05 < P < 0.1$, Wilcoxon test). Similarly, the difference in fPlg between both groups was not significant (0.28 ± 0.02 and 0.35 ± 0.09 U/mL, respectively; $P < 0.15$, Wilcoxon test). The four patients with reocclusion had higher post-thrombolysis HRG values than the other patients (for example 1.18 ± 0.22 *vs* 1.05 ± 0.30 U/mL at 12 h), but this was not significant ($P = 0.14$).

HRG and systemic fibrinogenolysis

Systemic fibrinogenolysis was assessed by the residual fibrinogen concentration, relative to each individual's pre-treatment value. There were seven patients with no or only limited fibrinogenolysis (mean residual fibrinogen 83 ± 24%), while fibrinogenolysis was more extensive in the other 27 patients (residual fibrinogen 16 ± 9%). The patients of the former subgroup had significantly lower pre-treatment HRG concentrations than the latter ones: 0.90 ± 0.25 and 1.13 ± 0.21 U/mL ($P < 0.025$ by analysis of variance; Fig 3). Likewise, the difference in fPlg between both subgroup was also significant: 0.39 ± 0.09 and 0.32 ± 0.08 U/mL, respectively ($P = 0.05$; Fig 3).

We found no further correlations between HRG or fPlg and other parameters reflecting systemic fibrinolytic activity such as euglobulin clot lysis time, α_2-antiplasmin consumption or total FDP generation (data not shown).

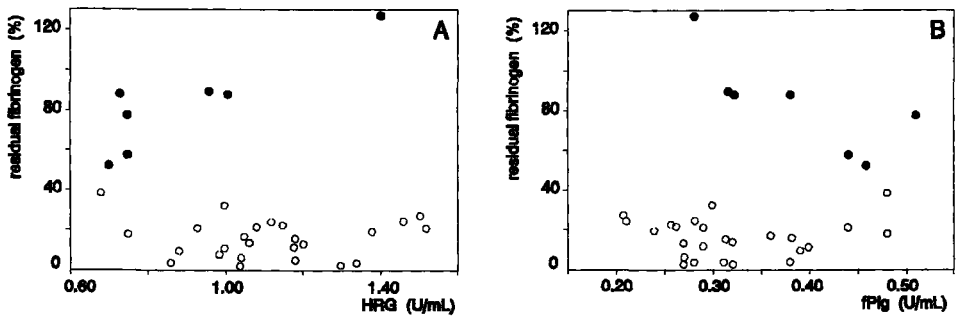

Figure 3 *Systemic fibrinogenolysis, expressed as percentage residual fibrinogen (at 90 min after dosing) as a function of pre-treatment HRG (A) and fPlg (B). Extensive (○) and no or limited fibrinogenolysis (●) is indicated.*

DISCUSSION

Although HRG might theoretically be important for the regulation of plasminogen activation[1-5] by reducing the incorporation of plasminogen into a developing thrombus and by limiting the amount of plasminogen available for activation by thrombolytic drugs,[1,2,5] there are no reports on the clinical relevance of HRG for thrombolytic therapy.

The HRG concentration in our patients with AMI was similar to that in healthy controls (Fig 1), and the reference range of HRG in our laboratory compared very well with published values.[7,8,16] Since the pre-treatment HRG was determined in samples collected in the first hours after the onset of AMI symptoms (within 4 h; mean 2.4 h[11]), it was probably too early to observe the negative acute phase reaction suggested by Jespersen *et al.*[16] The slight decrease in HRG after thrombolytic therapy, originally described by Lijnen *et al.* in three patients,[9] could now be confirmed in a much larger group (Fig 1). The question whether HRG is proteolytically degraded by plasmin during thrombolysis, which is still controversial in the literature,[9,10] was not within the scope of our present

study, which was only oriented towards the clinical relevance. However, if HRG was indeed degraded by plasmin, it is not unreasonable to suppose that its binding properties to plasminogen would be different from intact HRG or would even be lost. To date, there is no literature available on this subject. For this reason we have used pre-treatment HRG concentrations throughout our present study.

In our opinion, the reasonably good correlation between plasminogen activity and antigen, together with the complete absence of a relation between plasminogen activity and calculated fPlg, suggests that HRG-bound plasminogen still retains its full enzymatic activity towards a small chromogenic peptide substrate after activation *in vitro*. Thus the fPlg activity is not reflected by such an assay; this finding has not been reported previously and merits further investigation.

A number of highly intriguing questions concerning thrombolytic therapy of AMI remain unsolved, despite the extensive research in this field. Why are the coronary thrombi not lysed in every patient treated; why do some initially recanalized arteries reocclude; and why do some patients not develop systemic fibrinogenolysis? The present study in AMI patients treated with SK or APSAC fails to give the slightest hint that HRG would be of any relevance for achieving successful reperfusion (Fig 2) or for the time to reperfusion. However, our results do not exclude the possibility that reocclusion within 24 h could be associated with high HRG concentrations and therefore low concentrations of fPlg; this finding would not have been essentially different if the HRG concentrations after 12 or 24 h had been used instead of pre-treatment values. The small number of patients with reocclusion precludes statistically valid conclusions and this therefore requires further investigation.

The finding of a statistical correlation between high HRG and extensive fibrinogenolysis (Fig 3) is quite unexpected and as yet unexplained; it would either reject the hypothesis of high HRG causing decreased plasminogen activation or indicate that a different mechanism is operative.

The conclusion from the present study is that there is no support for the hypothesis that HRG is important for achieving successful reperfusion. It remains possible that HRG is relevant for the occurrence of reocclusion, but additional evidence is certainly necessary. The statistically significant association between high HRG concentrations and profound systemic fibrinogenolysis was a surprising finding for which no explanation can be offered. Finally, we feel tempted to conclude that HRG is not relevant for the clinical outcome of thrombolytic therapy of AMI with SK or APSAC, as we have previously demonstrated for anti-SK antibodies.[17]

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Chapter 8

Plasma markers of thrombin activity during coronary thrombolytic therapy with saruplase or urokinase: no prediction of reinfarction

JJML Hoffmann, HR Michels[1], J Windeler[2] and H. Barth[3]

[1] Department of Cardiology, Catharina Hospital, Eindhoven

[2] Department of Medical Statistics, Ruhr University, Bochum, Germany

[3] Department of Clinical Research, Grünenthal GmbH, Aachen, Germany

submitted for publication

SUMMARY

One of the principal problems associated with thrombolytic therapy is rethrombosis of vessels which were initially patent. Although platelets as well as coagulation activation have been implicated in rethrombosis, the specific mechanisms leading to this complication are largely unclear. Available evidence is limited to smaller studies using the current thrombolytic agents. Here we report on the multicentre SUTAMI trial comparing recombinant saruplase and urokinase in 543 patients with acute myocardial infarction, in 33 of whom early reinfarction was documented. Plasma from these patients and 33 matched patients without reinfarction was investigated for thrombin-antithrombin III complex and prothrombin activation fragments 1+2 as markers of activated coagulation, during 72 hours after starting the lytic therapy.

The median concentrations of both parameters significantly increased 3-6 fold after the therapy, indicating extensive activation of the coagulation system. Following heparin administration, both thrombin-antithrombin III complex and prothrombin fragments 1+2 returned towards normal in most patients. At none of the time points studied there was a significant difference in these coagulation parameters between the patients with and those without reinfarction. In contrast to other authors, thrombin-antithrombin III complex concentration was no reliable indicator of reinfarction in the patients studied and neither was the concentration of prothrombin activation fragments 1+2.

INTRODUCTION

Early thrombolytic treatment of coronary thrombosis has been proven to significantly reduce the mortality of patients with acute myocardial infarction (AMI).[1-3] Rapid and sustained restoration of the patency of the infarct-related arteries is necessary for salvaging viable myocardial tissue, but rethrombosis of initially patent vessels can occur in up to 30% of the patients. The mechanisms leading to reocclusion have not yet been fully elucidated, but there is growing evidence that activation of the coagulation system and of platelets might cause ongoing thrombosis, which exceeds the fibrinolytic activity induced with the thrombolytic agents.[4] This is supported by clinical and experimental observations that adjunctive therapy with anticoagulants or anti-platelet drugs accelerates or enhances thrombolysis.[4,5] Moreover, increased thrombin activity has been shown during thrombolytic therapy using sensitive techniques.[6-9]

Coagulation activation can reliably be demonstrated by determining one of the activation-specific substances in plasma, for which accurate and sensitive assays are available. Fibrinopeptide-A (FpA) is a small fibrinogen fragment, which is released when thrombin converts fibrinogen into fibrin. Thrombin-antithrombin III complex (TAT) is rapidly formed in the circulation between active thrombin and its natural inhibitor antithrombin III. Prothrombin fragments 1 and 2 (F_{1+2}) are split off by factor Xa when it activates prothrombin. Because all these molecules are rapidly eliminated from the circulation, they are well suited as sensitive markers of *in vivo* activation of the coagulation system.

One group of investigators has reported that an elevated plasma concentration of TAT during thrombolytic therapy was highly predictive of coronary reocclusion within 24-36 h.[9] These results are based on observations in only 13 patients with reocclusion and they have not yet been confirmed by others. This prompted us to perform the present study, which is part of a clinical trial comparing the efficacy and safety of saruplase (recombinant pro-urokinase) and urokinase in patients with AMI.

The aim of our investigation was to document the course of TAT and F_{1+2} as markers of *in vivo* thrombin activity during thrombolytic therapy with saruplase or urokinase, in patients who had clinical signs of reinfarction and in an equal number of matched patients, who experienced an uneventful clinical course. In addition, we examined whether TAT and F_{1+2} could reliably be used as indicators of reinfarction after thrombolysis in this patient group.

PATIENTS AND METHODS

Patients and treatment

The patients were selected from a multicentre, randomized, double-blind trial comparing saruplase (recombinant pro-urokinase) and urokinase in the treatment of acute myocardial

infarction (SUTAMI).[12] Patients between 20 and 75 y presenting within 6 h after the onset of chest pain indicative of AMI, were included into this trial if they met the usual criteria. They were randomized to treatment with either saruplase or urokinase. Saruplase was the non-glycosylated, single-chain form of urokinase-type plasminogen activator (pro-urokinase), produced by recombinant DNA technology in *E.coli* (Grünenthal GmbH, Aachen, FRG) and urokinase was purified from human urine (Behringwerke AG, Marburg, FRG). Saruplase was given as a 20 mg bolus, followed by 60 mg as an intravenous infusion over 1 h. Urokinase was administered by intravenous infusion, first 1.5 MU in 5 min and then 1.5 MU over 1 h. Each regimen included a placebo medication for the other arm of the study (double-dummy technique). In both groups, heparin was started 30 min after completion of the active drug infusion (thus, 90 min after starting the thrombolytic therapy) by continuous infusion of 15 U/kg/h and the dose was adapted according to the activated partial thromboplastin time. Heparin was continued until the coronary angiography, 24-72 h after start of the therapy. In-hospital reinfarction was assessed using clinical and electrocardiographic signs before discharge, 7-14 days after admission.

Blood collection

Before administering the thrombolytic medication and after 1 h (end of infusion), 2 h (after 30 min heparin infusion), 6-12 h (during heparin), 24-36 h (prior to angiography) and 60-72 h (after angiography), blood was taken for haemostatic assays. At each time point, two 9 mL samples were collected in plastic syringes using a 19 gauge needle. One sample was drawn into 1 mL citrate (0.01 M final concentration) and the other one into 1 mL citrate supplemented with aprotinin (250 KIU/mL blood) in order to instantly inhibit *in vitro* plasmin activity. The samples were immediately placed in melting ice and centrifuged as soon as possible for at least 10 min at 2000 *g*. The plasma was pipetted off, divided into three aliquots and frozen at -20 °C until transfer to the central laboratory. There, the samples were stored at -70 °C; each sample was thawed only once.

Assays

TAT and F_{1+2} were determined in duplicate in aprotinin-citrate plasma, using commercially obtained ELISA kits (Enzygnost®; Behringwerke AG, Marburg, FRG), as described before.[10,11] In our hands, the lower limit of detection of the TAT assay was 0.7 μg/L, the normal range < 4.0 μg/L and the inter-assay coefficient of variation (CV) 10.0 % at 9.1 μg/L (n = 47). For F_{1+2} these data were: detection limit 0.02 nmol/L, normal range 0.4 - 1.2 nmol/L and CV 10.9% (mean 0.45 nmol/L; n = 20).

Statistics

For each of the patients with reinfarction, a matching patient without reinfarction was selected on the basis of successively: treatment group, sex, infarct localization, body weight and age. The matching for the first four items was complete; the median difference in age within a pair was 3 years (range 0-21 years).

Median and interquartile ranges of TAT and F_{1+2} values at each of the six time points were calculated. Groups of patients with and without reinfarction were compared using analysis of variance in a repeated measures model; the analysis was restricted to patients with complete data ($n \geq 54$). The values of F_{1+2} were log-transformed prior to analysis. *P* values < 0.05 were considered significant.

After it had been verified that the type of thrombolytic treatment had no significant effect on the TAT and F_{1+2} results, we pooled the data in order to form two groups with and without reinfarction, respectively.

RESULTS

TAT during thrombolysis

The median concentration of TAT and the 25-75% range at each of the six time points is given in Figure 1 for the group of patients with reinfarction as well as those without. At admission, the TAT concentration was elevated in 78% (45/58) of the patients. After 1 h, just after completing the thrombolytic infusion, TAT had further increased in 40 of the 55 evaluable patients and in the 15 others it was slightly lower than before thrombolysis, but above the normal range in all patients. At 2 h, when the patients were on heparin for 30 min, TAT decreased significantly in the majority of patients (39/54) and at 6-12 h it had decreased further, but was still elevated in most patients (43/56). The decrease continued in most patients during the infusion of heparin. However, there were 19/53 patients in whom TAT increased in either of the two samples between 6 and 36 h and one single

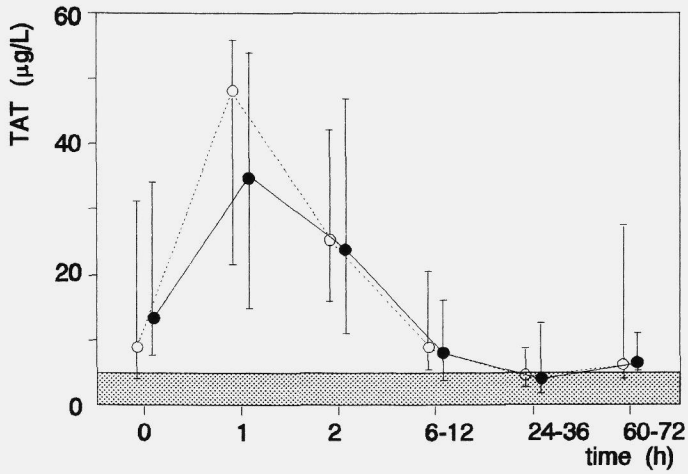

Figure 1 *Course of median TAT concentration in patients without (○) and with (●) reinfarction. Vertical lines represent interquartile range; shaded area is the normal range.*

patient displayed a persistent increase in TAT under heparin. After 24-36 h, before the coronary angiography, TAT was still above the upper limit of normal in 50% of the patients (Fig 1). After the angiogram had been made, TAT increased again in most (39/53) patients and remained normal in only 12 patients. There was no significant difference in TAT between the patients with reinfarction and those without over the entire study period (P = 0.38 by analysis of variance), as illustrated in Figure 1.

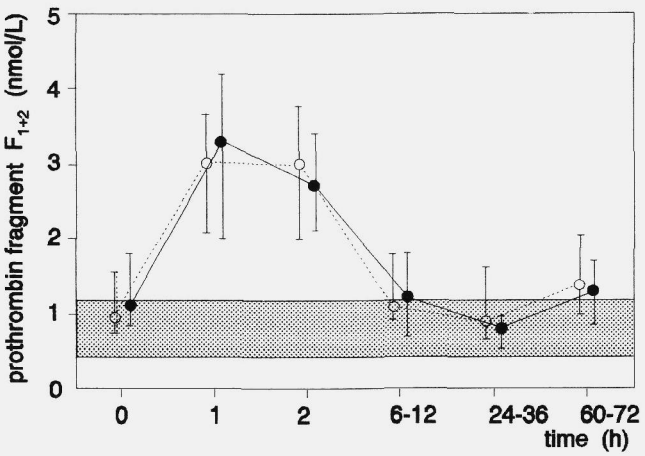

Figure 2 *Course of median F_{1+2} concentration in patients without (○) and with (●) reinfarction. Vertical lines represent interquartile range; shaded area is the normal range.*

F_{1+2} during thrombolysis

Figure 2 shows the course of the F_{1+2} concentration (median and interquartile range) during the treatment period. At presentation, 18 of the 58 patients (31%) had a F_{1+2} concentration above the upper limit of normal. After thrombolysis, F_{1+2} increased in 48/55 patients and decreased in 7, who all had an increased F_{1+2} at admission. Shortly after starting the heparin infusion, at 2 h, median F_{1+2} changed only negligibly, but it had normalized in more than half the patients by 6-12 h and continued to decrease thereafter (Fig 2). The decrease was continuous in 37 of the evaluable patients, whereas in the other 17 either of the two samples had a higher value than the previous one. Before angiography, the F_{1+2} concentration was within the normal range in 30/55 patients and only slightly higher in another 12 patients. After angiography, F_{1+2} increased again in the vast majority of patients (45/53): in 29 it remained within normal limits whereas 25 patients had elevated F_{1+2}. Like for TAT, there was no significant difference in F_{1+2} concentration between the patients with and without reinfarction (P = 0.33).

TAT and F_{1+2} as indicators of reinfarction risk

When using a cut-off value for TAT of 6 μg/L,[9] neither the values obtained at 1 h, nor those at 2 h did reliably indicate reinfarction. At 1 h, the relative risk for reinfarction associated with high TAT was 0.963 (95% confidence interval: 0.89-1.04). The sensitivity of increased TAT for reinfarction was 93% and the specificity 0% (Table 1).

Similarly, as shown in Table 1, F_{1+2} at 1 h did not indicate reinfarction when 2.0 nmol/L was used as a cut-off value: relative risk 1.04 (95% CI: 0.77-1.39), sensitivity 76% and specificity 25%. Also at the other time points, neither F_{1+2} nor TAT were of value as indicators of the outcome in terms of reinfarction (data not shown).

Table 1 *Contingency tables of TAT and F_{1+2} concentrations at 1 h according to reinfarction.*

	TAT		F_{1+2}	
	< 6.0 μg/L	≥ 6.0 μg/L	< 2.0 nmol/L	≥ 2.0 nmol/L
reinfarction	1	26	6	21
no reinfarction	0	28	7	21

DISCUSSION

There is accumulating evidence that thrombolytic therapy is associated with generation of thrombin activity.[6-9] Increased FpA in plasma has been demonstrated during treatment with streptokinase[6,7] as well as tissue-type plasminogen activator,[7,8,13] so the type of thrombolytic agent probably plays no role in this phenomenon. In theory, FpA may not be specific for thrombin activity, since plasmin and tissue-type plasminogen activator can cleave small peptides, containing the FpA sequence, off fibrinogen.[8,14] *In vitro* experiments have confirmed that plasmin will increase the FpA concentration in platelet-rich plasma, but Winters *et al.* have shown that this effect is mediated by thrombin, since it can be markedly suppressed by thrombin inhibitors.[15] Moreover, there is supplementary evidence for the involvement of thrombin activation in thrombolysis. Concomitant hirudin and heparin could accelerate and enhance the lysis of thrombi by tissue-type plasminogen activator and prevent reocclusion in animal models or *in vitro*,[16-18] while in clinical studies heparin increased the patency rate after alteplase treatment.[13]

Technical requirements for blood collection and assay of FpA are rather stringent, contrary to two novel assays, which measure other aspects of thrombin activation, namely TAT and F_{1+2}.[10,11] At present, data on the clinical usefulness of TAT and F_{1+2} during thrombolytic therapy are still scanty and unconfirmed.[9,19] This inspired us to perform the study described here.

Although the protocol of the SUTAMI trial included only a single coronary angiography and thus did not allow us to record reocclusion, there were 33 patients in

whom reinfarction was diagnosed on the basis of clinical and electrocardiographic data. Since reinfarction after thrombolysis may be regarded as the clinical analogue of angiographically proven reocclusion, we studied the course of TAT and F_{1+2} in these patients and their value for indicating reinfarction. Confirming previous reports, we found that activated thrombin is present *in vivo* at admission in AMI patients and that thrombolytic therapy amplifies the generation of thrombin.[6-9,18]

The course of TAT and F_{1+2} in our patients showed a marked increase until heparin infusion was started (between 1 and 2 h). This is in close agreement with the results of Munkvad *et al.*,[19] who used a similar scheme of heparinization after rtPA. In our patients the median peak concentrations of TAT and F_{1+2} were 1.5 to 2 times higher, which might reflect the more pronounced fibrinogenolysis in our patients as compared with their study.[19] The results of Gulba *et al.* are quite different, however.[9] These authors described an immediate decrease in TAT in those patients with sustained patency, whereas a significant increase in mean TAT was observed only in patients with non-successful lysis or those with reocclusion.[9] Because Gulba and coworkers started a heparin infusion before the thrombolytic agent, their results are difficult to compare with ours. For this reason, the application of TAT assays as an indicator of early reocclusion, as recommended by Gulba *et al.*,[9] may be restricted. We found TAT and F_{1+2} to be essentially identical in the patients who experienced an early reinfarction and those who did not (Figs 1,2). Therefore TAT and F_{1+2} were not indicative of reinfarction (Table 1).

Further, a conspicuous difference in the course of TAT and F_{1+2} became apparent. Although the median TAT decreased immediately after starting heparin administration, it lasted until 24-36 h before it was within the normal range in 50% of the patients (Fig 1). Median F_{1+2}, on the contrary, did hardly decrease shortly after beginning the heparin infusion, but it normalized much faster than TAT. The quicker initial decrease in TAT as compared to F_{1+2} can be explained by the difference in half-life (about 3 min and 90 min, respectively), but this does not account for the fact that F_{1+2} normalized more rapidly than TAT. The same discrepancy has been noted previously during heparin treatment of deep venous thrombosis,[20] but remains as yet unexplained.

It is intriguing to dwell on the mechanisms involved in the activation of coagulation during thrombolytic therapy. For long it has been known that fibrin-bound thrombin, which is enzymatically active and is inaccessible to heparin-antithrombin III complex[21] can be released upon plasmin-mediated degradation of fibrin.[22] While this might explain the rise in TAT, it does not account for the increase in F_{1+2}, which represents newly activated thrombin rather than release of already active thrombin. Alternatively, it has been shown that a partially lysed thrombus is highly thrombogenic itself and that further dissolution of the clot in the coronary artery might re-expose the underlying vessel wall injury, which provides an ongoing thrombogenic stimulus.[23] Recently it has been suggested that lysis of fibrin exposes procoagulant activity on endothelial cells and thus causes coagulation activation not only at coronary sites, but on the vascular intima in the systemic circulation.[19] It is still too early to favour either of these possible mechanisms.

In conclusion, we have demonstrated that during thrombolytic therapy with saruplase and urokinase, the coagulation system is extensively activated and that this increased *in*

vivo thrombin generation can be suppressed with heparin. It proved to be impossible to get an indication of early reinfarction from the concentrations of TAT and F_{1+2} measured during thrombolytic therapy, at least when utilizing the current practice of administering heparin after the thrombolytic drug.

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Chapter 9

Blood viscosity during thrombolytic therapy with anistreplase in acute myocardial infarction

JJ Hoffmann, JJ Bonnier[1], PG Melman[1] and I Bartholomeus[2]

[1] Department of Cardiology, Catharina Hospital, Eindhoven

[2] Medical Department, SmithKline Beecham, Rijswijk

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SUMMARY

It has been postulated that a reduction in blood viscosity, due to degradation of plasma fibrinogen, might be of benefit to patients with acute myocardial infarction (AMI), who are treated with thrombolytic agents. The aims of this study were to investigate the time course of rheological parameters, to identify the principal factors determining blood viscosity and to find possible correlations between viscosity and cardiac function during thrombolytic therapy with anistreplase. Therefore, the viscosity of whole blood and plasma and the haematocrit were measured before and at 10 time points after thrombolysis in 10 AMI patients. In addition, plasma fibrinogen and fibrin(ogen) degradation products were determined.

Immediately after the start of thrombolysis, the viscosity of blood (both at high and low shear rate) and plasma decreased significantly and continued to do so for 24 hours. The mean haematocrit also decreased markedly, and even after correction for these haematocrit changes, the reduction in blood viscosity remained significant: it decreased to 72% of pretreatment (measured at low shear rate), whereas the high-shear viscosity fell to 95% of baseline. The viscosity of plasma significantly decreased from 1.39 ± 0.13 mPa.s (mean ± SD) before thrombolysis to 1.22 ± 0.08 mPa.s after 2 hours. There was a rapid, nearly complete depletion in fibrinogen, followed by a striking rebound after the second day. The decrease in blood viscosity lasted for 2 days after anistreplase and was mainly accounted for by the reduction in haematocrit. The contribution of fibrinogen to blood viscosity appeared less prominent. In spite of these rheological changes, no improvement of the cardiac output was noticed in the patients.

INTRODUCTION

In the early phase after acute myocardial infarction (AMI), the rheological conditions in coronary vessels may be rather unfavourable. Apart from the impaired flow in the microcirculation distal to the occlusive thrombus, the viscosity of blood is increased due to a high haematocrit[1] and an increase in plasma fibrinogen concentration.[1-4] This increase in viscosity may contribute considerably to the evolving myocardial necrosis, since oxygen supply to the myocardial tissue inversely depends on blood viscosity.[2] By restoring the patency, thrombolytic therapy improves the flow conditions in the coronary circulation. Additional improvement of blood rheology by lowering the increased viscosity might be advantageous in terms of oxygen supply and salvage of viable myocardial tissue. Because fibrinogen is a major determinant of blood viscosity, thrombolytic drugs which cause extensive fibrinogen breakdown could offer this additional benefit. In the past, streptokinase was shown to decrease plasma viscosity[4-6] and more recent data confirm the higher reduction in plasma viscosity obtained with the less fibrin-selective thrombolytic agents as compared with tissue-type plasminogen activator.[7-10] However, the alleged clinical benefit of reduced viscosity has not yet been proven. The present study documents the time course of blood and plasma viscosity in AMI patients treated with anistreplase. Further, we sought to identify the major factors influencing blood viscosity during thrombolysis and to correlate changes in viscosity with cardiac output after thrombolytic therapy.

PATIENTS AND METHODS

Patients

Ten patients, nine males and one female, with a mean age of 60 years (range 50-68 y), presented with typical signs of AMI and were included into the study within 4 h from the onset of chest pain. There were nine patients with an inferior AMI and one had an anterior infarction. None of them had experienced a previous AMI. The usual criteria for inclusion and exclusion were applied, except that the use of aspirin or other anti-platelet drugs was reason for exclusion, because these patients simultaneously participated in a study on the influence of thrombolysis on platelet function.

On the average 2.4 h (range 1.2 - 4.2 h) after beginning of chest pain, anistreplase (APSAC; Eminase® 30 U) was administered by intravenous injection over 2-5 min. Heparin was started 3 h later by continuous intravenous infusion; its dose was adjusted according to the activated partial thromboplastin time. At 60, 75 and 90 min after starting the thrombolysis, coronary angiography was performed for assessing coronary patency and at 90 min cardiac output was measured using the thermodilution technique. Assessment of patency and cardiac output were repeated after 48 h.

Before and after anistreplase, blood was collected for determining the rheological and haemostatic parameters as specified below. Blood sampling was complete in 4 patients, 1 sample was missing in each of 4 other patients and 2 patients had 2 missing samples; at all time points ≥ 8 observations were available. The study protocol had been approved by our hospital's ethical committee and the patients gave their informed consent prior to inclusion.

Methods

Blood for viscosity determinations was collected into K_3-EDTA (final concentration 1 mg/mL), supplemented with aprotinin (final concentration 250 KIU/mL) in order to prevent *in vitro* fibrinogenolysis. Viscosity was measured within 1 hour from blood sampling using a rotational viscosimeter (LS-30; Contraves AG, Zürich, Switzerland). Native blood viscosity, *i.e.* at the actual haematocrit, was determined at two different shear rates, 128.5 s^{-1} (high shear) and 1.3 s^{-1} (low shear), according to the ICSH recommendations.[11] Standardized blood viscosity at either shear rate was corrected to a haematocrit of 0.45, as described by Matrai *et al.*[12]

For determining coagulation parameters, blood was drawn into citrate, which was supplemented with aprotinin (final concentrations 0.01 M and 250 KIU/mL, respectively), and centrifuged for 15 min at 3000 g at 4 °C; the plasma was snap-frozen in liquid nitrogen and stored at -70 °C until analysis. Commercially obtained reagents and standard methods were used.[13] Fibrinogen was determined using the Clauss clotting rate method, and fibrin degradation products (FbDP) and fibrinogen degradation products (FgDP) by specific enzyme immunoassays based on monoclonal antibodies (Fibrinostika; Organon, Boxtel, the Netherlands).

Statistics

Statistical evaluation of changes over time points was done using a univariate repeated measurements analysis of variance. In case of significance this was followed by a paired t-test between data at baseline and the other time points. Multiple regression analysis was carried out using the pooled data across all time points. P values < 0.05 were considered significant.

RESULTS

Clinical

Coronary patency was observed after 90 minutes in 9/9 patients (one patient had coronary angioplasty after 60 minutes) and after 48 hours no reocclusion was seen. These results are not essentially different from what is usual for anistreplase therapy.

Rheology

The pre-treatment blood viscosity was within the normal range in all patients, both at low and at high shear rates. After administration of the thrombolytic drug there was a rapid

drop in native blood viscosity, which reached its lowest value 24 h later. At that time, the native blood viscosity was 77% of pre-treatment ($P < 0.001$) at high shear rate and 49% of baseline ($P < 0.001$) at low shear rate (Fig 1). Hereafter, the mean blood viscosity gradually increased but it was still lower than the initial value before discharge, 7-9 days after thrombolysis. The mean haematocrit decreased steadily during the entire observation period (Fig 1).

When the blood viscosity was standardized to haematocrit 0.45, a different picture was seen. The low-shear blood viscosity was minimal after 12 h (72% of baseline; $P < 0.02$) and the high-shear viscosity decreased to 95% of pre-treatment ($P < 0.05$), also at 12 h (Fig 1). Both parameters returned to their baseline values after the second day and increased significantly above pre-treatment values before discharge.

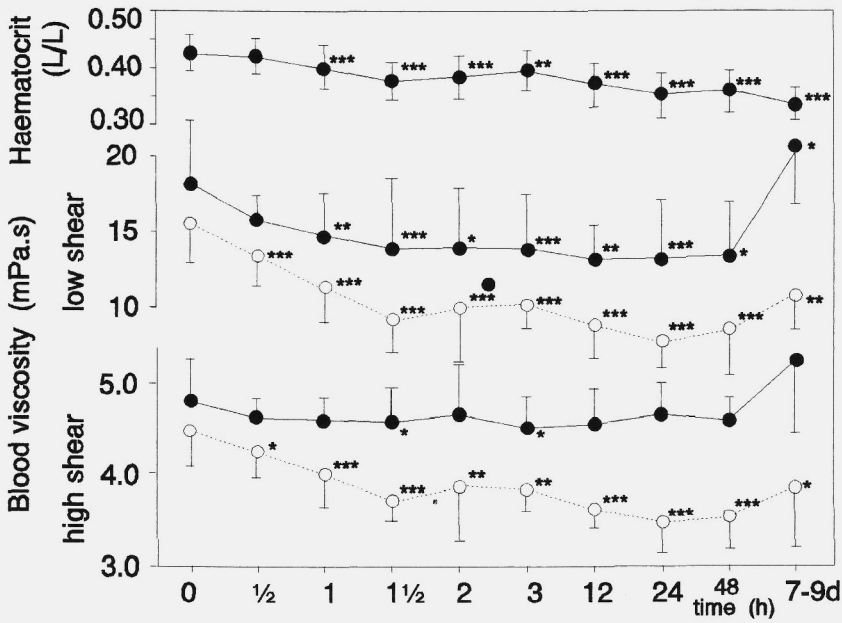

Figure 1 *Course of rheological parameters during thrombolytic therapy. In the middle and lower frames, the dotted lines represent the native blood viscosity (○) and the solid lines the standardized viscosity, corrected to haematocrit 0.45 (●). Mean ± SD (vertical lines). Difference from pre-treatment values: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.*

The viscosity of plasma was initially normal in all patients but one, in whom it was elevated; mean (± SD) 1.39 ± 0.13 mPa.s. It sharply dropped in all patients immediately after administration of anistreplase (Fig 2). After 2 h the mean plasma viscosity was 1.22 ± 0.08 mPa.s, or 88% of baseline; this difference was significant ($P < 0.01$). From 90 min

up to 24 h the mean plasma viscosity remained more or less stable and then started to increase. Before discharge it had raised to 104% of the baseline value: 1.45 ± 0.14 mPa.s (P = 0.033; Fig 2).

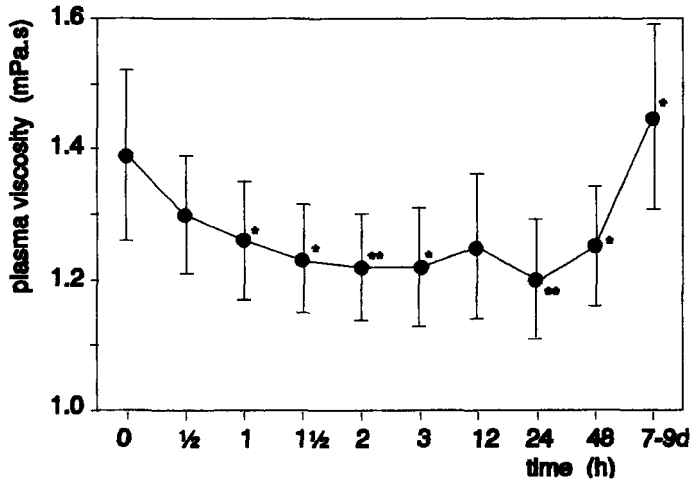

Figure 2 *Plasma viscosity as a function of time after thrombolysis; mean ± SD. Difference from baseline: * $P < 0.05$ and ** $P < 0.01$.*

Fibrinogen

All patients had a normal fibrinogen before treatment, ranging from 2.9 to 4.0 g/L (mean 3.33 ± 0.45 g/L). Already after 30 min a considerable decrease in fibrinogen was apparent amounting to 13% of pre-treatment ($P < 0.001$). The nadir, at 4% of baseline, was observed after 2 h and fibrinogen remained very low until 12 h (Fig 3). Then, a rapid increase became manifest, crossing the pre-treatment value after the second day. This fibrinogen rebound was still present before discharge, when the mean concentration was 36% above the pre-treatment value (4.53 ± 0.52 g/L; $P < 0.002$).

Fibrin(ogen) degradation products and coagulation factors

The drop in fibrinogen was accompanied by a rapid, marked increase in the concentration of FgDP. It reached its maximum after 60 min (mean 429, range 80 - 820 mg/L) and hereafter slowly decreased. At day 7-9, the FgDP concentration was still above the normal range in the majority of patients (Fig 3).

The degradation products of crosslinked fibrin (FbDP) also significantly increased, but with a profile different from that of FgDP. The highest value was noted after 12 h (mean 11.3 ± 5.3 mg/L) and the rate of increase was only moderate. Before discharge, FbDP was still elevated in all patients in whom it was measured.

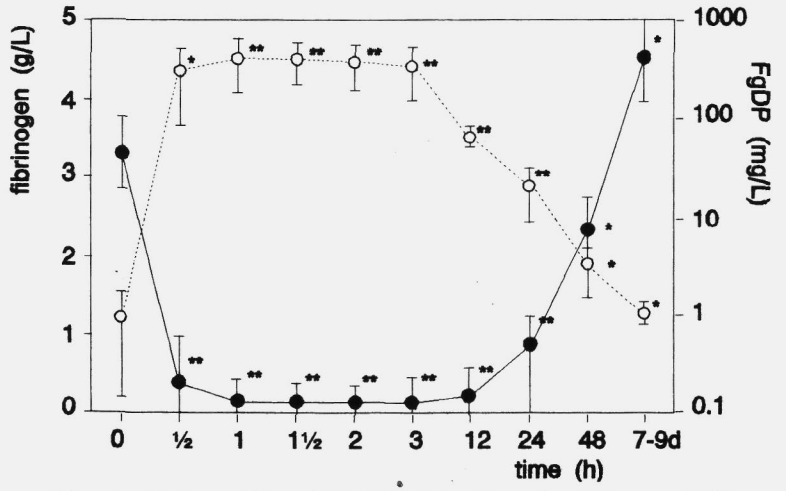

Figure 3 *Fibrinogen (●) and FgDP (○) during the study period; mean ± SD. Difference as compared with pre-treatment values: * $P < 0.01$ and ** $P < 0.001$.*

Other coagulation proteins exhibited the expected pattern. Plasminogen activity significantly decreased (to about 12% of pre-treatment at 3 h), α_2-antiplasmin activity became very rapidly entirely depleted (mean < 0.10 U/mL after 30 min) and the euglobulin clot lysis time was extremely shortened (<15 min) in all patients 60 min after administration of anistreplase ($P < 0.001$). The profiles were closely comparable to that of fibrinogen. These parameters had essentially returned to their baseline values by day 2 (data not shown).

Cardiac function

Cardiac output was 6.5 ± 2.0 L/min at 90 min and it decreased to 4.9 ± 1.2 L/min by 48 h after start of the thrombolysis. The mean left ventricular ejection fraction was 75 ± 10% (range 63-87%) at 90 min and 66 ± 16% (range 36-84%) after 48 h.

Regression models

We analyzed the pooled data of all patients in order to find the major determinants of viscosity during thrombolysis and their relative importance. The native blood viscosity (low shear) was significantly correlated with the haematocrit ($r = 0.681$; $P < 0.0001$) and with fibrinogen ($r = 0.249$; $P < 0.02$), but not with the FgDP concentration ($r = 0.01$; n.s.). The total variance in low shear blood viscosity was explained for 50% by the variance in the haematocrit and for another 13% by that in fibrinogen; addition of FgDP did not further improve the regression model.

Similarly, the correlation between high shear blood viscosity and the haematocrit was significant ($r = 0.739$; $P < 0.0001$). However, the high shear blood viscosity was only weakly correlated with fibrinogen ($r = 0.166$; $P = 0.112$) and not at all with FgDP (r

= 0.08; n.s.). The haematocrit was the main determinant of blood viscosity at high shear rate, explaining 57% of its variance, followed by fibrinogen, which added 8% to the variance.

The most important parameter determining the plasma viscosity under the circumstances studied was fibrinogen. It contributed 28% to the variance in plasma viscosity and was significantly correlated with it ($r = 0.537$; $P < 0.0001$). Again, the FgDP concentration conveyed no explanation to the variance in plasma viscosity.

There was no correlation of cardiac output with blood viscosity ($r = -0.154$; n.s.) and neither with plasma viscosity ($r = -0.216$; n.s.) or fibrinogen ($r = -0.508$; n.s.).

DISCUSSION

Thrombolytic therapy can nowadays be regarded as the standard treatment of patients with AMI. Among the numerous studies on various aspects of thrombolytic therapy, little attention has been given to its rheological effects. For long it has been postulated that lowering the viscosity of blood by extensive degradation of fibrinogen by means of thrombolytic drugs might be beneficial to the patient,[4-6] but this hypothesis has never been substantiated.

In order to adequately appreciate the relevance of blood rheology for AMI and its thrombolytic treatment, some basic concepts of viscosity should be addressed.[14] The movement of blood in a vessel is not homogenous: the flow pattern is best represented by an infinite number of concentrical fluid layers, each of which moves with a discrete velocity. The velocity of the central layer is higher than that of more eccentric layers; so, a faster layer slides over an adjacent, more slowly moving layer of fluid. This process is called shearing. The velocity gradient over the fluid, *i.e.* the difference in velocity in relation to the distance between layers, is the shear rate. The force necessary to make a layer sliding over an adjacent one at a given shear rate is only dependent on a material property of the fluid, called its dynamic viscosity coefficient or its viscosity. In ideal, so-called Newtonian, fluids the viscosity is independent of the flow conditions and thus independent of the shear rate. However, due to its high content of red cells, blood is a non-Newtonian fluid, and hence its viscosity is dependent on the prevailing shear rate. At high shear rates, the contribution of red cells to blood viscosity is relatively low, because they have only a slight tendency to form aggregates. Under low shear conditions however, the red cells are predisposed to aggregate and this causes a dramatic increase in viscosity. Thus, it is not possible to define *the* viscosity of blood, but only to measure it at a given shear rate or range of different shear rates. *In vivo*, high shear rates (200 s^{-1} or higher) prevail in arteries and large veins, whereas low shear rates (20-50 s^{-1}) are encountered in small venules.[1] In AMI, the shear rate in coronary vessels distal to the occlusive thrombus may be extremely low, 20 s^{-1} or less.[1] By convention, blood viscosity is reported at a low and a high shear rate.[14] Accordingly, we have chosen to measure blood viscosity at 1.3 and 128.5 s^{-1}, respectively. Since the haematocrit is one of the major determinants of blood viscosity, we calculated the standardized viscosity in addition to the native viscosity; the

standardized viscosity is corrected for and therefore no longer dependent on the actual haematocrit.[12]

Our results confirm that thrombolytic therapy with a drug that causes a profound systemic lytic state, significantly reduces the viscosity of blood and plasma. The decrease in standardized blood viscosity found here, by 28 % (low shear) and 5 % (high shear), is comparable with the values found by Arntz *et al.*,[8] who described 16 % and 9 % decrease, respectively, after the same dose of anistreplase, but at slightly different shear rates, namely 5.75 and 115 s^{-1}. The 12% reduction in plasma viscosity found by us (Fig 2), is very close to their finding a 15 % decrease.[8] These values are equivalent to the drop in plasma viscosity by 7-19 %, described after high dose streptokinase,[4-6,8,10] and are 2 to 3 times higher than after treatment with tPA.[10] This is not surprising in view of the limited systemic fibrinogenolysis by tPA as compared with streptokinase and anistreplase. The latter caused an extensive depletion of plasma fibrinogen in the present study (Fig 3).

The high correlation between plasma viscosity and fibrinogen corroborates the importance of this protein as a determinant of the viscous properties of plasma.[14] It should be kept in mind, however, that this correlation was challenged by two factors: we performed the regression analysis on pooled data (which ignores the interdependence of observations from a single patient and might lead to overestimation of a correlation), whereas the sometimes undetectable fibrinogen values prohibited estimating its exact contribution to the variance in viscosity (a potential source of underestimation).

It appeared that the contribution of FgDP to plasma and blood viscosity was not significant, even not at the very high concentrations prevailing during the early phase after anistreplase. This is distinct from the findings by Rampling *et al.*,[15] who in an *in vitro* study on the rheological effects of fibrinogen and its degradation products, found that all FDP caused measurable effects on viscosity, although significantly less than intact fibrinogen. Even the small, late FDP had still viscometric effects.[15] However, in their experiments aqueous solutions were used to create red cell suspensions, while we used plasma. It is conceivable that effects of FgDP could not be distinguished from the effects of other plasma proteins on the plasma viscosity. Although fibrinogen fragments may have certain effects on the viscosity of plasma and blood, our data suggest that these effects are probably not large enough to make a relevant contribution to *in vivo* blood rheology.

The discordant changes in native and standardized blood viscosity indicate that the decrease in haematocrit observed during the treatment plays an important role in the improvement of blood rheology. This was further accentuated by the regression analysis, which showed that the contribution of the haematocrit to blood viscosity was 4 to 7 times greater than that of fibrinogen (again, the influence of fibrinogen has possibly been underrated, as indicated above). Since a thrombolytic agent itself is unlikely to cause a drop in haematocrit, the haemodilution noticed must be explained by other factors such as fluid balance, medication and infusion policy. From earlier studies it is known that a decrease in haematocrit is commonly seen after AMI, even without thrombolytic treatment.[1,2,6] Moreover, it has previously been shown that contrast agents used in coronary angiography caused haemodilution due to their osmotic effect.[16] In the present

study, repeated angiographies were performed and therefore the haemodilution induced in the early phase may have been even larger than during standard thrombolytic treatment.

Extrapolating our results might mean that the improvement of haemorheology during thrombolytic treatment is only partially the consequence of the fibrin selectivity of the thrombolytic agent administered. Some support for this inference, although partial and indirect, is provided by a study, recently reported by Bassand *et al.*[17] They found no significant differences in left ventricular ejection fraction, regional wall motion, infarct size and in-hospital mortality between anistreplase and tPA, despite large differences in fibrinogen breakdown. Had the degree of systemic fibrinogenolysis (and thus blood viscosity) had important effects on these cardiac parameters, perceptible differences might expectedly have been found in this trial using two drugs with dissimilar effects on fibrinogen. In the present study, no correlation was found between the reduction in viscosity and cardiac output, but the number of observations might have been too low for detecting such a relation.

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PART 3

PLATELET FUNCTION IN THROMBOLYTIC THERAPY

Chapter 10

Platelet function during thrombolytic therapy with anistreplase

JJML Hoffmann, JJRM Bonnier[1], PG Melman[1], I Bartholomeus[2], R Standring[3] and R Fears[3]

[1] Department of Cardiology, Catharina Hospital, Eindhoven

[2] Medical Department, SmithKline Beecham, Rijswijk

[3] SmithKline Beecham, Research Division, Great Burgh, Epsom, England

submitted for publication

SUMMARY

Platelets are likely to play a role during thrombolytic therapy of acute myocardial infarction, but their exact involvement is not fully understood at present. The effects of thrombolytic agents on platelet function have mainly been studied using in vitro experiments; data from in vivo studies are scarce. Available results are rather controversial. We studied 10 patients with acute myocardial infarction, who were treated with intravenous anistreplase. Use of aspirin or other anti-platelet drugs was not allowed before and during the study period. Before and after thrombolysis, blood was collected for determining ex vivo platelet aggregation and platelet factor 4, β-thromboglobulin and thromboxane-B_2 in plasma.

Immediately after anistreplase therapy, the aggregation of platelets was significantly inhibited: aggregation induced with ADP decreased to 67 ± 36% (mean ± SD) of pre-treatment ($P < 0.05$), with arachidonic acid to 29 ± 29% ($P < 0.005$) and with collagen to 58 ± 46% ($P < 0.05$). This aggregation defect was transient; aggregation had returned to normal after 6-12 h in all patients. Then, a significant stimulation of ADP-induced aggregation became apparent, lasting until 24-48 h after thrombolysis. The platelet proteins and thromboxane-B_2 were significantly increased before thrombolysis and showed a steady decrease after anistreplase. During the first hours after anistreplase therapy, there were no indications of activation of platelets. Additional in vitro studies confirmed these findings. Antibodies to streptokinase appeared not to affect platelet function in these patients.

In conclusion, treatment with anistreplase caused a marked immediate, but transient inhibition of ex vivo platelet aggregation without any signs of systemic platelet activation during the first 24 h after thrombolysis. However, after 1-2 days some activation of platelets was observed; this effect had disappeared after 1 week.

INTRODUCTION

Thrombolytic therapy as nowadays clinically applied in patients with acute myocardial infarction (AMI) is associated with two major complications: reocclusion of the recanalized arteries and bleeding.[1] It is likely that platelets play an important role in both these processes. In clinical trials it has been established that the efficacy of a thrombolytic regimen can be significantly enhanced by concomitant administration of drugs which inhibit the activity of platelets.[2,3] Although activated platelets are associated with reocclusion this does not necessarily mean that thrombolytic drugs cause platelet activation directly. It has been proposed that the damaged vessel wall or atherosclerotic plaque which initially caused thrombus formation will again activate platelets and the coagulation system after the thrombus has been dissolved by the thrombolytic drug.[4]

Studies which have examined the effects of thrombolytic drugs on platelets remain controversial. In animal models infusion of tissue-type plasminogen activator (tPA) or streptokinase (SK) resulted either in platelet activation[5-7] or in inhibition of platelet function.[8] Most studies with human platelets which were exposed *in vitro* to therapeutic amounts of tPA, SK, urokinase or anistreplase (anisoylated plasminogen-SK activator complex; APSAC), showed a significant decrease in platelet aggregation,[9-12] but there are also reports describing stimulation of platelet aggregation after incubation with urokinase, tPA or SK.[13-15] Studies on platelet function after *in vivo* administration of thrombolytic drugs in humans are rather scanty and they too yield discordant results. Increased platelet activity has been described after urokinase and SK,[13,14] while transient inhibition of platelet aggregation has also been found.[16]

It is generally accepted that most if not all effects of thrombolytic drugs on platelets are mediated by plasmin.[7-12] Anistreplase is the acylated complex of plasminogen with streptokinase and causes marked systemic plasmin generation in patients, as previously shown.[17] Therefore, this therapy is well suited for investigating the effects on platelets. We describe a study of platelet aggregation and several other parameters of *in vivo* platelet activity in ten patients, who were treated for AMI with anistreplase and who did not use aspirin or other anti-platelet drugs prior to and during the study period.

MATERIALS AND METHODS

Study procedure

Ten patients, nine males and one female, with a mean age of 60 y (range 50-68 y), who presented with typical signs of AMI were included into the study within 4 h from the onset of chest pain. None of them had experienced a previous AMI. The usual criteria for inclusion and exclusion were applied, as described before,[17] with one exception. Recent use of aspirin or other anti-platelet drugs within 10 days before admission was reason for exclusion from the present study and the protocol also prohibited aspirin or anti-platelet

drugs during the study period. On the average 2.4 h (range 1.2-4.2 h) after beginning of chest pain, anistreplase (Eminase®, 30 U) was administered by slow intravenous injection over 5 min. Intravenous heparin was started 3 h later; its dose was adjusted according to the activated partial thromboplastin time. At 60, 75 and 90 min and 48 h after starting thrombolysis, coronary angiography was performed to assess coronary patency, using the TIMI criteria. Before and after giving the thrombolytic drug, blood was collected for determining the haemostatic parameters as specified below. The study protocol had been approved by the hospital's ethical committee and every patient gave his or her informed consent in writing before inclusion.

Blood collection

Before administering anistreplase and 30, 60, 90 min and 2, 3, 6, 12, 24 and 48 h hereafter, blood was collected through a venous catheter or by venepuncture. An additional sample was taken after 7-9 days, before discharge from the hospital. The blood was collected into 0.1 volume of trisodium citrate (final concentration 0.01 M), supplemented with aprotinin (final concentration 250 KIU/mL) in order to prevent ongoing *in vitro* fibrinogenolysis. For measuring platelet release products, blood was collected into prechilled tubes containing citrate, theophylline, adenosine and dipyridamole (CTAD-tubes; Boehringer-Mannheim, Almere, the Netherlands). Blood anticoagulated with K_3-EDTA was obtained for platelet counting.

Immediately after blood collection, the CTAD-samples were centrifuged for 15 min at 3000 *g* at 4 °C and the plasma was snap-frozen in liquid nitrogen and stored at -70 °C until analysis. Platelet-rich plasma (PRP) for aggregation studies was prepared by low-speed centrifugation (180 *g* for 5 min at 21 °C) and the platelet count of PRP was adjusted to 200/nL.

Reagents and methods

Platelet aggregation was carried out within 1 h from blood collection using a four-channel optical aggregometer (PAP-4; Biodata, Hatboro, PA, USA). The reagents for inducing aggregation were obtained commercially and their final concentrations were as follows: adenosine diphosphate (ADP) 0.6, 1.2, 2.4 and 3.6 μM, collagen (from bovine tendon) 1.0 and 5.0 μg/mL, arachidonic acid 1.6 mM and ristocetin 1.5 mg/mL. Aggregation responses were recorded as percentage of maximum light transmission; the minimal and maximal transmission values were set for each sample using PRP and platelet-poor plasma, respectively.

The platelet release products platelet factor 4 (PF4) and β-thromboglobulin (βTG) were determined in CTAD-plasma using enzyme immunoassays (ELISA; Stago, Boehringer-Mannheim) and the prostaglandin metabolite thromboxane-B_2 (TxB_2) was measured in plasma after chromatographic extraction, using a radioimmunoassay kit (Amersham International Plc., Amersham, UK). The reference ranges are 1-10 IU/mL for PF4, 11-60 IU/mL for ßTG and 6-59 pg/mL for TxB_2, respectively.

The determination of IgG antibodies to SK in plasma was performed using a radioimmunoassay method.[18] Degradation products of fibrinogen were measured using a

specific enzyme immunoassay, based on two monoclonal antibodies (Fibrinostika®-FgDP; Organon Teknika, Boxtel, the Netherlands).

In vitro experiments

Citrated blood from normal volunteers was processed to give PRP containing 200 platelets per nL and the PRP was incubated with anistreplase (final concentration 10 mU/mL, or approximately 70 nM) at 37 °C. At the times indicated, aliquots were removed for determining platelet aggregation as described above. Similarly, aliquots of PRP incubated with anistreplase were added to CTAD in order to stop any platelet activation, the platelets were removed by high-speed centrifugation and the plasma stored at -70 °C for analyzing PF4 and βTG (see above). PRP incubated with saline served as a control.

In order to study whether the effects of anistreplase on platelets were primarily associated with platelets or with plasma, a crossover experiment was done. Blood was collected from four healthy donors and PRP was incubated with anistreplase and a saline control as described above. After 30 min at 37 °C, the platelets were pelleted by high-speed centrifugation and the plasma carefully pipetted off. Then, the anistreplase-treated platelets were resuspended in the control plasma and the control platelets in the plasma incubated with anistreplase. A complete aggregation profile was obtained from both preparations.

Statistics

Statistical evaluation of the data was performed using the paired t-test, if indicated after transformation of the original data, considering P values < 0.05 significant. For measures having a normal distribution, mean ± SD is given and in case of non-normal distributions, median and range are given. The relation between platelet function and FgDP or fibrinogen was investigated using multiple linear regression analysis.

RESULTS

Clinical findings

Nine of the ten patients suffered from an inferior AMI and one had an anterior infarction, as judged from the pretreatment ECG. At coronary angiography, 60 min after administering anistreplase, the infarct-related vessel was patent (TIMI grade 3) in seven patients, partially patent (grade 2) in one patient and not patent (grade 0) in the remaining two. One patient had to be excluded after 60 min because of starting treatment with anti-platelet agents. At 90 min, the remaining nine patients all had patent (grade 3) arteries and none of these was reoccluded at angiography after 48 h (all TIMI grade 3). Due to logistical reasons, there were only four patients in whom blood collection was possible at 6 h; therefore, this time point was omitted from further analysis.

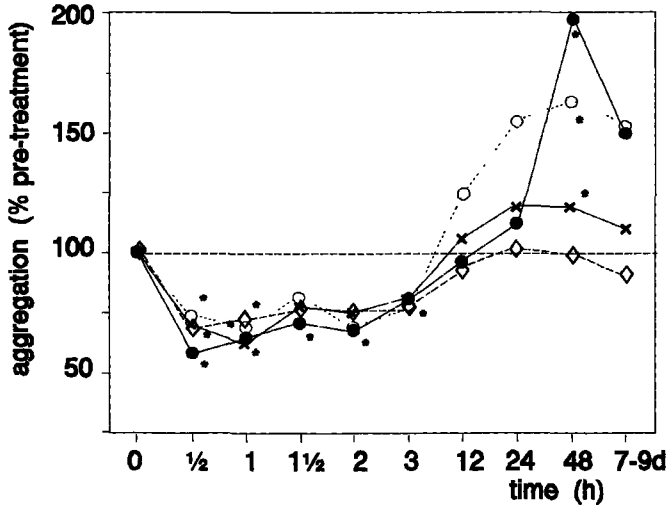

Figure 1A *Course of ADP-induced platelet aggregation after anistreplase, expressed as a percentage of pretreatment aggregation intensities. ADP concentrations: 0.6 μM (●); 1.2 μM (○); 2.4 μM (x); 3.6 μM (◇). Means of 8-10 observations. Differences from baseline: * P < 0.05.*

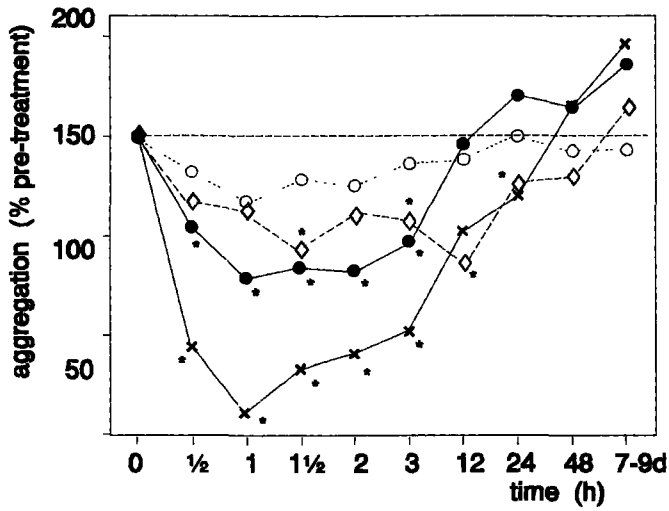

Figure 1B *Course of platelet aggregation, induced with 1 μg/mL collagen (●); 5 μg/mL collagen (○); 1.6 mM arachidonic acid (x); and 1.5 mg/mL ristocetin (◇). See legend to figure 1A.*

Platelet count

The concentration of platelets in blood before treatment was normal in all patients (mean ± SD: 263 ± 78 /nL) and showed only minimal, statistically insignificant changes after starting the thrombolysis. After 7-9 days, the mean platelet count had significantly increased above the baseline value in most patients (319 ± 68 /nL; $P < 0.02$). None of the patients showed thrombocytopenia during the observation period.

Platelet aggregation

Before therapy the aggregation profile of all patients was normal or nearly normal as tested with the four inducing agents. In three patients there was clear aggregation observed with the lowest concentration of ADP; in one of them this aggregation was irreversible and in the other two the aggregates were rapidly dispersed. Two patients had low aggregation with arachidonic acid.

Immediately after administration of anistreplase, the ADP-induced aggregation was inhibited to a significant degree (Fig 1A). This inhibition occurred with all four ADP concentrations used and it was transient. In most patients the pre-treatment aggregation was fully restored after 6-12 h. Then, there was a clear stimulation of ADP-induced aggregation, when using the three lower ADP concentrations; evidently, 3.6 μM ADP was a too strong inducer to reveal the state of activation of the platelets. The hyperaggregation reached its maximum at 1-2 days after thrombolysis and completely disappeared before discharge from the hospital. The aggregation profile of a typical patient from this study is shown in Figure 2, depicting the most relevant time points only.

Collagen-induced aggregation also rapidly decreased after anistreplase. This effect was most pronounced using the lower collagen concentration (Figs 1B,2), although the rate of aggregation induced by the higher collagen concentration was significantly reduced after anistreplase, too. After 12 h, the collagen-induced aggregation had normalized again in all patients. There was no rebound of collagen-induced aggregation observed.

The aggregation induced with arachidonic acid was markedly inhibited in all patients within 1 h from the beginning of thrombolytic therapy (Figs 1B,2). The restoration to pre-treatment values took 1-2 days in most of the patients. The platelet agglutination by ristocetin was also significantly diminished between 90 min and 24 h after anistreplase (Fig 1B).

The course of the concentration of the degradation products of fibrinogen (FgDP) in plasma is shown in Figure 3. There was a sharp increase in FgDP immediately after anistreplase in all patients; the peak values were reached after 60 min. Then, the mean FgDP concentration decreased with an estimated half-life of approximately 5-6 hours (Fig 3). There was a significant positive correlation between platelet aggregation induced with ADP (both intensity and rate) and plasma fibrinogen ($r = 0.34$ to 0.57; $P < 0.001$) and a negative one between ADP-induced aggregation and FgDP concentration ($r = -0.46$ to -0.60; $P < 0.001$).

The pre-treatment concentration of IgG antibodies reactive with streptokinase ranged from 0.15 - 3.44 μg/mL, the median value being 0.82 μg/mL; these results are closely comparable to our previous findings.[19] There was no significant relationship between the

pre-treatment IgG anti-SK concentration and any of the aggregation parameters described at any time examined. For example, using the 2 h timepoint as representative of the platelet response (Figs 1A,1B) and using data for agonist concentrations 1.2 μM ADP and 1 μg/mL collagen as sensitive indices, no significant correlations were observed (Fig 4).

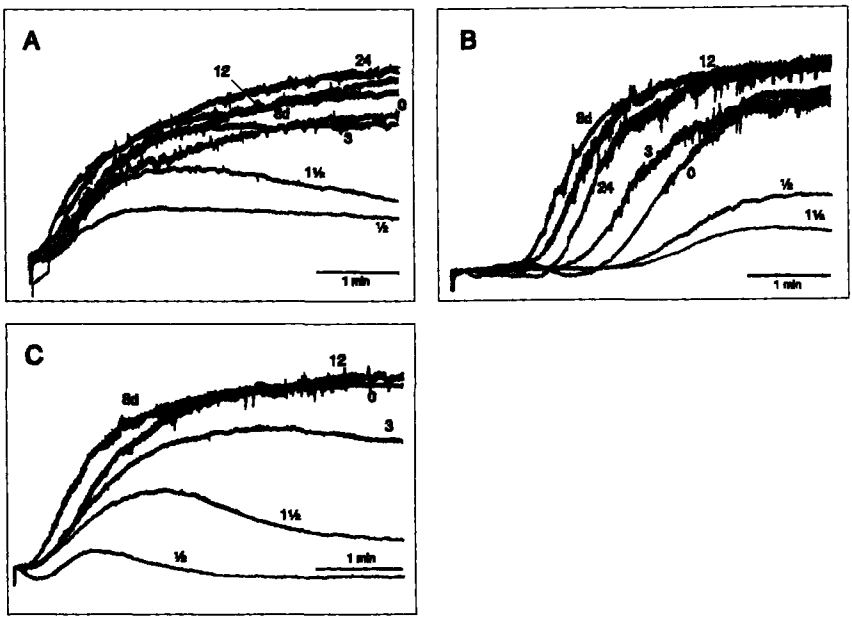

Figure 2 *Typical profile of platelet aggregation at different time points (in hours; d: days) after anistreplase. Aggregation induced with 3.6 μM ADP (A), 1 μg/mL collagen (B) and 1.6 mM arachidonic acid (C). Light transmission (y-axis) versus time (x-axis); bars represent 1 min of aggregation.*

Platelet release products

The plasma concentrations of the α-granule proteins PF4 and βTG were increased in all patients before treatment. After anistreplase, slightly different patterns emerged between the patients, but the individual courses of PF4 and βTG were closely parallel. The mean concentrations of these proteins gradually decreased after thrombolysis, but significantly increased again between 12 and 24 h after anistreplase (both $P < 0.05$), as shown in Figure 5A. After 7-9 days the majority of patients still had somewhat increased PF4 and ßTG. The plasma concentration of TxB_2 was increased in nine of the ten patients before thrombolysis (median 102 pg/mL, range 58-402 pg/mL). Immediately after anistreplase, TxB_2 increased to median 221 pg/mL (range 71-1312 pg/mL; not significantly different from baseline) and then gradually decreased. The TxB_2 concentration showed a significant increase between 12 and 24 h after thrombolysis (Fig 5B; $P < 0.05$) and then continued to decrease towards normal values before discharge.

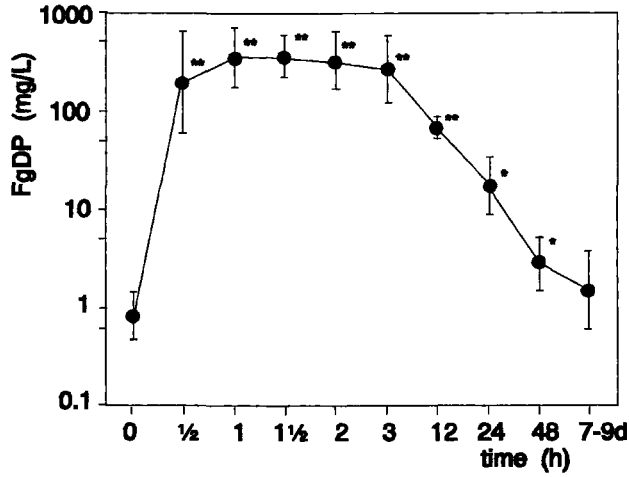

Figure 3 *FgDP concentration after anistreplase; mean ± SD; n = 8-10. Differences from pretreatment: * P < 0.01; ** P < 0.001.*

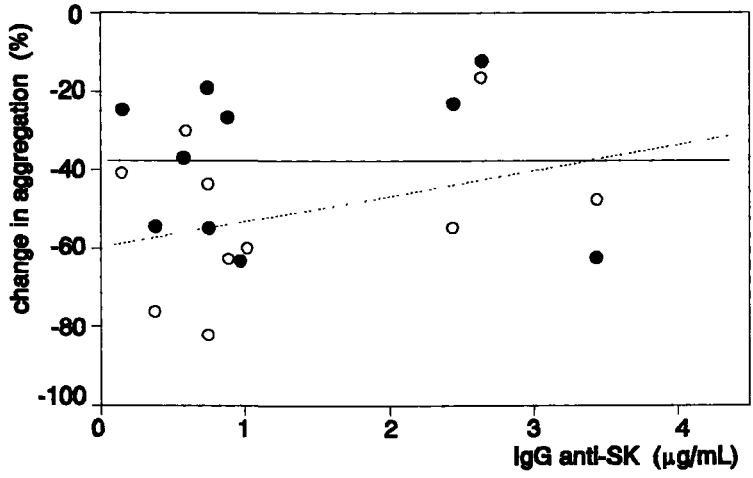

Figure 4 *Change in platelet aggregation after 2 h as a function of pre-treatment IgG anti-SK concentration. Results are calculated for each patient as % inhibition of platelet aggregation compared to pre-treatment value using 1.2 µM ADP (●; regression line [solid]: slope = -0.064; r = 0.0004; P = 0.99) and 1 µg/mL collagen (○; regression line [dashed]: slope = -6.44; r = 0.343; P = 0.33).*

In vitro studies

When normal PRP was incubated with a pharmacological amount of anistreplase the platelet aggregation induced by ADP was very rapidly inhibited; the inhibition became apparent already within 5 min (not shown). The aggregation induced by arachidonic acid and collagen was also inhibited, but slower than with ADP; significance was reached only after 15 min (Table 1). Ristocetin-induced agglutination was even slower inhibited (after 30 min 73.2 ± 10.0% of control, $P < 0.05$). After incubating PRP with anistreplase for 60 min, the aggregation by all four agonists was strongly reduced in comparison with the control experiments (Table 1). During these incubations there was a time-dependent, statistically significant release of α-granule proteins into the PRP. After 60 min, βTG had risen to 326 ± 187% of the untreated control, while the increase in PF4 was more moderate to 166 ± 49% of control (both $P < 0.05$ by paired t-test; n = 5).

The crossover experiments showed that anistreplase-treated platelets suspended in untreated plasma had maintained their aggregation response to collagen completely and to ADP partially (on the average 63% of control; range 55-70%; n = 4). However, when control platelets were suspended in anistreplase-treated plasma, the ADP-induced aggregation disappeared completely and the aggregation response to collagen was slightly diminished to 66% and 90% of control for low and the high collagen, respectively (not shown).

Table 1 *Platelet aggregation as a function of time after in vitro incubation with anistreplase, in % of maximal response. Mean ± SD; n = 5.*

	0 min	15 min	60 min
ADP 0.6 μM	23.8 ± 6.4	12.4 ± 2.2***	4.0 ± 1.5***
ADP 1.2 μM	46.8 ± 24.6	14.0 ± 7.7*	1.6 ± 3.1*
ADP 2.4 μM	63.0 ± 25.5	33.0 ± 19.0*	10.2 ± 7.5*
ADP 3.6 μM	59.6 ± 24.5	41.6 ± 21.1**	18.8 ± 15.3***
collagen 1 μg/mL	73.2 ± 8.1	51.2 ± 28.0	27.6 ± 28.9*
collagen 5 μg/mL	80.0 ± 7.5	74.8 ± 6.8*	70.4 ± 9.3***
arachidonic acid 1.6 mM	71.6 ± 6.6	32.8 ± 32.3*	24.2 ± 34.1*
ristocetin 1.5 mg/mL	82.8 ± 6.6	73.8 ± 7.0	69.4 ± 10.4***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$ *vs* control (saline only)

DISCUSSION

The complications of bleeding and reocclusion after thrombolytic therapy probably both involve platelets. However, there is considerable controversy in the literature regarding the effects of thrombolytic agents on platelet function, both in experimental animal models[4-7]

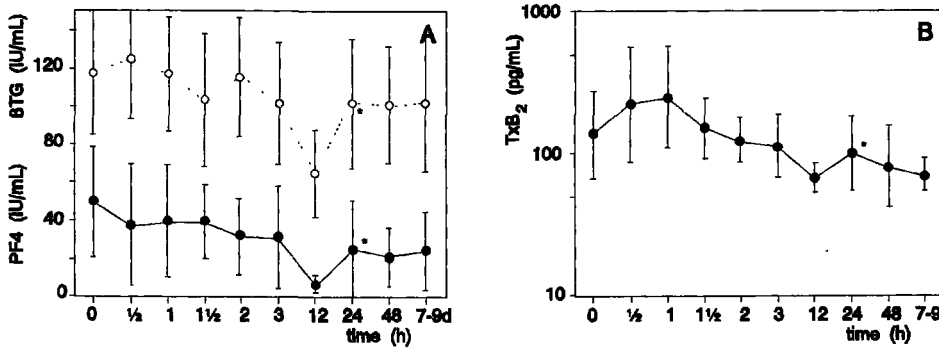

Figure 5 *Concentration of platelet release products in plasma after anistreplase. (A) PF4 (●) and BTG (○). (B) TxB_2 in plasma. Mean ± SD, n = 8-10; difference from preceding timepoint: * P < 0.05.*

and in man.[9-16] Possible explanations for these discrepancies are species differences, experimental design (*in vitro* incubations of washed platelets or PRP opposed to platelet studies after *in vivo* administration of the thrombolytic drug) and differences in drug concentrations and thus in plasmin generation. Schafer *et al.* have demonstrated that the effect of plasmin on human platelets *in vitro* is concentration-dependent: at low concentration, plasmin attenuates platelet aggregation, whereas a high concentration of plasmin induces aggregation.[20,21] Recently, a similar effect has been shown for tPA *in vitro*.[22] Investigations on platelet function *in vivo* after thrombolysis are limited in number and studies with anistreplase, which causes marked systemic plasminaemia, have not been published before.

The results of the present *in vivo* study clearly show that platelet aggregation is markedly reduced immediately after starting the thrombolytic treatment (Fig 1). This inhibitory effect of anistreplase was transient, since the platelets had regained their full aggregation response within 12-24 h after thrombolysis. In none of the patients was there any indication observed for a stimulation of platelet aggregation during the first day of treatment; the responses to sub-threshold amounts of ADP were very low and remained so after anistreplase. Because patients having taken aspirin or other anti-platelet drugs were excluded from the study, which was confirmed by the normal pre-treatment aggregation profiles, we conclude that anistreplase did not induce systemic activation of platelets. This result is in contrast with some *in vivo* findings after SK or urokinase.[13,14] Beginning on the second day after thrombolysis there was a reactive increase in aggregation, which had completely disappeared before discharge, about one week after the AMI. The course of aggregation inhibition observed was parallel with that of increase in FgDP concentration; this is compatible with a causal relation, to be discussed further. The biphasic pattern of aggregation (inhibition in the early hours after thrombolysis and hyperaggregation during the next few days) has been observed before after streptokinase and tPA.[16] It is also in accordance with the second period of platelet hyperaggregation observed 24 h after

urokinase therapy by Griguer *et al.*[13] However, the first period of hyperaggregation found by these authors, which occurred between 4 and 8 h after thrombolysis with urokinase,[13] could not be confirmed in the present investigation. These results in patients are essentially different from a number of animal studies. In rabbits, converse temporal effects of tPA and SK were described: there was an early stimulation of ADP-induced aggregation within 30 min, while some hours later the aggregation response was significantly decreased as compared with the control animals.[7] Since the design of the latter study and the methods used are comparable to ours, apparently the difference in species must explain the contrary effects on platelets.

The platelet release products PF4 and βTG, which were increased before therapy, showed a gradual decrease thereafter. TxB_2 showed a small peak immediately after anistreplase, but this was not statistically different from pre-treatment. This increase, although small, is not unlike the findings of Fitzgerald *et al.*, who found the urinary metabolites of thromboxane-A_2 to be significantly increased as compared with patients not receiving SK.[14] Our failure to detect systemic platelet activation and significant increases in plasma TxB_2 just after anistreplase does not exclude the possibility of a limited activation process, presumably confined to the location of the initial infarction where after dissolution of the thrombus the newly exposed vascular lesion restimulates platelet activation and thrombin generation.[23] On the other hand, there are recent indications from *in vitro* studies that the synthesis of TxB_2 is severely impaired by several thrombolytic drugs,[24] which could offer an additional explanation for the lack of an increase in plasma TxB_2 in our patients.

The significant increases in PF4, ßTG and TxB_2 between 12 and 24 h after thrombolysis (Fig 4) appear to coincide with the stimulation in ADP-induced platelet aggregation (Fig 1). These findings are suggestive of a more generalized platelet activation, which could offer an explanation for the clinically well-known risk of reocclusion in this period after successful thrombolysis. The trigger for this activation remains unclear but the onset of hyperaggregability is presumably revealed at a time when the systemic plasminaemia is decreasing and fibrinogen concentration is increasing. Possible stimuli to increased aggregation in these conditions include damaged vessel wall at the site of the coronary lesion, catecholamine concentration and, in particular, heparinization in view of the evidence indicating that heparin can induce hyperaggregability of platelets.[25]

Results from the present study indicate that the concentrations of pre-treatment anti-SK antibody found in this population of AMI patients do not influence the extent of platelet inhibition by anistreplase. This conclusion contradicts the finding of a recent *in vitro* study[26] which, however, employed concentrations of anistreplase and SK much higher than those achieved therapeutically. Our demonstration of a lack of antibody effect *in vivo* supports the conclusion from another *in vitro* study which used therapeutically relevant concentrations of thrombolytic agents.[12]

The findings from the *in vitro* part of the present investigation with anistreplase lend full support to the results in our patients concerning the inhibition of platelet activity. The existing controversy as to the effects of thrombolytic drugs on platelets appears to originate mainly from *in vitro* experiments rather than from studies in patients.[9-16,21] When

interpreting results from *in vitro* experiments as models for the *in vivo* situation it should be taken into account that platelet aggregation in PRP probably only partially reflects the aggregation process in blood because of the presence of red blood cells and differences between static and flow systems. These findings stress the preference for platelet studies after thrombolysis *in vivo* over model systems in the laboratory.

Because the platelet count was constant over the first part of the study period and the restoration of platelet aggregability is too fast to be explained by the release of newly formed platelets from the bone marrow, the transient response is suggestive of a non-platelet factor as the cause of the inhibition. This is corroborated by the results from our *in vitro* crossover experiment, which indicates that the aggregation defect is likely to be associated with plasma rather than with platelets. Therefore, it is less probable that the degradation of platelet surface receptors by plasmin plays a major role in the aggregation defect. Presumably, the high concentration of FgDP, which correlated significantly with the decreased aggregation induced by ADP, is the principal factor influencing platelet aggregation after thrombolysis. The competition between these degradation products and fibrinogen for binding to the platelet glycoprotein IIb/IIIa receptor, which is involved in ADP-induced aggregation, could provide a suitable explanation for the transient inhibition observed.[11,27]

The possibility that the observed effects are completely or in part caused by the cardiac catheterization and coronary angiography, can not entirely be ruled out. In our opinion, such a possibility is quite unlikely because the changes in platelet function are not synchronous with the timing of the angiographies. Moreover, we previously had ascertained that platelet function is not inhibited by the angiographic procedure and the contrast agent used (report in preparation).

The main limitation of our study is the relatively low number of patients who presented with symptoms of AMI, were eligible for thrombolytic treatment and, most importantly, had not used aspirin or other drugs affecting platelet function during the previous two weeks. However, exclusion of aspirin users allowed us to make firm conclusions on the effects of anistreplase on platelet function. After the ISIS-2 trial[2] AMI patients not taking aspirin are seen much less frequently and yet such patients are required for performing studies like the present one.

Since it has now been found that platelet function is affected for at least 6-12 h after anistreplase, it would be interesting to reconsider the simultaneous administration of aspirin and thrombolytic drugs. There is some evidence from *in vitro* studies that the inhibitory effect of anistreplase and, to a lesser degree, SK on ADP-induced platelet aggregation is additive with aspirin;[12] this might afford potential benefit to the patient. On the other hand, such an interaction might increase the risk of bleeding. There are, however, no strong data supporting increased bleeding from clinical trials using the combination of aspirin and a thrombolytic drug (SK) creating a systemic lytic state.[2] On the contrary, increased bleeding due to this interaction has been suggested for thrombolytic agents causing less systemic lysis.[8,28] The interaction between thrombolytic agents and aspirin deserves further study, also in view of the concept that a systemic lytic

state, as induced by anistreplase and SK, might provide protection against ongoing thrombosis by inhibiting platelet activation during thrombolysis.[29,30]

In conclusion, we found that anistreplase induced an immediate but transient defect in *ex vivo* platelet aggregation in patients treated for AMI, without signs of systemic platelet activation in the early phase after thrombolysis. Local activation of platelets could not be excluded, but would be of very limited extent. Our results in patients, together with experiments using platelets treated *in vitro* with anistreplase, suggest that this temporary platelet inhibition is mediated by fibrin(ogen) degradation products, although the hypothesis of a direct effect of plasmin on platelet surface receptor proteins cannot be completely rejected yet. Additional investigations are required to obtain definite proof of the mechanisms, by which thrombolytic drugs bring about this inhibition of platelet function.

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Chapter 11

Interactions between thrombolytic agents and platelets: effects of plasmin on platelet glycoproteins Ib and IIb/IIIa

JJML Hoffmann and WCM Janssen

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SUMMARY

The mechanisms by which thrombolytic agents affect platelet function are not yet fully elucidated. The aim of the present study was to investigate the effects of plasmin, generated by thrombolytic agents in plasma, on platelet glycoproteins (GP) Ib and IIb/IIIa. Platelet-rich plasma was incubated with pharmacological amounts of streptokinase, anistreplase and tissue-type plasminogen activator and the platelet surface GPs were investigated with a panel of monoclonal antibodies using flow cytometry. As assessed from the mean fluorescence intensity of incubated and control platelets, no significant changes in the binding of antibodies to GP Ib and GP IIb/IIIa were found. The functional integrity of these glycoproteins was severely impaired by treatment with the thrombolytic agents, as shown by significant inhibition of ADP- and ristocetin-induced platelet aggregation. Experiments with purified plasmin and washed platelets indicated significant degradation of GP IIb/IIIa and upregulation of GP Ib, which is in agreement with previous findings. In addition, platelet activation by plasmin was shown using two monoclonal antibodies to activation-specific antigens.

We conclude that degradation of platelet GPs by plasmin offers no likely explanation for the defect in platelet function, which is induced by thrombolytic agents in platelet-rich plasma.

INTRODUCTION

Plasminogen activators used clinically as thrombolytic drugs convert plasminogen to plasmin and the dosages used generate so much plasmin that the inhibiting potential of α_2-antiplasmin and other protease inhibitors is surpassed. This results in the so-called systemic lytic state and platelets are known to be affected by the systemic lytic state. Plasmin can inhibit platelet aggregation in platelet rich plasma (PRP) *in vitro*[1,2] and recently we have shown that plasmin generated after the administration of anistreplase to patients caused a marked decrease in platelet aggregation *ex vivo*, which lasted for about 12 hours.[3] The mechanism by which platelet function is inhibited is not precisely known. Three possible explanations can be proposed: extensive depletion of plasma fibrinogen, competition of fibrin(ogen) degradation products and intact fibrinogen for binding sites on the platelets and finally, degradation of platelet membrane receptor proteins.[4]

Some of the glycoproteins on the platelet surface are necessary for adequate platelet function. Among them are glycoprotein Ib (GP Ib), the receptor for the von Willebrand factor, and GP IIb/IIIa, the fibrinogen receptor. It has been shown *in vitro* that plasmin can degrade GP Ib on washed platelets,[5-7] but it is questionable whether this plasmin-induced proteolysis occurs also in PRP.[6,8] Recently, evidence has been presented that GP Ib remains intact after *in vivo* administration of thrombolytic drugs.[8,9] Concerning the effect of plasmin on GP IIb/IIIa the available data are highly controversial: decrease, increase and no variation in this GP complex have all been reported.[5,6,8,10-12]

The aim of the present study was to investigate the effects of thrombolytic agents on platelet surface antigens as defined by different monoclonal antibodies. We used incubations of PRP with pharmacological concentrations of three thrombolytic drugs, as well as treatment of washed platelets with plasmin. Binding of the monoclonal antibodies to the surface GPs was visualized using fluorescence flow cytometry.

MATERIALS AND METHODS

Materials

Purified human plasmin was purchased from Sigma, St.Louis, MO, USA. The thrombolytic drugs used were of clinical grade: streptokinase (Kabikinase®) was obtained from KabiVitrum, Amsterdam, the Netherlands; anistreplase (APSAC; Eminase®) was a gift from SmithKline Beecham Farma, Rijswijk, the Netherlands and recombinant tPA (Actilyse®) was donated by Dr. Karl Thomae GmbH, Biberach, FRG. Aprotinin (Trasylol®; 10,000 KIU/mL) was purchased from Bayer AG, Leverkusen, FRG.

The monoclonal antibodies were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam (thromb/1, thromb/7, thromb/8, gran/12), from Dako A/S, Glostrup, Denmark (Y2/51 and AN51), and Immunotech SA, Marseille, France (thromb/6). The specificity and references of these

antibodies are given in Table 1. With the exception of AN51 and thromb/6 antibodies, which were directly labelled with fluorescein isothiocyanate (FITC), the antibodies were in the purified, unconjugated form. We used $F(ab')_2$ fragments of goat anti-mouse IgG, FITC-conjugated, as the second antibody (Caltag Laboratories, South San Francisco, CA, USA). For platelet aggregation we used adenosine diphosphate (ADP; Sigma) and ristocetin (Lundbeck, Copenhagen, Denmark).

Table 1 *Specificity of the monoclonal antibodies with designation of clusters of differentiation (CD number) according to the International Workshop on Human Leukocyte Differentiation Antigens.*[13]

antibody (clone)	CD number	antigen	reference
AN51	42b	GP Ib	14
thromb/1 (C17)	61	GP IIIa in the GP IIb/IIIa complex	15
Y2/51	61	GP IIIa (not complex-specific)	16
thromb/7 (6C9)	41	GP IIb/IIIa complex	17
thromb/6	62	GMP-140 on activated platelets (PADGEM)	18
gran/12	63	lysosomal p53 antigen on activated platelets	19
thromb/8	9	p24 antigen	20

Blood collection

Blood was drawn between 8 and 10 a.m. from healthy volunteers through a clean puncture of an antecubital vein after short, minimal stasis. The first few millilitres were discarded. The blood was collected into plastic syringes or siliconized glass tubes containing trisodium citrate (0.01 M final concentration). Platelet rich plasma was prepared by low-speed centrifugation (180 *g* for 5 min at 21 °C) and platelet poor plasma by centrifugation at 3000 *g* for 15 min. The platelet count of PRP was set to 200/nL with platelet poor plasma.

Platelets were washed in phosphate buffered saline (PBS; 150 mM NaCl and 0.01 M phosphate, pH 7.4), containing 20 mM EDTA and resuspended in the same buffer. When using the AN51 monoclonal antibody, an additional aliquot was washed and resuspended in PBS without EDTA.

Incubations

Platelets in PRP were treated with the thrombolytic drugs, using concentrations equivalent to those attained therapeutically: SK 300 U/mL, APSAC 10 mU/mL (about 70 nM) and tPA 5 μg/mL (all final concentrations). Solutions of these drugs were added to PRP containing 200 platelets per nL and incubated for 90 min at 37 °C. At the end of the incubation, aprotinin was added (final concentration 250 KIU/mL) in order to inhibit ongoing plasmin activity. In parallel to the experiments with active drugs, control incubations were carried out using saline. In some experiments, samples of whole blood

anticoagulated with citrate or heparin (10 U/mL) or PRP prepared from the latter were tested with the thrombolytic agents.
Washed platelets (200/nL), resuspended in PBS-EDTA, were treated with plasmin in the concentrations indicated for 90 min at 37 °C and the incubation was terminated by adding aprotinin (final concentration 1000 KIU/mL). Then the platelets were washed and stained as described below. The generation of plasmin by SK in PRP was measured using the chromogenic substrate S-2251 and compared to a calibration curve using purified plasmin.

Immunofluorescence
Incubated platelets were washed twice and divided into 50 μL aliquots containing 10^7 platelets each. Each aliquot was incubated with 50 μL of a saturating amount of monoclonal antibody for 30 min at 4 °C. In order to prevent platelet activation by the antibodies, a mixture of citrate, theophylline, adenosine and dipyridamole (CTAD) was added together with the antibody. For indirect staining, the platelets were washed twice and incubated with 50 μL of a 1:30 diluted solution of FITC-labelled goat anti-mouse IgG, also for 30 min at 4 °C and then suspended into 0.5 mL PBS for measuring. In experiments with directly labelled monoclonal antibodies, the platelets were suspended in 0.5 mL PBS immediately after the incubation.

For measuring platelet-bound fluorescence, we used a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA), which was operated according to the instructions of the manufacturer. The optical system was calibrated with fluorescent microbeads (Calibrite beads; Becton-Dickinson) and checked daily. The fluorescence measurement was confined to platelets by means of electronic gating on their forward and side scatter signals. This procedure had been validated before and indicated that over 99% of the events within this gate reacted with platelet-specific antibodies and were negative with a red cell specific monoclonal antibody. In each sample at least 10,000 platelets were counted and their fluorescence signal was recorded with logarithmic amplification. Signal analysis was accomplished with the use of the Facscan Research software (Becton-Dickinson). The fluorescence signal was expressed as the mean fluorescence intensity (MFI; in channel number units).

Platelet aggregation
PRP was treated with each of the thrombolytic drugs and a saline control, as described above, and aggregation measured in an optical aggregometer (PAP-4; Biodata, Hatboro, PA, USA). The minimum and maximum values of light transmission were set using PRP and platelet poor plasma, respectively. To 0.45 mL of treated PRP we added 50 μL of agonist solution, giving final concentrations of 3.6 μM ADP or 1.5 mg/mL ristocetin.

Statistics
Results are given as mean ± SD. The paired t-test was used for comparing data, considering P values < 0.05 significant.

RESULTS

Effects of thrombolytic drugs in PRP

Platelets from healthy subjects were treated in PRP with pharmacological concentrations of the thrombolytic agents and then stained with monoclonal antibodies to GP Ib, GP IIb/IIIa and some other surface antigens (Table 1). None of the three thrombolytic drugs caused significant changes in the density, expressed as MFI, of the epitopes recognized by the antibodies, as shown in Table 2. Subpopulations of platelets with higher or lower fluorescence intensity than the main population never emerged after treatment with the thrombolytic drugs. When testing platelets every 15 min during the incubation period of 90 min, no changes in MFI were observed, either. Under these conditions 0.9 ± 0.05 CU/mL plasmin was generated ($n \approx 6$).

Even when PRP was treated with a 25,000 U/mL SK, which is about 100-fold the maximun therapeutic concentration, or with plasmin (up to 2.0 CU/mL) there were no significant changes in MFI (data not shown). The expression of the activation markers thromb/6 and gran/12 did not increase during incubation with any of the thrombolytics (Table 2), indicating no substantial platelet activation by these drugs. Finally, there was no change in the intensity of the CD9 antigen, which was used as a control.

Table 2 *Effect of treatment of platelets in PRP with SK (300 U/mL), anistreplase (10 mU/mL) and tPA (5 μg/mL) on the mean fluorescence intensity (MFI) of antibodies binding to platelets. PRP incubated with saline served as a control. Mean ± SD; n = 8.*

	MFI (channel)			
Antibody	control	SK	APSAC	tPA
AN51	256 ± 17	258 ± 18	252 ± 17	266 ± 24
AN51, no EDTA	438 ± 15	427 ± 17	426 ± 13	433 ± 26
thromb/1	489 ± 18	495 ± 25	492 ± 22	506 ± 38
Y2/51	506 ± 22	503 ± 21	504 ± 22	507 ± 27
thromb/7	554 ± 12	551 ± 12	553 ± 14	555 ± 19
thromb/6	196 ± 12	203 ± 12	200 ± 14	201 ± 29
gran/12	199 ± 15	207 ± 14	202 ± 15	209 ± 28
thromb/8	437 ± 27	436 ± 25	441 ± 21	443 ± 28

Effects of thrombolytic drugs in whole blood

In order to investigate whether the presence of erythrocytes or calcium ions had any influence on the effect of thrombolytic agents on platelet antigens, incubations were performed using citrated whole blood, heparinized whole blood and heparinized PRP as

substitutes for citrated PRP. The results were identical to those presented above for citrated PRP (data not shown).

Effects of plasmin on washed platelets

Washed platelets were treated with increasing concentrations of plasmin for 90 min and then stained with monoclonal antibodies. Typical examples are given in Figure 1 for anti-GP IIb/III (thromb/7 and Y2/51) and anti-GP Ib (AN51). It appeared that significant changes were only obtained when using ≥ 1.0 CU/mL plasmin ($P < 0.01$ *vs.* control) and therefore the subsequent incubations were carried out using 2.0 CU plasmin per mL of washed platelets.

Figure 2 shows that the GP IIb/IIIa epitopes recognized by the antibodies thromb/1 (CD61) and thromb/7 (CD41) nearly completely disappeared, while the Y2/51 (CD61) epitope on the same molecule remained intact. The expression of the GP Ib epitope AN51 (CD42b) did not significantly change. The activation antigens thromb/6 and gran/12 were clearly discovered (Fig 2).

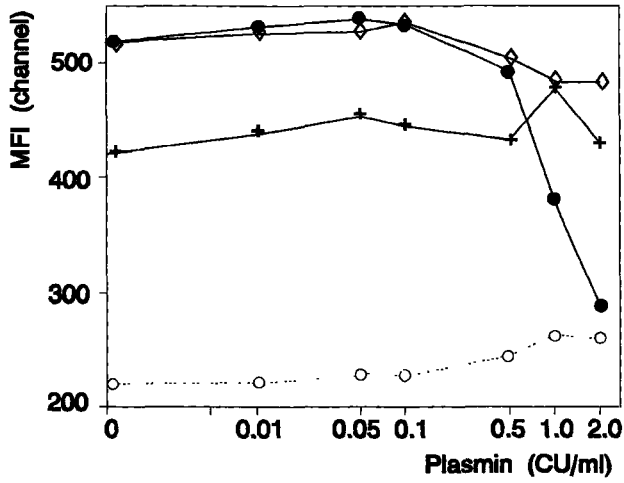

Figure 1 *Effect of increasing concentrations of plasmin in the MFI of GP Ib and two GP IIb/IIIa epitopes. AN51 (+), thromb/7 (●), Y2/51 (◇) and control (○). Means of 2-4 determinations.*

Platelet aggregation

After incubating PRP with therapeutic concentrations of SK, APSAC and tPA, the aggregation of platelets, induced by ADP and ristocetin, was progressively inhibited. A typical profile of ADP-induced aggregation of normal PRP treated with SK is shown in Figure 3. The effects of tPA and APSAC on aggregation were similar, but the degree of

inhibition showed some variation. APSAC caused about the same grade of inhibition as SK did, whereas tPA had a more moderate effect (data not shown).

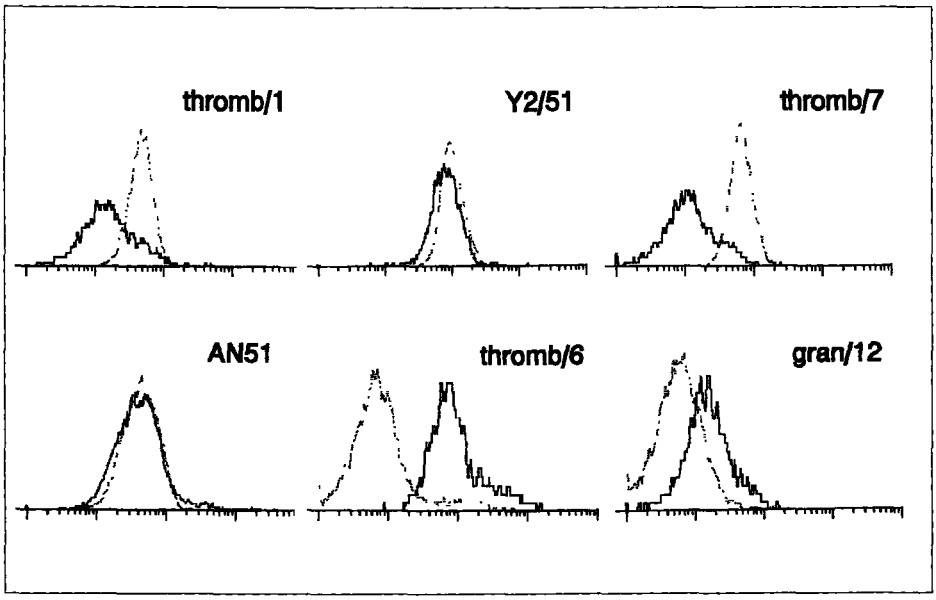

Figure 2 *Fluorescence histograms of washed platelets, treated with plasmin (2.0 CU/mL) and stained with the antibodies indicated (solid lines). Saline control (dashed lines).*

DISCUSSION

In an attempt to elucidate the mechanisms involved in the platelet dysfunction arising during thrombolytic therapy,[3] we have focused on the integrity of platelet surface glycoprotein receptors known to play a role in platelet aggregation. In order to imitate the conditions occurring *in vivo* as closely as possible, we used platelets in PRP and pharmacological concentrations of thrombolytic drugs. Under these circumstances, platelet aggregation defects are found which are very similar to those seen in patients (Fig 3).[3]

The effect of plasmin generated in PRP on GP Ib expression has not been described in detail before. Balduini *et al.* using a monoclonal antibody to GP Ib and flow cytometry, reported briefly that treatment of PRP with SK caused no modification in platelet composition.[8] This is in accordance with the lack of effect of SK, APSAC and tPA on platelets in PRP found here (Table 2), but not with previous findings in washed platelets.[5] We showed that plasmin had no effect on MFI in PRP, but 1.0 CU/mL significantly affected GP Ib on washed platelets (Fig 1); this excludes the possibility of an ineffective

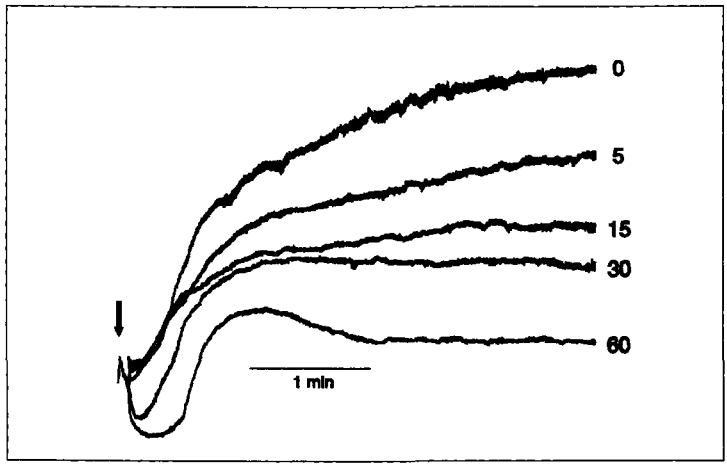

Figure 3 *Tracings of platelet aggregation induced with 3.6 μM ADP, in PRP which was treated for the times indicated (min) with 300 U/mL SK.*

amount of plasmin. In theory, replenishment of surface GP Ib from intracellular stores[7,9] could obscure GP Ib degradation, but our finding of no change in GP Ib expression for 90 min rejects this possibility. The reported failure to find significant differences in GP Ib density after *in vivo* thrombolysis in patients[8,9] despite a reduction in ristocetin-induced platelet agglutination during such therapy,[3,8] is fully compatible with our *in vitro* findings. Therefore it is more likely that this defect is caused by proteolysis of plasma von Willebrand factor,[21] rather than of platelet GP Ib.

Data on the effect of thrombolytic agents or plasmin on GP IIb/IIIa are highly controversial, but most results are from studies with washed platelets.[5,6,10,12] Two preliminary studies carried out in a plasma milieu showed concordant results, namely no or at most subtle changes in GP IIb/IIIa expression, in spite of significant reduction in GP IIb/IIIa-mediated fibrinogen binding and platelet aggregation.[8,11] While we demonstrated significant effects of plasmin on washed platelets (Figs 1,2), no such effects were found in platelets in PRP treated with the same concentration of plasmin or with the thrombolytic drugs investigated (Table 2). This agrees with the results of other investigators.[8,11] The same holds true for the appearance of activation antigens after plasmin treatment of washed platelets.

The conspicuous difference between effects in PRP and in purified systems is almost certainly due to the presence in plasma of plasmin inhibitors and large quantities of other suitable plasmin substrates like fibrinogen and some clotting factors. Moreover, even after complete activation of all available plasminogen in plasma, the concentration of plasmin is still not sufficient to overcome the threshold for an effect on platelet GPs. We could generate only 0.9 CU/mL plasmin in normal PRP by exhaustive activation with SK, a value

similar to previous data,[5,22] whereas Figure 1 indicates that at least 1.0 CU/mL is necessary for a detectable decrease in GP IIb/IIIa epitopes. Thus, studies on the effects of plasmin using washed platelets should be interpreted with reserve and the results not be extrapolated to more physiological conditions, like some authors seem to suggest.[5]

It is conceivable that the effects of thrombolytic agents on platelets are different in PRP and in whole blood,[23] but from the lack of changes in surface GPs we infer that neither red blood cells nor calcium ions play a significant role in the interaction between plasmin and platelets.

The significant decreases in surface GP IIb/IIIa of washed platelets treated with plasmin, as found with the antibodies thromb/1 and thromb/7 and, to a more limited extent, with Y2/51 (Figs 1,2) disagree with the activation and upregulation of the GP IIb/IIIa complex found by others.[10,22,24,25] Apparently, the incubation time used here was long enough to degrade even upregulated GP IIb/IIIa. On the other hand, low plasmin concentrations failed to induce any notable increase in the epitopes reacting with thromb/7 and Y2/51 (Fig 1). A small but significant increase was present, however, using the anti-GP Ib antibody (AN51) if a relatively high concentration of plasmin, 1.0 CU/mL, was used (Fig 1). This supports the concept of GP Ib redistribution.[7,9,12]

Finally, the main limitations of the current investigations must be considered. Firstly, it is an *in vitro* study, the results of which can not be directly extrapolated to the *in vivo* situation. In the second place it should be kept in mind that the immunological technique used here can only provide information on the structure of the GP receptors, rather than on their function. Whereas the anti-GP Ib and anti-GP IIb/IIIa monoclonals employed are known to react with epitopes which are necessary for adequate platelet function,[14-17] there remains the possibility that other functionally critical epitopes on the receptors could be degraded by plasmin and still lead to impaired platelet function. However, recent findings by others that fibrinogen receptors on activated platelets are resistant to plasmin digestion[26] and that there remains sufficient functional GP IIb/IIIa to support fibrinogen-dependent aggregation after treatment of platelets with tPA,[25] sustain our findings. Furthermore, we could not demonstrate any effect of the thrombolytic agents on the thromb/8 antigen, which has no known receptor function in haemostasis and therefore served as an indifferent control.

In conclusion, we have shown that plasmin degradation of the platelet surface receptors GP Ib and GP IIb/IIIa plays no relevant role in the development of the platelet dysfunction, which is induced by thrombolytic drugs in platelet rich plasma. Therefore this platelet defect must be explained by other mechanisms and this is currently under investigation in our laboratory.

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Chapter 12

Interactions between thrombolytic agents and platelets: effects of fibrinogen degradation products on platelet aggregation

JJML Hoffmann

in preparation

SUMMARY

Thrombolytic therapy is frequently associated with impaired platelet function, which may result in clinical bleeding, a prolonged bleeding time and decreased platelet aggregation. The exact cause for this platelet dysfunction is not well understood. In order to investigate one of the possible mechanisms, the present study addresses the effects of fibrinogen degradation products on platelet aggregation. Both purified FDP fragments and crude, plasmic digests of fibrinogen were used.

In normal platelet-rich plasma, the fragments had no effect on ADP-induced aggregation. However, when the concentration of fibrinogen in platelet-rich plasma was reduced to values occurring during thrombolytic therapy, platelet aggregation was clearly inhibited by fragments Y and D, but not by fragment E. Similar inhibition of aggregation was observed when crude fibrinogen digests were added to platelet-rich plasma with a low fibrinogen concentration. Besides the decrease in magnitude of aggregation, the FDP fragments also caused a reduction in the rate of aggregation.

It is concluded that high concentrations of FDP compete with fibrinogen in binding to its platelet receptor and thus inhibit platelet aggregation. This offers a likely explanation for the transient platelet defect observed during thrombolytic therapy.

INTRODUCTION

Thrombolytic therapy has been proven valuable for restoring the patency of infarct-related coronary arteries in patients with acute myocardial infarction (AMI) and for improving survival after AMI.[1-3] Bleeding, mainly confined to vascular puncture sites, remains one of the major adverse events associated with this type of treatment and it is independent of the clot-selectivity of the thrombolytic agent.[4-6] The exact mechanisms which lead to bleeding are not known, although the dissolution of fibrin in haemostatic plugs is thought to be the predominant cause. Other potential contributing factors are the consumption of fibrinogen, the generation of high concentrations of fibrin(ogen) degradation products (FDP) and impairment of platelet function.[4,7] There is insufficient evidence that increased fibrinogen degradation products (FgDP) by their action as antithrombins or a low fibrinogen concentration can explain the bleeding tendency. Currently, many investigators believe that platelets play an important role as a cause of the impaired haemostasis associated with thrombolytic therapy.

A number of hypotheses for the interaction of thrombolytic agents with platelet function have been presented. Plasmin generated on the platelet surface by the plasminogen activating drugs could directly degrade the glycoprotein (GP) receptors necessary for platelet function (GP Ib and GP IIb/IIIa). Alternatively, platelet activity might be affected indirectly by the high concentrations of FgDP, which inhibit fibrinogen binding to its receptor (GP IIb/IIIa) on the platelet surface; fibrinogen binding is essential to support platelet aggregation.[8] Finally, under the lytic state the degradation of circulating fibrinogen might be large enough to deplete fibrinogen to a concentration below the critical value for supporting platelet function. In spite of converse findings from experiments with washed platelets, there is now accumulating data that thrombolytic agents have no direct effect on platelets, neither *in vivo*, nor in plasma *in vitro*.[9-11] It is therefore likely that the transient platelet dysfunction seen after thrombolysis[12,13] is caused by FgDPs interfering in the binding of fibrinogen to platelets. This hypothesis is supported by the finding that increased FgDP concentrations are significantly correlated with bleeding after thrombolytic therapy with tPA.[14]

It has been previously shown that the different FDP fragments have different effects on the binding of fibrinogen to platelets: fragments Y and D can bind to GP IIb/IIIa or inhibit ADP-induced aggregation, whereas fragment E has no such effect.[15-17] Because the composition of FgDPs is rather variable during thrombolytic therapy,[18] it is interesting to investigate which fragments compete with fibrinogen for the platelet binding sites and thus affect platelet aggregation.

The purposes of the present study were to document the effects of thrombolytic drugs on platelet aggregation *in vitro* and to investigate the effects of different FDPs, either as a fibrinogen digest comparable to what occurs during thrombolytic therapy or as purified fragments, on fibrinogen-supported platelet aggregation.

MATERIALS AND METHODS

Materials

The aggregation agonists used were adenosine diphosphate (ADP; Sigma Chemical Co., St.Louis, MO, USA) and collagen from bovine tendon (Hormon Chemie GmbH, Munich, FRG). Purified human fibrinogen and streptokinase (Kabikinase®) were purchased from KabiVitrum, Stockholm, Sweden and aprotinin (Trasylol®) from Bayer AG, Leverkusen, FRG. Anistreplase (Eminase®) was a gift from SmithKline Beecham, Rijswijk, the Netherlands and recombinant tissue-type plasminogen activator (rtPA; Actilyse®) from Dr. Karl Thomae, Biberach, FRG.

Crude, whole FgDP was prepared by digesting plasminogen-rich fibrinogen with streptokinase in the absence of calcium. The digestion was terminated by adding aprotinin (final concentration 250 KIU/mL) after 3, 20 and 60 min for early, intermediate and late digests, respectively.[19] The composition of these digests was verified using polyacrylamide gel electrophoresis. The FDP fragments Y, D_{cate}, D_{EGTA} and E_{EGTA} were isolated and purified as described previously.[20,21]

Platelet preparation

Blood was drawn from healthy volunteers through a clean venepuncture after short, minimal stasis. The first few millilitres were discarded. The blood was collected into plastic syringes or siliconized glass tubes containing trisodium citrate (0.01 M final concentration). Platelet rich plasma was prepared by low-speed centrifugation (180 *g* for 5 min at 21 °C) and platelet poor plasma by centrifugation at 3000 *g* for 15 min. The platelet count of PRP was adjusted to 200/nL with platelet poor plasma or with modified Tyrode's buffer (130 mM NaCl, 2.6 mM KCl, 12 mM $NaHCO_3$, 2 mM $CaCl_2$, 5 mM glucose, 2 g/L bovine serum albumin, pH 7.3).

Treatment of platelets with thrombolytic agents

Platelets in PRP (200 /nL) were supplemented with pharmacological amounts of the thrombolytics, giving final concentrations of SK 300 U/mL, anistreplase 10 mU/mL (about 70 nM) and tPA 5 μg/mL and incubated at 37 °C for the times indicated. An aliquot was removed and added to aprotinin (final concentration 250 KIU/mL) in order to inhibit ongoing plasmin activity. It had been previously verified that this concentration of aprotinin did not affect platelet aggregation induced by ADP and collagen. In parallel to the experiments with active drugs, control incubations were carried out using saline.

Platelet aggregation

Platelet aggregation was carried out in a four-channel optical aggregometer (PAP-4; Biodata, Hatboro, PA, USA), stirring speed 800 rpm and with ADP and collagen as the agonists. PRP was measured at 200 platelets /nL. To 0.45 mL of PRP we added 50 μL of agonist solution, giving final concentrations of 0.6, 1.2, 2.4 and 10.8 μM ADP and 1.1 μg/mL collagen. Aggregation responses were recorded as percentage of maximal light transmission for each sample. The rate of aggregation was calculated by the instrument as the steepest slope in arbitrary transmission units per minute (AU/min).

The effects of crude or purified FDP preparations on platelet aggregation was tested by adding 5 μL aliquots of concentrated FDP solutions to 0.45 mL PRP. The mixture was warmed to 37 °C for 5 min prior to the addition of the agonist. Control samples were made using saline alone.

Statistics

All experiments were performed in quadruplicate and the results are given as mean ± SD. Paired student t-test was used for comparing the data. P values < 0.05 were considered significant.

RESULTS

Table 1 *Effect of thrombolytic agents on platelet aggregation (% of maximal response) at different time points. Mean ± SD; n = 5.*

	incubation time (min)				
	0	5	15	30	60
ADP 0.6 μM					
tPA	22 ± 3	18 ± 3*	16 ± 6	13 ± 4*	7 ± 3***
streptokinase	20 ± 4	14 ± 5*	9 ± 3**	8 ± 4*	1 ± 1***
anistreplase	24 ± 3	13 ± 2***	12 ± 1***	9 ± 2***	4 ± 1***
ADP 3.6 μM					
tPA	64 ± 16	45 ± 12**	34 ± 15**	27 ± 15**	16 ± 9***
streptokinase	73 ± 22	49 ± 18	36 ± 26	17 ± 12**	8 ± 9**
anistreplase	60 ± 12	54 ± 13**	42 ± 11**	26 ± 4*	19 ± 8***
collagen 1.1 μg/mL					
tPA	79 ± 9	80 ± 9	75 ± 7	63 ± 20*	59 ± 11*
streptokinase	70 ± 6	57 ± 14	35 ± 27	22 ± 23*	5 ± 28*
anistreplase	73 ± 4	70 ± 4	51 ± 14	33 ± 11*	28 ± 15*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$ compared with untreated platelets

Effect of thrombolytic agents

Upon incubation of PRP with any of the thrombolytic drugs, there was a rapid, progressive decrease in aggregation response observed. Inhibition of aggregation became apparent already after 5 min of incubation. Qualitatively, the effects were similar for the three

thrombolytic agents, but the degree of aggregation inhibition varied. SK caused the strongest decrease in aggregation, closely followed by anistreplase, whereas tPA had a more moderate effect (Table 1).

Effect of purified FDP fragments

Purified FDP fragments Y, D_{cate}, D_{EGTA} or E_{EGTA}, when added to PRP prepared from normal plasma in a final concentration of 1 mg/mL, did not significantly influence the aggregation response to ADP (0.6 - 10.8 μM) and collagen (1.1 μg/mL) in comparison with control PRP to which no FDP was added (not shown).

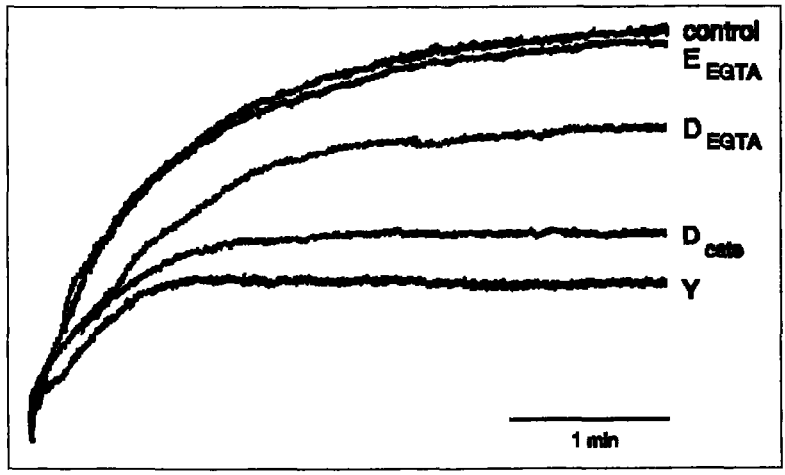

Figure 1 *Influence of different FDP fragments (100 µg/mL) on platelet aggregation induced with 1.2 µM ADP.*

When these FDP fragments were added to PRP from a patient with reactive thrombocytosis, whose PRP was diluted with Tyrode's buffer and therefore had a fibrinogen concentration of only 0.6 g/L, evident changes in aggregation were obtained. At 25 μg/mL, FDP-Y reduced the extent of aggregation to 40% and 77% of control and the rate to 61% and 96% (1.2 and 10.8 μM ADP, respectively). FDP-D_{cate} (100 μg/mL final concentration) decreased the aggregation response to 31% and 80%, respectively, whereas 100 μg/mL FDP-D_{EGTA} only slightly inhibited the aggregation induced by 1.2 μM ADP (to 82%) and no longer affected aggregation by the higher ADP concentration. The aggregation rate was not markedly influenced by these two fragments. FDP-E_{EGTA} at 100 μg/mL exhibited no effect on ADP-induced aggregation. Typical aggregation profiles of this case are shown in Figure 1.

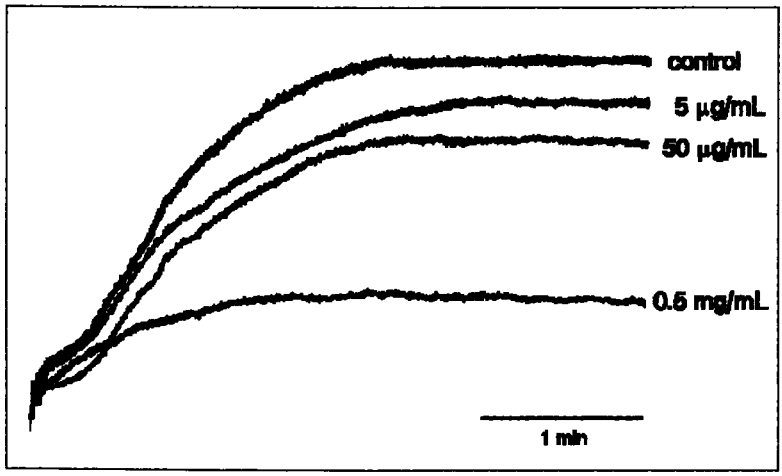

Figure 2 *Dose-dependent inhibition by the late (60 min) fibrinogen digest of ADP-induced (1.2 µM) platelet aggregation.*

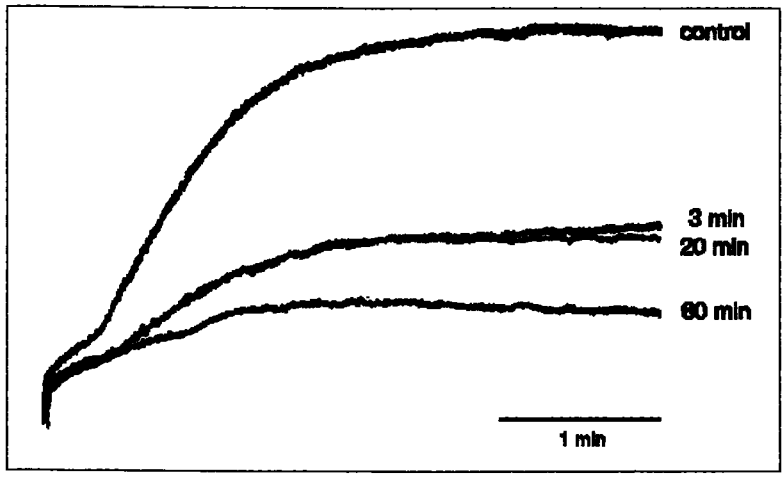

Figure 3 *Comparison of the effect of the three fibrinogen digests (at 0.5 mg/mL fibrinogen equivalent) on the ADP-induced (1.2 µM) platelet aggregation.*

Effect of fibrinogen digests

Crude fibrinogen digests similarly inhibited and retarded platelet aggregation in a dose-dependent way when they were added to PRP with the low fibrinogen concentration, as shown in Figure 2.

At equal concentrations the inhibiting effect of the late (60 min) digest was stronger than that of the intermediate (20 min) and early (3 min) digests (Fig 3).

DISCUSSION

The results presented in Table 1 show that platelet aggregation is very rapidly and significantly inhibited by the action of plasmin, generated in PRP by the three thrombolytic agents. At concentrations equivalent to those attained clinically, streptokinase nearly completely inhibited ADP- and collagen-induced aggregation, whereas the effect of anistreplase was approximately identical but that of tPA was relatively mild (Table 1). This is comparable to findings by others.[11,17,22] More importantly, these *in vitro* results are closely similar to the platelet dysfunction observed *in vivo* after treating patients with anistreplase. This functional defect was shown to be associated with plasma rather than with platelets and the impairment of platelet function showed a good correlation with the concentration of FgDP during thrombolytic therapy.[13] Therefore it is reasonable to suppose that platelet aggregation is inhibited by FgDP.

We failed to demonstrate an inhibitory effect of purified FDP fragments or a crude fibrinogen digest on the aggregation of platelets in normal plasma. Most probably this is explained by the relatively high concentration of fibrinogen (approximately 7 μM in normal plasma) compared with that of the FDP fragments added (about 0.2-1 μM). For, there was evident inhibition of aggregation when we used PRP with a much lower fibrinogen, about 2 μM (Fig 1). Such concentrations of fibrinogen are frequently observed in coronary thrombolytic therapy with non-fibrin selective agents, but it proved to be very difficult to obtain plasma samples with low fibrinogen in the absence of high FgDP; this considerably hampered the present study. Under the low-fibrinogen conditions, fragment Y caused the strongest inhibition, followed by fragments D_{cate} and D_{EGTA}, whereas fragment E_{EGTA} had no inhibitory effect at all. As for fragments Y, D_{cate} and E this is in agreement with the other findings,[15,16] although these authors investigated binding and inhibition of ADP-induced aggregation of washed platelets with a 30-50 fold excess of fragments over fibrinogen. The supply of platelets in our case was too limited to perform extensive dose-response studies; nevertheless, the degree of inhibition by fragment Y (40% at a FDP-Y : fibrinogen ratio of 1:10) seemed much larger than found by Thorsen *et al.* (50% at a 1:1 ratio)[16] or by Tomikawa *et al.* (50% at a 10:1 ratio).[23] Because these workers used gel filtered platelets, it is conceivable that the platelets had lost part of their responsiveness to ADP in their experiments. Unlike Thorsen and coworkers did,[16] we noted a slight inhibition of aggregation by D_{EGTA} (Fig 1).

The plasmic degradation of fibrinogen *in vivo* gives rise to complex mixtures of various FDP fragments, while the composition varies with time after dosing.[18] In order to simulate these conditions, we prepared FDP mixtures by digesting fibrinogen for different times. Our results suggest that the late digest, which contains mainly FDP-D and E,[19] gives a higher degree of aggregation inhibition than the early (FDP-X and Y) and intermediate (FDP-Y and D) digests. As shown in Figure 2, the inhibition is dose-dependent. The

differences between the digests are not exactly in line with our findings with the purified fragments, but agree with previous reports using non-quantitative techniques.[24,25] The degree of aggregation inhibition found by us is comparable to the results of a recent study.[17]

Apart from inhibition of the magnitude of aggregation, the FDPs also affected the rate of aggregation (Fig 3). This effect has previously been noticed after *in vivo* fibrinogenolysis.[13] In view of the results of Landolfi *et al.*, this might not only be a consequence of the competitive action between FDP and fibrinogen, but also of the decrease in fibrinogen concentration.[26] According to their data, the consumption of fibrinogen seen in patients treated with streptokinase or anistreplase can easily be accompanied by a decrease in aggregation velocity to half its maximal value. Although it is unclear whether such a decrease in fibrinogen also diminishes platelet function *in vivo*, it could present an additional factor in reducing platelet function.

We conclude from our results that the high FgDP concentrations generated during thrombolytic therapy seriously impair platelet function. Since the time course of the transient platelet defect is not compatible with an intrinsic platelet cause,[13,27] the best explanation for the impaired platelet function is the competitive inhibition by FDP fragments of the binding of fibrinogen to its platelet receptor. Quantitative binding studies are required to further explicate this concept of FDP-induced impairment of platelet function during thrombolytic therapy.

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General Discussion

In each of the preceding chapters, the results obtained in our own studies have been discussed and compared with those of major investigations published by others. Therefore, a comprehensive general discussion is regarded dispensable. Instead, the present section will be devoted to a number of more general problems associated with thrombolytic therapy, to some important questions which are still unresolved and finally, an outlook will be presented on new developments, which are expected to come within the scope of clinical thrombolysis within the next few years.

PRACTICAL PROBLEMS IN THROMBOLYTIC THERAPY

Thrombus resistance

In 20-25% of AMI patients, thrombolytic therapy with the current plasminogen activators fails to rapidly lyse the thrombus in the infarct-related artery. Surprisingly, this percentage is more or less equal for all five thrombolytic drugs which are in clinical use (see Introduction), with the possible exception of tPA in a modified regimen using so-called front-loaded administration.[1] The reasons why clots are resistant to lysis are largely unknown. Recently it has been suggested that the initial cause of thrombus formation might have a significant influence on the composition of the thrombus and that the lysability of a thrombus is dependent on its composition. Histological examinations have indicated that relatively mild types of injury to coronary arterial endothelium give rise to clots rich in erythrocytes and fibrin, whereas deep injury would result in platelet-rich thrombi.[2] Erythrocyte-rich thrombi can easily be lysed, but platelet-rich thrombi may be more resistant to lysis.[3] It has been demonstrated that the lysability of *ex vivo* thrombi by SK is dependent on the concentration of plasminogen in the clot[4] and since this concentration changes with time, it could explain the well-known variability in lysis of older thrombi.

Although it has been postulated that release of PAI-1 from platelets trapped in the thrombus might inactivate tPA, there is also evidence that platelet-induced clot retraction decreases the binding of tPA to fibrin in the clot and thus inhibits thrombolysis.[5] Though this may provide an explanation for resistance to lysis with tPA, it does not for resistance to other thrombolytic drugs. However, the involvement of platelets in thrombus resistance may be regarded as established, since several groups have shown that inhibition of platelet activity considerably enhances thrombolysis.[6,7]

Effective dose of thrombolytic drug

There is no agreement on the necessity of individual dose-adjustment of thrombolytic drugs. Some drugs are frequently given in a fixed dose (SK 1.5 MU; APSAC 30 U), but this practice has recently been questioned.[1,8] There are other factors, which can markedly influence the biological effect of a given dose, for example the mode of administration,[1] the presence of antibodies in the case of SK and APSAC (see below) and the elimination rate of the drug. This seems essentially important for tPA, which is cleared from plasma by

the liver. It has been shown that the liver blood flow is a major parameter determining the clearance rate of tPA.[9] In patients with AMI the liver blood flow may be rather variable as a consequence of impaired cardiac output. The consequence hereof for the elimination rate of tPA and for its clinical effect, if any, has not been acknowledged until recently.[9] Further studies are required to assess whether part of the therapeutic failures are caused by this factor.

Coronary angiography during thrombolysis

In the earlier years of thrombolysis, many reperfusion trials have been carried out in which a pretreatment coronary angiogram was requested in order to document coronary occlusion and one or more post-treatment angiograms in order to assess recanalization. During angiography, relatively large volumes of contrast agents are usually administered, but the possible effects of such agents on thrombolysis and on haemostatic parameters in plasma have been neglected. Apart from the fact that they are considered as pro-thrombotic, certain contrast agents can modify the structure of fibrin fibrils and impair fibrinolysis.[10] In addition, we have recently shown that a non-ionic contrast agent will lead to marked haemodilution.[11] The ionic contrast agents generally will cause even larger effects than the non-ionic ones, due to their higher osmotic effects.[12,13] The haemodynamic and haemostatic effects of contrast agents should be better realized by workers in the thrombolytic field (see also chapter 8).

Heparin monitoring after thrombolysis

In current clinical practice, heparin is always used as an adjunctive to coronary thrombolysis. The time of starting the heparin infusion, dosage and duration are empirically determined. There are no systematic clinical trials on these aspects of heparin administration and neither are there reliable data on the laboratory control of heparin therapy after thrombolysis. The APTT is almost universally used for monitoring heparin therapy.

Particularly after thrombolysis with drugs causing an extensive systemic lytic effect, such as SK, APSAC and urokinase, patients amy have high concentrations of FgDP, which cause prolongation of the APTT due to their *in vitro* anticoagulant effects. The anticoagulant effect of heparin *in vitro* is additive to this FgDP effect,[14] so the APTT does not accurately reflect the heparin activity. Information as to the extent of the *in vivo* anticoagulant effect of FgDP is lacking. Therefore, it is conceivable that shortly after thrombolysis, when large amounts of FgDP are still circulating, titration of heparin dosage using an APTT assay can lead to incorrect dosing. It would be advisable to perform a controlled clinical study on the dosage and laboratory monitoring of heparin, as long as heparin remains the drug of first choice after coronary thrombolytic therapy.

CONTROVERSIAL QUESTIONS IN THROMBOLYSIS

Type of thrombolytic agent: is there a "best" drug ?

Now the results of the first large clinical studies, which have directly compared the different thrombolytic agents in AMI, are known it may be concluded that none of the available thrombolytic agents is *the* best drug.[15-18] Unquestionably, there are differences between agents and sometimes they may even be marked, but they seem to depend largely on what measure is taken as a parameter of effect. For example, the widely used angiographic coronary arterial patency after 90 min may be a less accurate reflection of the final outcome of therapy: survival or death. Although rtPA appears to recanalize occluded vessels more rapidly than other drugs,[1,18] definite proof that this will translate into lower mortality is still lacking.[15,18] Therefore, future thrombolytic agents should be judged for their ability to achieve real targets (gain of lives and reduction in post-AMI morbidity), rather than surrogate clinical endpoints.

Systemic lytic state: benefit or disadvantage ?

The issue of systemic lysis caused or not caused by a thrombolytic agent, has received much emphasis in the early years. Initially it was thought that severe fibrinogenolysis, due to non fibrin-selective drugs (SK, APSAC), would contribute to increased bleeding risk and was therefore to be avoided. However, therapy with more fibrin-selective drugs (tPA) is equally accompanied by haemorrhage and nowadays it has been recognized that there are no large differences in the frequency of bleeding complications among the different agents.[19-21]

Recently, it has been suggested that a prolonged systemic lytic state, as occurs with APSAC, might be even beneficial, because the high concentrations of FgDP limit the incidence of reocclusion.[22-24] As pointed out in this thesis (chapters 10-12), FgDPs probably impair the activity of platelets and thus contribute to a lower reocclusion rate.

Antibody formation after SK and APSAC: clinical impact ?

As discussed before (see chapter 6), antibodies reacting with SK are ubiquitously found as a consequence of immunization with streptococcal proteins after infections. In addition, SK is a non-human protein and thus can it elicit an immune response. Recently, several authors have demonstrated that therapy with SK or APSAC will give rise to high titres of anti-SK antibodies, which may persist for years.[25-30] It is still unknown whether the "natural" antibodies are similar or identical to the ones produced after SK therapy, but there is ample evidence that both types of antibodies do neutralize SK and APSAC *in vitro*,[25,27,28] bind to SK and APSAC *in vivo*,[27-29,31] and rapidly disappear from plasma after the drugs have been administered (see also chapter 6).[27] Based on these findings and on single case reports,[30,31] some authors have suggested that high anti-SK concentrations can cause failure of thrombolytic therapy.[25,28-33] However, such contentions have never been adequately proven. Indeed, our results (chapter 6) and those of others[34] have indicated no correlation between coronary patency and lack of a systemic lytic state due to high anti-SK titres in groups of patients treated with APSAC or SK. Prospective studies in patients with

their second AMI, who have previously been treated with SK or APSAC, are obviously required for assessing the clinical relevance of antibodies reacting with SK.

FUTURE PERSPECTIVES

Thrombolytic therapy in other thrombo-embolic disorders than AMI

Pulmonary embolism

Thrombolytic treatment of pulmonary embolism has been well investigated over the past three decades, both with the first-generation agents SK and UK as well as more recently with tPA. It has been proven that thrombolytic agents can markedly accelerate clot lysis and restoration of the pulmonary circulation.[35,36] Although non-fatal sequelae of pulmonary embolism can be prevented or reduced, the question whether thrombolytic therapy leads to a reduction in mortality is still not resolved and therefore it remains a controversial issue.[37] Anyhow, it may be considered the therapy of choice in patients with acute, massive pulmonary embolism with an otherwise poor prognosis.[35-37]

Deep vein thrombosis

Deep vein thrombosis may be treated with thrombolytic drugs in the relatively few eligible patients. Available data from controlled, randomized trials indicate that long-term infusion (for 24 to 72 h) of SK effectively lyses thrombi, but often at the expense of increased bleeding.[36,37] In a recent randomized trial comparing two doses of long-term intravenous tPA in deep venous thrombosis, it was concluded that this type of therapy could not be recommended.[38] A special problem in deep venous thrombosis is that the thrombus is generally aged and thus may be more resistant to lysis (see above).

There are numerous reports of successful thrombolytic therapy in single cases or small series of patients with rare forms of venous thrombosis, such as in the central retinal, hepatic, renal and mesenteric veins.[36] Obviously, the lack of large, controlled studies makes it difficult to exactly assess the merits of thrombolytic treatment in these conditions.

Peripheral arterial thrombosis

Thrombi in peripheral arteries can be lysed in at least two in three patients; the angiographic success seems to be independent of the type of thrombolytic drug used.[39] Thrombolytic therapy in peripheral arterial occlusions is still controversial, because advantages over conventional strategies have not yet been demonstrated in randomized trials.[37]

Stroke

Thrombolysis in patients with acute stroke may be accompanied by serious or even fatal side-effects as intracranial bleeding and brain oedema[40] and therefore must be still regarded as experimental. Perhaps the newer thrombolytic agents with a more favourable benefit/risk ratio will prove to be useful in these patients.

Unstable angina pectoris
Basically, unstable angina pectoris resembles AMI in nature (see also Introduction), and therefore it is logical that this condition has been the subject of various thrombolytic trials. Some small studies suggested marginal clinical and angiographic benefits, but thrombolysis is not yet indicated for routine use.[37,41,42]

Other indications
A special application of thrombolysis is the treatment of catheter-induced thrombosis, which can occur during angiography, angioplasty or round indwelling vascular access catheters.[36,39]

New thrombolytic agents
Dosage of plasminogen activators
The introduction of new thrombolytic agents will be preceded by modifications in the dosage schemes of the currently available drugs. There is a trend towards accelerated infusions of SK, UK and tPA,[1] sometimes even approaching bolus administration.[43] The results of dose-finding studies with an old drug as SK have only recently learned that further dosage optimalization is still possible.[44]

Combinations of plasminogen activators
Studies with combinations of different thrombolytic drugs have shown the potential for higher clinical efficacy than with single drug regimens. The rationale is to combine drugs which have mutually additive properties, for example tPA (rapid lysis) and SK (sustained lytic state and low reocclusion rate).[45] The promising synergism between the recombinant agents tPA and uPA, which had been found in animal models,[46] has not been found in man, yet.[47] Large-scale trials using combinations of thrombolytic drugs are still lacking.[43]

Mutants and variants of plasminogen activators
Modern molecular biology has enabled the production of genetic mutants and variants of tPA and uPA with more favourable properties.[15,47-50] Many mutants of rtPA with deletions in the domains involved in its clearance from plasma and in its reaction with the main inhibitor, or rtPA variants with enhanced fibrin binding or increased resistance to plasmin have been constructed and tested for their efficacy in animal models. The first reports on proteins with promising thrombolytic properties in man have recently been published.[51] Single-chain uPA has also been modified by genetic engineering and sometimes with apparent success, but it is still too early for clinically useful alternatives to the parent molecule.[15,43,50]

Chimeric plasminogen activators
Recombinant technology also has made it possible to construct hybrid molecules, the so-called chimeric plasminogen activators. These agents are composed of domains of different natural fibrinolytic molecules, mainly tPA and single chain uPA.[15,50] Although a number of

chimeric agents have been shown *in vitro* to possess the properties of the domains used, data on their *in vivo* properties are not available yet.

Antibody-plasminogen activator complexes
An alternative novel approach is that of antibody-targeted plasminogen activators. Monoclonal antibodies, which are specific for constituents of the clot, such as anti-fibrin or anti-platelet antibodies, can be coupled chemically to a plasminogen activator. The goal is to concentrate the plasminogen activator at the site of the thrombus.[48,50] This can also be achieved using bispecific antibodies, which bind with one half to a plasminogen activator and to the thrombus with the other half.[52] Like most of the other developments discussed in this section, the experiments are still in a preliminary, pre-clinical phase.

Bat plasminogen activator
A plasminogen activator from saliva of the vampire bat *(Desmodus rotundus)* has been isolated recently and the gene cloned. It is highly homologous to human tPA, but it is about 200 times more fibrin-selective and resistant to cleavage by plasmin. Initial studies in animal models of thrombosis indicated that this vampire bat tPA is a potent and markedly fibrin-specific thrombolytic agent.[53]

Staphylokinase
One of the latest "new" plasminogen activators is actually known for more than 40 years: staphylokinase. It is a non-enzymatic protein from *Staphylococcus aureus*, which in its action resembles SK. In animal models its thrombolytic potency was comparable to that of SK, but it is more fibrin-selective.[54] Now it can be prepared by recombinant DNA methodology, it can be further investigated in clinical trials. The first experience with two patients receiving staphylokinase confirms the expected potential as a promising new thrombolytic agent (D. Collen; communication at the 11th International Congress on Fibrinolysis, Copenhagen; july 1992).

Conjunctive therapy
The necessity for conjunctive drug treatment to thrombolytic therapy has been previously discussed.[55] Thrombolytic drugs can cause, either directly or indirectly, activation of the coagulation system and of platelets. Without proper action, there is a considerable risk of reocclusion of a vessel which had been patent immediately after thrombolysis (see Introduction).

Anti-thrombins
Because of the pivotal role of thrombin in both coagulation and platelet activation, the major area of interest in conjunctive drug therapy is that of anti-thrombins. Heparin is currently the most widely used drug during and after thrombolysis, but it is associated with increased bleeding risk. Moreover, heparin is relatively ineffective in preventing reocclusion, because it does not inhibit the activity of fibrin-bound thrombin and thus allows accretion of the thrombus.

Hirudin, from saliva of the leech *Hirudo medicinalis*, is a very potent thrombin inhibitor, which acts independently of antithrombin III.[56] At present, hirudin and a number of hirudin analogues can be produced by DNA technology. Recombinant hirudin has been shown in animal studies to be very effective as an antithrombotic agent.[57,58] The first clinical studies on hirudin concomitantly with thrombolysis are currently under way.
Other approaches include synthetic peptides, which selectively block the active site of thrombin, such as argatroban,[59] or which mimic critical sequences in fibrinogen and thus inhibit thrombin activity, such as P-PACK or small FgDP-like peptides. The knowledge on these substances is still preliminary and data from clinical studies are lacking.

Anti-factor Xa
Inhibition of coagulation factor Xa provides an alternative way for antithrombotic medication. Recently, several selective factor Xa inhibitors have been isolated from leeches and from ticks. One of these substances, tick anticoagulant peptide, has been tested in its recombinant version in a dog model of arterial thrombosis and was found to have potent antithrombotic effects.[60]

Anti-platelet drugs
After the ISIS-2 trial, the use of aspirin as an anti-platelet drug after thrombolysis has rapidly become standard practice, because it further decreased the mortality from AMI (see chapter 10). Aspirin is certainly not the best anti-platelet drug imaginable, since it does not prevent the adhesion of platelets to the residual thrombus or to the re-exposed arterial injury. More potent agents than aspirin are needed to ensure stable and sustained coronary patency.
In vivo experiments with anti-platelet prostaglandins have shown that the rate and degree of thrombolysis can markedly be enhanced. Examples are thromboxane A_2 receptor antagonists, thromboxane synthetase inhibitors and stable prostacyclin analogues.[43,61-63]
Two new classes of agents which inhibit platelet aggregation are monoclonal anti-glycoprotein IIb/IIIa antibodies[6,59] and peptides, either synthetic or isolated from snake venoms, which block the binding of fibrinogen to platelets by acting as GP IIb/IIIa receptor antagonists.[64]

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Summary

Samenvatting

SUMMARY

In the **introduction** to this thesis, a review is presented of the literature which forms the biochemical and clinical basis of thrombolytic therapy. The action of the fibrinolytic system and its major components are discussed. Then, the current pathophysiological concepts of atherosclerosis in general and acute myocardial infarction in particular are reviewed, followed by a short historical overview on thrombolytic therapy. Hereafter the pharmacology of the currently available thrombolytic agents is comprehensively discussed and their most important clinical effects and side-effects presented. Subsequently a number of haemostatic tests are discussed which are important for thrombolytic therapy and the ensuing chapters in this thesis. The section is concluded with an outline of the aims of the investigations, comprising three major items: analytical and pre-analytical aspects, application of haemostatic assays in thrombolytic therapy and finally the role of platelet function in this type of treatment.

PART 1: (pre-)analytical aspects of haemostatic assays

In **chapter 1** methods were investigated to prevent ongoing lysis of fibrinogen and degradation of haemostatic proteins after blood collection during thrombolytic therapy with streptokinase or APSAC. It was found that an anticoagulant mixture containing at least 200 KIU/mL of the protease inhibitor aprotinin was necessary to completely inhibit breakdown of fibrinogen *in vitro*. Alternative inhibitors could be applied as well, but these were less effective than aprotinin.

In **chapter 2** a novel determination of fibrinogen was evaluated, which is based on nephelometric monitoring of the clotting plasma in the prothrombin time assay using the ACL coagulation analyzer. The method performed adequately in normal or nearly normal plasma samples, but in abnormal plasmas the accuracy was poor. The fibrinogen concentration was markedly overestimated in samples containing high concentrations of fibrinogen degradation products, with fragment Y causing the largest deviation from e reference method.

Chapter 3 describes a comparative study on four fibrinogen assays, which was mainly focused on determinations during thrombolytic therapy. The assays were the clotting rate method (Clauss), the total clottable protein method (Blombäck), the automated nephelometric method discussed in chapter 2 and a new enzyme immuno-assay detecting only fibrinogen molecules with at least one intact Aα chain. The four methods showed excellent correlation in samples without fibrinogen degradation products. However, in the presence of high concentrations of these products the three clot-based assays gave spurious fibrinogen values; the direction of the deviation depended on the exact composition of the fibrinogen degradation products. The immunological method was virtually not affected by

these fragments. This implicates that the degradation of fibrinogen during thrombolytic therapy may be significantly less extensive than previously supposed.

PART 2: haemostatic assays in thrombolytic therapy

Chapter 4 includes the report of a clinical trial comparing the at the time new thrombolytic drug APSAC (anistreplase), given intravenously, and streptokinase given by the intracoronary route. Major endpoints were angiographic reperfusion and early reocclusion. There were no significant differences found between both drug regimens with respect to reperfusion, reocclusion, bleeding, other side-effects and most haemostatic parameters. After APSAC, the euglobulin clot lysis time was shortened for a significantly longer period than after streptokinase. This more sustained fibrinolytic activity of APSAC could probably account for lower rate of reocclusion.

In **chapter 5** the systemic effects observed in the clinical study of chapter 4 are described. All but a few patients showed a systemic lytic state characterized by a significant decrease in fibrinogen, plasminogen, α_2-antiplasmin and euglobulin clot lysis time and an increase in fibrin(ogen) degradation products and global clotting times. Although there was a trend for the systemic lysis to be more profound after APSAC, no relevant differences were found with streptokinase, except the longer sustained fibrinolytic activity of APSAC.

In **chapter 6** the significance of antibodies to streptokinase was examined, using the same patient group as in the two preceding chapters. There was a significant correlation between the pre-treatment concentration of IgG antibodies to streptokinase and the degree of systemic lysis after dosing. However, the clinical effect (reperfusion rate) was not affected by anti-streptokinase antibodies.

Although the amount of streptokinase in the dose of APSAC used is much higher than that of streptokinase given in the study, both regimens caused an identical consumption of antibodies, indicating that APSAC might be poorly bound by these antibodies.

Chapter 7 reports a study on the relevance of histidine-rich glycoprotein for thrombolytic therapy. This protein is considered to regulate the activation of plasminogen. No correlations were found between the concentration of histidine-rich glycoprotein and any of the clinical parameters studied. Surprisingly, the occurrence and degree of the systemic lytic state was associated with a histidine-rich glycoprotein, but inversely to the theoretical expectations.

Chapter 8 deals with the detection of thrombin activity after thrombolytic therapy with urokinase or pro-urokinase (saruplase). It was a case-control study on all patients who experienced a clinically manifest reinfarction in a large multicentre clinical trial. Although there were evident signs of thrombin activity, namely increases in the concentrations of thrombin-antithrombin III and prothrombin fragments 1+2, these parameters were not predictive of reinfarction under the regimens studied.

In **chapter 9** a study on haemorheology during thrombolytic therapy is described, performed in a small group of patients receiving anistreplase for acute myocardial

infarction. Significant changes in blood and plasma viscosity were found in all patients and the effects lasted for at least 24 h. There was a decrease in haematocrit but the reduction in blood viscosity remained significant even after correction for changes in haematocrit. The most important determinant of blood viscosity proved to be haemodilution, whereas the contribution of fibrinogen was more limited. In spite of marked rheological changes, no improvement of cardiac output was noticed.

PART 3: platelet function in thrombolytic therapy

Chapter 10 contains the results of a study on the effects of APSAC on platelet function; it only included patients with acute myocardial infarction who abstained from taking anti-platelet medication before and during the study. The *ex vivo* aggregation of platelets was significantly inhibited immediately after thrombolysis and remained decreased for 6-12 h. In this phase there were no signs of platelet activation. The inhibition of platelet aggregation was correlated with the concentrations of fibrinogen and fibrinogen degradation products. The transient inhibition was followed by stimulation of aggregation, lasting until 24-48 h after dosing. Determinations of substances released from platelets (platelet factor 4, ß-thromboglobulin and thromboxane-B_2) confirmed the lack of platelet activation by APSAC.

The next two chapters are devoted to the mechanisms of the interaction between thrombolytic agents and platelets. In **chapter 11** a flow cytometric *in vitro* study is described on the effects of plasmin and thrombolytic agents on a number of platelet surface glycoproteins that are necessary for proper platelet function. It was found that under circumstances comparable to the *in vivo* situation, *i.e.* platelets in a plasma environment, neither thrombolytic drugs nor plasmin significantly affected the integrity of these glycoproteins. In a purified system without plasma, however, marked degradation of some glycoproteins by plasmin was detected. Therefore, direct interaction between plasmin and the platelet glycoproteins offers no likely explanation for the functional defect observed in chapter 10.

In **chapter 12** preliminary results are presented on the role of fibrinogen degradation products in the mechanism of plasmin-associated platelet inhibition. It was found that fibrinogen degradation products could seriously inhibit platelet aggregation, at least when the fibrinogen concentration in plasma was low. These data support the concept that the transient platelet dysfunction observed in patients during thrombolytic therapy is caused by high concentrations of degradation products of fibrinogen, which inhibit the binding of fibrinogen to platelets.

The **general discussion** addresses a number of practical problems in thrombolytic therapy for which additional investigations should provide a solution. It also includes a discussion of controversial questions, which are not directly of interest for patient treatment, but are related to the mechanisms involved in achieving the therapeutic success. Future perspectives of thrombolytic therapy are finally presented, including the development of

new thrombolytic drugs, applications of thrombolysis outside the field of myocardial infarction and progress on drugs which are given as adjunctive treatment in order to prevent or delay thrombin-mediated reocclusion and reinfarction.

SAMENVATTING

In de **inleiding** van dit proefschrift wordt een overzicht gepresenteerd van de literatuur, die de biochemische en klinische basis vormt voor thrombolytische therapie. De werking van het fibrinolytische systeem en de belangrijkste componenten hiervan wordt besproken. Daarna volgt een overzicht van de patho-fysiologie van atherosclerose in het algemeen en van het acute myocardinfarct in het bijzonder, gevolgd door een kort overzicht van de historie van thrombolytische therapie. Vervolgens worden de farmacologische eigenschappen van de thans in gebruik zijnde thrombolytische middelen besproken, samen met hun belangrijkste klinische effecten en bijwerkingen. Daarna komen enkele stollingsbepalingen aan de orde, die van belang zijn voor thrombolytische therapie en voor de volgende hoofdstukken. De inleiding wordt besloten met een samenvatting van de doelstellingen van het onderzoek, dat in drie stukken kan worden verdeeld: de analytische en pre-analytische aspecten van enkele stollingsbepalingen, de toepassing van stollingsbepalingen tijdens thrombolytische therapie en tenslotte de rol van bloedplaatjes bij deze behandeling.

DEEL 1: (pre-)analytische aspecten van stollingsbepalingen

In **hoofdstuk 1** werden methoden onderzocht om het voortgaan van de afbraak van fibrinogeen en andere stollingseiwitten tegen te gaan na de afname van bloed bij patiënten tijdens een thrombolytische behandeling met streptokinase of APSAC. Er werd vastgesteld, dat toevoeging van de protease-remmer aprotinine aan het anticoagulans noodzakelijk was; een concentratie van minstens 200 KIE per mL bloed kon de afbraak van fibrinogeen *in vitro* volledig remmen. Andere remmers konden ook wel gebruikt worden, maar deze waren steeds minder effectief dan aprotinine.

In **hoofdstuk 2** werd een nieuwe bepalingsmethode voor fibrinogeen in plasma geëvalueerd. Deze is gebaseerd op de nefelometrische meting van het stollende plasma tijdens de prothrombinetijd bepaling in de ACL stollingsautomaat. De analytische eigenschappen van deze nieuwe methode waren voldoende in normale of vrijwel normale plasma monsters, maar in sterk afwijkende monsters was de nauwkeurigheid slecht. De concentratie van fibrinogeen werd sterk overschat in monsters met een hoge concentratie van fibrinogeen afbraak produkten, waarbij fragment Y de grootste afwijking veroorzaakte in vergelijking met de referentie methode.

Hoofdstuk 3 beschrijft een vergelijkend onderzoek van vier verschillende technieken om fibrinogeen te meten; het was vooral gericht op bepalingen tijdens thrombolytische therapie. De vier methoden waren: de meting van de stollings-snelheid volgens von Clauss, de bepaling van totaal stolbaar eiwit volgens Blombäck, de geautomatiseerde nefelometrische methode uit hoofdstuk 2 en een nieuwe immuno-enzymatische bepaling van fibrinogeen, waarvan de moleculen minstens één intacte Aα keten bezitten. De vier

methoden kwamen uitstekend overeen in monsters met weinig fibrinogeen afbraak produkten. Echter, in monsters met hoge concentraties van deze produkten gaven de drie stollingsmethoden vals te hoge of vals te lage uitslagen; de richting van de afwijking was afhankelijk van de precieze samenstelling van de fibrinogeen afbraak produkten. De immunologische bepaling werd nauwelijks door deze fragmenten gestoord. Dit houdt in, dat de afbraak van fibrinogeen tijdens thrombolytische therapie veel geringer kan zijn dan voorheen algemeen werd aangenomen.

DEEL 2: stollingsbepalingen tijdens thrombolytische therapie

Hoofdstuk 4 bevat de resultaten van een klinische studie waarin het destijds nieuwe thrombolytische middel APSAC (anistreplase), dat intraveneus werd toegediend, werd vergeleken met streptokinase, dat intracoronair gegeven werd. De belangrijkste eindpunten van de studie waren de angiografisch vastgestelde reperfusie van de coronairvaten en vroege reocclusie. Wij vonden geen wezenlijke verschillen tussen beide behandelingen in termen van reperfusie, reocclusie, bloedingen, andere bijwerkingen en stollingseffecten. De euglobuline stolsellysis tijd bleef langer verkort na APSAC dan na streptokinase en deze langdurige fibrinolytische werking van APSAC zou kunnen samenhangen met het minder voorkomen van reocclusie.

In **hoofdstuk 5** werden de systemische effecten bestudeerd van beide middelen uit hoofdstuk 4. Het bleek dat de meeste patiënten een toestand van systemische lysis doormaakten, gekenmerkt door een zeer sterke daling van fibrinogeen, plasminogeen, α_2-antiplasmine en de euglobuline stolsellysis tijd, een toename van de concentratie van fibrinogeen afbraak produkten en verlengde stollingstijden. Hoewel er wat aanwijzingen waren dat de mate van systemische lysis sterker zou zijn in de patiënten die met APSAC werden behandeld, waren de verschillen met streptokinase niet statistisch van belang, met uitzondering van de langdurige fibrinolytische activiteit van APSAC.

Hoofdstuk 6 is gewijd aan een onderzoek naar de betekenis van antistoffen tegen streptokinase in dezelfde groep patiënten als in de voorgaande twee hoofdstukken. Er bleek een statistisch significant verband te bestaan tussen de plasma concentratie van IgG antistoffen tegen streptokinase voor de behandeling en de mate van systemische lysis na toediening van het thrombolytische middel. Het klinische resultaat (reperfusie) werd echter niet beïnvloed door de streptokinase antistoffen.

Hoewel de hoeveelheid streptokinase in de toegediende dosis APSAC veel groter is dan de streptokinase dosis die de andere helft van de patiënten kreeg, was de daling in de concentratie van antistoffen even groot. Dit betekent dat deze antistoffen slechts matig binden aan APSAC.

In **hoofdstuk 7** werd de betekenis onderzocht van histidine-rijk glycoproteine voor thrombolytische therapie. Dit eiwit wordt beschouwd als een regulator van de activering van plasminogeen. Er werd geen verband gevonden tussen de concentratie van histidine-rijk glycoproteine en een of meer van de onderzochte klinische resultaten. Verrassend was dat de concentratie van histidine-rijk glycoproteine een verband vertoonde met de

aanwezigheid en de mate van de systemische lysis, maar het verband was precies tegengesteld aan hetgeen theoretisch werd verwacht.

Hoofdstuk 8 behandelt een onderzoek waarin gezocht werd naar activiteit van thrombine tijdens thrombolytische behandeling van acuut myocardinfarct met urokinase en pro-urokinase (saruplase). Het betrof een grote klinische studie waarin alle patiënten, bij wie klinisch een re-infarct was waargenomen, werden vergeleken met gepaarde patiënten, die geen complicaties doormaakten tijdens de behandeling. Hoewel er zeer duidelijke tekenen werden gevonden van activering van thrombine, namelijk verhoogde plasma concentraties van thrombine-antithrombine III complex en van prothrombine fragmenten 1+2, bleken deze bepalingen geen voorspellende waarde te hebben voor het optreden van re-infarct tijdens deze behandeling.

In **hoofdstuk 9** wordt een studie beschreven naar bloedrheologie tijdens thrombolytische behandeling met anistreplase in een kleine groep patiënten met acuut myocardinfarct. Bij alle patiënten werden belangrijke verschillen gevonden in de viscositeit van bloed en plasma na het toedienen van anistreplase; deze veranderingen hielden minstens 24 uur aan. Verder daalde de hematocriet sterk, maar de veranderingen in viscositeit bleven statistisch van belang, zelf als rekening gehouden werd met de afname van de hematocriet. De belangrijkste factor voor de bloedviscositeit was de verdunning van bloed, terwijl de bijdrage van fibrinogeen aan de viscositeit beperkt was. Ondanks de grote veranderingen in bloedrheologie werd geen verbetering van de pompfunctie van het hart waargenomen.

DEEL 3: bloedplaatjesfunctie tijdens thrombolytische therapie

Hoofdstuk 10 beschrijft de resultaten van een onderzoek naar de effecten van APSAC op de functie van bloedplaatjes; het betrof hier uitsluitend patiënten met een acuut myocardinfarct, die geen aspirine of vergelijkbare medicijnen hadden gebruikt voor hun opname en die ook tijdens de studie niet kregen. De aggregatie van bloedplaatjes *ex vivo* werd onmiddellijk na toediening van APSAC sterk geremd en bleef gedurende 6-12 uur verminderd. In deze periode werden geen tekenen van activering van bloedplaatjes gezien. De mate, waarin de aggregatie werd geremd, hing nauw samen met de concentratie van fibrinogeen en de afbraak-produkten ervan in plasma. De tijdelijke remming van de aggregatie werd gevolgd door een fase van gestimuleerde aggregatie, die duurde tot 24-48 uur na begin van de behandeling. Bepaling van stoffen, die vrijkomen uit bloedplaatjes wanneer deze geactiveerd worden (plaatjes factor 4, ß-thromboglobuline en thromboxaan-B_2), bevestigde dat er geen activering van bloedplaatjes door APSAC kon worden waargenomen.

In de volgende twee hoofdstukken wordt nader ingegaan op de manier, waarop thrombolytische middelen de functie van bloedplaatjes beïnvloeden. In **hoofdstuk 11** worden *in vitro* experimenten beschreven, waarbij de effecten van thrombolytische middelen en plasmine op enkele glycoproteinen op de buitenzijde van bloedplaatjes werden gemeten met behulp van flow cytometrie. Sommige van deze glycoproteinen zijn

noodzakelijk voor het naar behoren functioneren van bloedplaatjes. Onder experimentele omstandigheden, die goed vergelijkbaar zijn met de toestand *in vivo*, namelijk bloedplaatjes in plasma, lieten zowel de thrombolytische middelen als plasmine de structuur van deze glycoproteinen intact. Echter, wanneer bloedplaatjes werden onderzocht in een systeem zonder plasma, werd een soms zeer belangrijke afbraak van deze glycoproteinen door plasmine waargenomen. Derhalve speelt een directe interactie tussen plasmine en bloedplaatjes-glycoproteinen waarschijnlijk geen rol bij het ontstaan van de voorbijgaande remming van de functie van plaatjes zoals die werd gezien in hoofdstuk 10.

In **hoofdstuk 12** worden de voorlopige resultaten besproken van een onderzoek naar de rol van fibrinogeen afbraak produkten bij de remming van plaatjesfunctie door thrombolytische middelen. Er werd vastgesteld, dat fibrinogeen afbraak produkten de aggregatie van bloedplaatjes sterk konden remmen, tenminste wanneer de concentratie van fibrinogeen in plasma voldoende laag was. Deze gegevens steunen de opvatting, dat de tijdelijke remming van de functie van bloedplaatjes die wordt gezien in patiënten, die met thrombolytica worden behandeld, het gevolg is van de hoge concentraties van de afbraak produkten van fibrinogeen, die verhinderen dat intact fibrinogeen bindt aan bloedplaatjes.

In de **algemene discussie** worden nog enkele praktische problemen besproken, die samenhangen met thrombolytische therapie en waarvoor nog geen goede oplossing is gevonden. Eveneens wordt aandacht geschonken aan een paar controversiële punten, die weliswaar niet direct van belang zijn voor de behandeling van patiënten, maar die samenhangen met de werking van thrombolytica en de wijze, waarop de resultaten van de behandeling tot stand komen. Tenslotte komt een blik in de toekomst van de thrombolytische therapie aan de orde, waarbij aandacht wordt geschonken aan de ontwikkeling van nieuwe thrombolytische middelen, toepassing van thrombolytische therapie bij andere aandoeningen dan acuut myocardinfarct en de voortgang bij het ontwikkelen van geneesmiddelen, die gebruikt zullen moeten worden om het optreden van reocclusie en re-infarct na geslaagde thrombolytische behandeling uit te stellen of, liever nog, geheel te voorkomen.

Addenda

LIST OF ABBREVIATIONS *

ADP	adenosine diphosphate
AMI	acute myocardial infarction
anti-SK	antibodies to streptokinase
APSAC	anisoylated plasminogen streptokinase activator complex (anistreplase)
APTT	activated partial thromboplastin time
BRL 26921	Beecham Research Laboratories compound 26921
CD	cluster of differentiation
CK	creatine phosphokinase
CK-MB	creatine phosphokinase, MB isoenzyme
CTAD	citrate-theophylline-adenosine-dipyridamole solution
CU	casein unit
DNA	desoxyribonucleic acid
EACA	ϵ-aminocaproic acid
ECG	electrocardiogram
ECLT	euglobulin clot lysis time
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F_{1+2}	prothrombin fragments 1 + 2
FbDP	fibrin degradation products
FDP/fdp	fibrin(ogen) degradation products
FgDP	fibrinogen degradation products
FITC	fluorescein isothiocyanate
FpA	fibrinopeptide-A
fPlg	free plasminogen
GISSI	Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto miocardico
GP	glycoprotein
HBDH	ß-hydroxy-butyrate dehydrogenase
HRG	histidine-rich glycoprotein
INR	international normalized ratio
ISIS	International Study of Infarct Survival
KIU	kallikrein inhibitor unit
MFI	mean fluorescence intensity
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PF4	platelet factor 4
PRP	platelet-rich plasma
PT	prothrombin time

TCP	total clottable protein
TCT	thrombin clotting time
TIMI	thrombolysis in myocardial infarction study
tPA	tissue-type plasminogen activator
TT	thrombin time
TxB_2	thromboxane-B_2
UK	urokinase
uPA	urokinase-type plasminogen activator
XDP	crosslinked fibrin degradation products
rscuPA	recombinant scuPA
RT	reptilase time
rtPA	recombinant tPA
scuPA	single chain urokinase-type plasminogen activator
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
SK	streptokinase
SKRT	streptokinase resistance titre
ßTG	ß-thromboglobulin
TA	tranexamic acid
TAT	thrombin-antithrombin III complex

* Units and chemical formulae are not included

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SAMENVATTING VOOR LEKEN

Het acute hartinfarct berust in vrijwel alle gevallen op het plotseling optreden van bloedstolling in een van de kransslagaders, die de hartspier voorzien van zuurstof. Het bloedstolsel (thrombus) ontstaat meestal op een plaats, waar de kransslagader reeds vernauwd was ten gevolge van aderverkalking (atherosclerose) en de slagader kan dan door het bloedstolsel gemakkelijk worden afgesloten, zodat het erachter liggende spierweefsel geen zuurstof meer ontvangt. Wanneer de afsluiting niet snel wordt opgeheven, zal het betreffende stuk van de hartspier afsterven en nooit meer herstellen. De functie van het hart wordt op deze wijze verminderd, soms zelfs zo sterk, dat de patiënt eraan overlijdt.

Ongeveer 10 jaar geleden werd ontdekt, dat bepaalde medicijnen, mits ze snel na het ontstaan van het hartinfarct worden toegediend, het bloedstolsel konden oplossen. Later is aangetoond, dat hiermee de nadelige gevolgen van het acute hartinfarct aanzienlijk konden worden verminderd: de hartfunctie van de patiënt kan voor een belangrijk deel behouden blijven en veel minder patiënten overlijden aan een hartinfarct dan in de tijd voordat de stolsel-oplossende (thrombolytische) middelen werden gebruikt.

De thrombolytische middelen werken niet rechtstreeks op het stolsel, maar ze activeren een bepaald systeem in bloed, waardoor uiteindelijk het werkzame enzym plasmine ontstaat. Plasmine breekt fibrine (dit vormt het essentiële bestanddeel van een stolsel) af, zodat dit oplost en de doorgankelijkheid van de kransslagader weer wordt hersteld. De werking van plasmine is niet uitsluitend gericht op fibrine, maar plasmine breekt ook veel andere eiwitten af. De effecten, die hierdoor ontstaan zijn ongewenste bijwerkingen van de thrombolytische middelen en omdat ze voorkomen in het gehele systeem van bloed en bloedvaten overal in het lichaam, worden ze systemische effecten genoemd. De aard van deze systemische effecten en in het bijzonder de manier, waarop ze ontstaan, zijn het onderwerp van dit proefschrift.

In de inleiding wordt de stand van zaken weergegeven, zoals die momenteel bekend is. Vooral de stollingsaspecten van thrombolytische behandeling worden besproken.

Deel 1 van het proefschrift belicht enkele laboratorium-onderwerpen, die samenhangen met bloedonderzoek tijdens een thrombolytische behandeling. Wanneer bloed stolt, wordt het in bloedplasma opgeloste eiwit fibrinogeen omgezet in het onoplosbare fibrine, dat als het ware het skelet van het bloedstolsel vormt. Fibrinogeen is dus van groot belang voor een goede bloedstolling. Fibrinogeen is echter ook een van de eiwitten, die door plasmine kunnen worden afgebroken, niet alleen in het lichaam (*in vivo*), maar ook erbuiten, bijvoorbeeld wanneer bloed geprikt is en zich in een buisje bevindt (*in vitro*). De brokstukken van fibrinogeen, die ontstaan als het door plasmine wordt afgebroken, kunnen allerlei effecten hebben; onder andere storen ze de meting van fibrinogeen in het laboratorium.

Het eerste hoofdstuk van het proefschrift handelt over het voorkómen van de vorming en de werking van plasmine na bloedafname, zodat het bloed nauwkeurig de situatie weergeeft zoals die *in vivo* was, zonder de veranderingen die *in vitro* ontstaan zijn na de bloedafname.

Vervolgens zijn er twee hoofdstukken (2 en 3) gewijd aan de eigenschappen van verschillende methoden om fibrinogeen in bloedplasma te meten, vooral tijdens thrombolytische behandeling.

Deel 2 beschrijft een aantal aspecten van de bloedstolling tijdens verschillende vormen van thrombolytische behandeling; vooral de systemische effecten komen aan de orde.

Dit deel begint met het verslag van een studie, waarin het destijds nieuwe thrombolytische middel APSAC (tegenwoordig anistreplase genaamd), is vergeleken met de standaardbehandeling met streptokinase. De klinische resultaten en die van het laboratoriumonderzoek zijn in aparte hoofdstukken (4 en 5) vermeld; geconcludeerd wordt, dat er geen belangrijke verschillen zijn en dat het nieuwe middel even werkzaam is als het oude.

Streptokinase, het meest gangbare thrombolytische middel, wordt gewonnen uit een bepaalde soort veel voorkomende bacteriën. Veel mensen hebben ooit eens een infectie met deze bacteriën doorgemaakt en hebben afweer opgebouwd in de vorm van antistoffen. Deze antistoffen kunnen reageren met streptokinase en theoretisch zouden ze zelfs streptokinase onwerkzaam kunnen maken. In hoofdstuk 6 wordt een onderzoek beschreven naar de betekenis van deze antistoffen voor thrombolytische therapie met streptokinase en APSAC (dit bevat ook streptokinase). Hoewel de systemische effecten duidelijk werden geremd door de antistoffen, konden wij niet aantonen dat het gewenste effect van de behandeling, namelijk het weer doorgankelijk maken van de kransslagader, erdoor werd beïnvloed.

In hoofdstuk 7 is, opnieuw bij dezelfde groep patiënten, nagegaan of het eiwit histidine-rijk glycoproteïne (HRG) invloed heeft op de werkzaamheid van streptokinase en APSAC. De precieze functie van HRG is weliswaar nog onbekend, maar er zijn aanwijzingen dat de vorming van plasmine vertraagd zou kunnen worden door hoge concentraties HRG. Wij vonden geen verband tussen de HRG concentratie in bloedplasma en de resultaten van de thrombolytische behandeling.

Een ander nieuw thrombolytisch middel, saruplase, is in een grote studie vergeleken met urokinase, een uit menselijke urine verkregen stof (hoofdstuk 8). Wij richtten ons vooral op het opnieuw optreden van een hartinfarct, kort na de geslaagde thrombolytische behandeling van het eerdere infarct. Er is beweerd, dat dit zogenaamde re-infarct voorspeld zou kunnen worden aan de hand van de uitslagen van een bepaalde test in het laboratorium (de TAT bepaling), die aangeeft of er recent bloedstolling is opgetreden. Wij vonden, dat noch met deze test, noch met een nieuwe test die vergelijkbare informatie geeft, bruikbare gegevens over het optreden van een re-infarct verkregen konden worden.

Het laatste hoofdstuk (9) van dit deel beschrijft een oriënterend onderzoek naar de stroperigheid (viscositeit) van bloed tijdens thrombolytische behandeling. Er was reeds bekend, dat fibrinogeen een belangrijke bijdrage levert aan de viscositeit van bloed en

vooral die van bloedplasma, en dat fibrinogeen grotendeels wordt afgebroken bij behandeling met anistreplase. Wij konden inderdaad bevestigen, dat de viscositeit van bloed en bloedplasma daalde tijdens de behandeling, maar het bleef onduidelijk of dit voor de patiënt een extra voordeel biedt, naast het oplossen van het stolsel in de kransslagader. Om deze belangrijke vraag te kunnen beantwoorden is onderzoek op meer patiënten noodzakelijk.

Het derde deel van het proefschrift handelt over de effecten van thrombolytische middelen op de functie van de bloedplaatjes. Bloedplaatjes zijn kleine celfragmenten in bloed, die van groot belang zijn voor een goede bloedstolling. Na iedere beschadiging van een bloedvat klonteren de bloedplaatjes samen en wordt de beschadiging afgesloten door de plaatjes-klonter. Ook bij dit proces is fibrinogeen van belang: het uit fibrinogeen gevormde fibrine vormt als het ware de lijm tussen de bloedplaatjes, waardoor ze stevig aan elkaar kleven. Aangezien fibrinogeen wordt afgebroken tijdens de thrombolytische behandeling, mag verwacht worden dat de klontering van bloedplaatjes ook verandert. In de literatuur zijn tegenstrijdige resultaten bekend over de effecten van thrombolytische middelen op bloedplaatjes; de meeste studies zijn echter niet verricht in patiënten, maar *in vitro* in laboratoriumproeven. Het is zeker niet zo, dat resultaten van *in vitro* experimenten zonder meer van toepassing verklaard kunnen worden op de *in vivo* situatie.

Dit blijkt ook uit onze bevindingen bij *in vivo* onderzoek (hoofdstuk 10), die lijnrecht staan tegenover sommige bekende gegevens uit *in vitro* proeven. Wij vonden, dat de samenklontering van bloedplaatjes duidelijk geremd werd gedurende de eerste 6-12 uur na thrombolytische behandeling en de mate van remming bleek nauw samen te hangen met de afbraak van fibrinogeen in bloed.

Vervolgens onderzochten wij, of verschillende thrombolytische middelen *in vitro* effect hebben op enkele structuren (de zogenaamde glycoproteïnes), die zich aan het oppervlak van bloedplaatjes bevinden. Deze glycoproteïnes zijn essentieel voor de werking van deze cellen, omdat ze de aanhechtingspunten vormen tussen fibrine en de bloedplaatjes. Onder omstandigheden, die vergelijkbaar zijn met die tijdens thrombolytische behandeling in patiënten, vonden wij geen veranderingen in glycoproteïnes (hoofdstuk 11).

Tenslotte hebben wij in het laatste hoofdstuk (12) geprobeerd, in laboratorium experimenten de *in vivo* omstandigheden na te bootsen, teneinde het effect van fibrinogeen afbraak op de samenklontering van bloedplaatjes nader te bestuderen. Hoewel het nog slechts voorlopige resultaten betreft, mogen wij vaststellen, dat de in hoofdstuk 10 gevonden tijdelijke remming van de bloedplaatjesfunctie wordt veroorzaakt door de hoge concentraties van bepaalde brokstukken van fibrinogeen, die tijdens de behandeling met anistreplase en streptokinase ontstaan door de inwerking van plasmine op fibrinogeen.

In de algemene discussie wordt aangestipt, welke ontwikkelingen er momenteel aan de gang zijn: nieuwe thrombolytische middelen zijn volop in onderzoek en daarnaast is er veel aandacht voor medicijnen, die hernieuwde stolling na een geslaagde behandeling moeten voorkomen. De rode draad in dit onderzoek is, dat de nieuwe medicijnen hun werking moeten doen zonder dat ze de nadelige bijwerkingen vertonen, die de huidige generatie

middelen nog wel heeft, zoals bijvoorbeeld de systemische effecten en de grote kans op een re-infarct, kort na een geslaagde behandeling. Opvallend bij deze nieuwe ontwikkelingen is, dat veel van de onderzochte middelen worden verkregen uit de dierenwereld: beesten als vleermuizen, bloedzuigers en teken blijken zeer werkzame stoffen te bevatten, die wellicht als medicijn voor de mens bruikbaar zijn.

CURRICULUM VITAE

De auteur werd op 30 januari 1952 geboren te Rotterdam, waar hij de lagere school doorliep. Vervolgens genoot hij voortgezet onderwijs aan het St. Norbertus Lyceum te Roosendaal en in 1970 behaalde hij hier het diploma gymnasium-ß. Daarna studeerde hij scheikunde aan de Katholieke Universiteit te Nijmegen. Het kandidaatsdiploma (richting S2) behaalde hij in 1973. In de doctoraalfase was zijn hoofdvak biochemie (prof. dr. H. Bloemendal) met klinische chemie (dr. A. Jansen), en de bijvakken organische chemie (prof. dr. R. Nivard) en farmacochemie (prof. dr. J. van Rossum). Tijdens de studie werd het C-diploma stralingsdeskundigheid behaald. In 1976 legde hij het doctoraalexamen scheikunde af en verkreeg hij tevens de onderwijsbevoegdheid.

In 1976 begon hij met de opleiding tot klinisch chemicus, eerst in het St. Elisabeths Gasthuis te Arnhem (opleider dr. P. Meulendijk) en tenslotte in het St. Radboud Ziekenhuis te Nijmegen (prof. dr. Th. Benraad). Op 1 augustus 1981 werd hij ingeschreven in het Register van Erkend Klinisch Chemici. Vervolgens was hij enige tijd waarnemend hoofd van het Algemeen Klinisch Chemisch Laboratorium van het Academisch Ziekenhuis van de Vrije Universiteit te Amsterdam en op 1 oktober 1982 trad hij als klinisch chemicus in dienst van het Catharina Ziekenhuis te Eindhoven. Hier werd hij belast met de professionele leiding van de hematologische secties van het Algemeen Klinisch Laboratorium (hematologie, morfologie, stolling en bloedtransfusie). In 1989 werd hij benoemd tot afdelingshoofd van het Algemeen Klinisch Laboratorium. Het in dit proefschrift beschreven onderzoek werd in dit laboratorium verricht.