Trombin activity in plasma : a study on its formation and inhibition

Trombin activity in plasma

A study on its formation and inhibition

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CONTENTS

CHAPTER I

INTRODUCTION

Hemostasis is a physiological process that enables the organism to protect itself against the loss of blood when injury occurs. The components that contribute to the hemostatic process involve the vessel wall, the blood platelet and other blood cells and humoral factors most of them pro-serine proteases present in the blood. Because of great advances in the field of biochemical techniques, the (pro-)serine proteases of the coagulation mechanism could be extensively studied over the past 20 years. The coagulation pathway originally thought to be a "cascade" sequence (see below) turned out to be more complex than originally supposed and several new elements and interrelationship between the cascade reactions have been added to the scheme. In this introducing chapter a short review on the present knowledge of blood coagulation and anticoagulant mechanism is given.

THE MECHANISM OF COAGULATION

During the process of coagulation a series of inactive serine protease zymogens is transformed into active enzymes by limited proteolysis. During this "cascade" process the first enzyme that is activated, activates a second, and this again activates a third enzyme etc. etc. and in this way a biochemical amplification of the process is obtained (1,2). The coagulation cascade eventually leads to the generation of a burst of thrombin, which is a biologically very active protein and the central enzyme of hemostasis. It acts on the vessel wall, it activates platelets and it

catalyzes the conversion of fibrinogen into fibrin and so stimulates the formation of a stable, fibrin-reinforced platelet plug. It also activates the coagulation factors V and VIII which are cofactors in the coagulation reactions, and factor XIII, the transglutaminase involved in the cross polymerization reaction of the fibrin meshwork. By its action on platelets, on factor V and on factor VIII it excerts an important positive feedback action on its own formation. The thrombin activities mentioned so far all have a procoagulant character. When, however, thrombin is bound to thrombomodulin, a protein present on the endothelial cell surface, the procoagulant activities are inhibited and fibrinolytic activities are initiated. Thrombomodulin-bound thrombin activates protein C, which in turn inactivates the coagulation cofactors V_a and $VIII_a$ and stimulates fibrinolysis by reducing the inhibition of plasminogen activator (3,4). Finally, thrombin acts as a chemoattractant for inflammatory cells and has a mitogenic effect on fibroblasts (5).

In figure 1 a simplified scheme of blood coagulation is given. It can be seen in this figure that blood coagulation can be triggered via two distinct pathways, the so-called intrinsic and extrinsic pathways of coagulation and that there is cross reaction between these pathways, the "Josso loop".

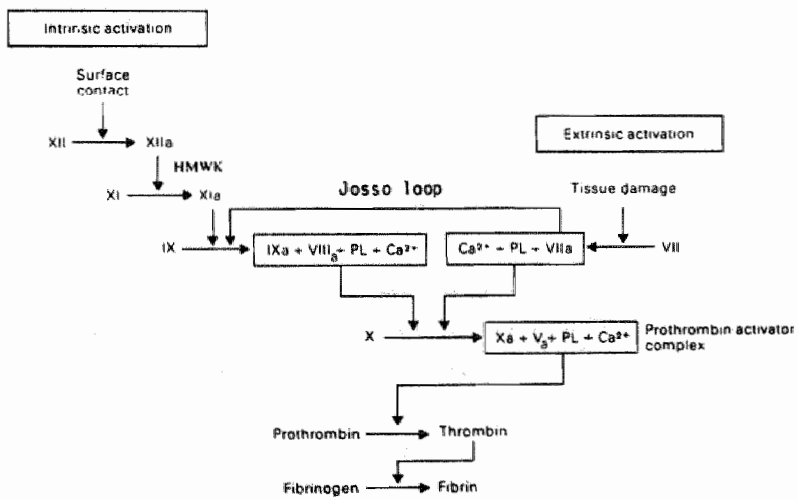

Fig. 1. Schematic representation of the coagulation pathways in plasma.

The intrinsic pathway

The intrinsic pathway of coagulation is triggered when blood comes into contact with negatively charged surfaces. This contact results in the conversion of factor XII to its active form factor XII_a. Factor XII_a then catalyzes the activation of prekallikrein to kallikrein and kallikrein in turn accelerates the activation of factor XII. So, contact with negatively charged surfaces establishes a reciprocal activation of factor XII and kallikrein (6-9). For an optimal activation of factor XII three factors are needed, i.e. prekallikrein, a negatively charged surface and high molecular weight kininogen (HMWK). The role of HMWK is to link the prekallikrein to the surface. Therefore, it functions as a non-enzymatic cofactor in the contact activation reactions. It has been proposed that activation of factor XII is associated with the formation of several active fragments (10). While one of these fragments has the primary function of activating the kallikrein-kinin system, another fragment initiates the intrinsic pathway of coagulation, which induces the activation of factor XI. Moreover, the contact activation system also initiates the fibrinolytic system and the complement system.

The activation of factor XI requires the presence of HMWK, which mediates the binding of both prekallikrein and factor XI to the surface, where both factors are activated by factor XII_a. The factors XII, XI, prekallikrein and HMWK are normally grouped together and are indicated as the contact activation factors.

The activation of factor IX by factor XI_a only requires the presence of Ca^{2+}-ions. However, factor VII_a in the presence of tissue thromboplastin can also activate factor IX and therefore factor IX is considered to be a point of linkage between the intrinsic and extrinsic pathways (see the paragraph on extrinsic activation for details). Factor IX_a, together with phospholipids, Ca^{2+}-ions and factor $VIII_a$, forms a factor X activating complex, the so called tenase complex (11). Factor IX_a binds to a negatively charged phospholipid surfaces via Ca^{2+}-bridges. In the tenase complex the non-enzymatic cofactor, factor $VIII_a$, substantially accelerates the activation of factor X to factor X_a (12). Factor X can also be activated via the extrinsic activation pathway (see below) and therefore in the originally proposed coagulation scheme factor X is the point of linkage between the intrinsic and the extrinsic pathways.

Activated factor X catalyzes the conversion of prothrombin to thrombin and again this activation takes place in a multimeric complex. Factor X_a, together with phospholipids, Ca^{2+}-ions and factor V_a forms the so-called prothrombinase complex which is the site of activation of prothrombin to thrombin. Like factor IX_a in the tenase complex factor X_a binds to negatively charged phospholipid surfaces via Ca^{2+}-bridges and this time factor V_a is the non-enzymatic cofactor, that substantially accelerates to activation of prothrombin (13-15). Recent studies have shown that factor X_a can also bind to a positively charged phospholipid surfaces in a Ca^{2+}-independent way (16). Apparently, also on a positively charged surface the conversion rate of prothrombin can be increased. The physiological significance of this phenomenon is as yet unknown.

Thrombin, the final product of the coagulation cascade, amplifies its own formation via the activation of the non-enzymatic cofactors V and VIII. It also stimulates the so-called platelet "flip-flop" which leads to the exposure of procoagulant phospholipids on the platelet surface. This procoagulant surface again supports the coagulation cascade reactions (17-19). Finally, thrombin catalyzes the conversion of fibrinogen to fibrin and activates factor XIII. It thus causes the formation of a stable, covalently linked network of fibrin. Thrombin, by its action on both platelets and fibrin thus is the central enzyme in the formation of a stable fibrin reinforced platelet plug.

The extrinsic pathway and the Josso loop

The extrinsic pathway is initiated when blood is exposed to tissue thromboplastin that is released from damaged tissue. Tissue thromboplastin is a lipoprotein to which unactivated factor VII can bind. This complex then has some proteolytic activity. It can activate factor X and activated factor X in turn can "retroactivate" factor VII and in this way the very active thromboplastin-factor VII_a complex results. In this complex the roles of phospholipid surface and non-enzymatic cofactor are played by the lipid and the protein part of tissue factor (20-22). The activation of prothrombin then proceeds in the way described in the preceding paragraph.

The factor VII_a-phospholipid complex is also involved in the activation of factor IX, and thus in the interaction between the intrinsic and ex-

trinsic pathways of coagulation (23-25). Recent studies indicate that this activation reaction might constitute a very important triggering mechanism in coagulation (26-33). However, kinetic in vitro studies have shown that the rate of factor X activation via a factor VII_a-thromboplastin dependent activation of factor IX is much smaller than that of a direct activation of factor X via the factor VII_a-thromboplastin complex. Therefore, it has hardly been accepted that the factor VII_a-thromboplastin dependent activation of factor IX has any importance in vivo.

However, some congenital coagulation disorders can not be explained properly by a defect in either the intrinsic or the extrinsic pathway. For example, why are, in haemophiliac patients, the deficiencies in the intrinsic activation pathway not compensated by the intact extrinsic pathway? Why do deficiencies of the contact factors not produce severe disturbances of the hemostatic process? In chapter II of this thesis, a study on the importance of factor IX activation in thromboplastin-dependent coagulation (the Josso Pathway) in plasma is presented.

NATURAL INHIBITORS

The activation of the coagulation cascade is controlled by number of regulatory mechanism, some of which entail plasma proteins that can neutralize or inactivate certain groups of activated coagulation factors at various sites. Such plasma proteins (inhibitors) include antithrombin III and heparin cofactor II, α_2-macroglobulin, α_1-antitrypsin, and C1 inhibitor. These proteins are serine protease inhibitors ("serpins") that attack activated clotting enzymes. A quite different type of inhibition is excerted by activated protein C that itself being a protease, attacks the protein co-factors (Factor V_a and $VIII_a$) of the activation complexes. Antithrombin III and protein C (with its cofactor protein S) seem to be the most important inhibitors, although the relative importance of other serpins for each different activated factor still remains a question of active research. The recently described vascular anticoagulant protein which interferes with the interaction of the clotting factors with a procoagulant surface provides an additional anticoagulant mechanism (34). In figure 2 the site of attack of natural inhibitors in plasma is given.

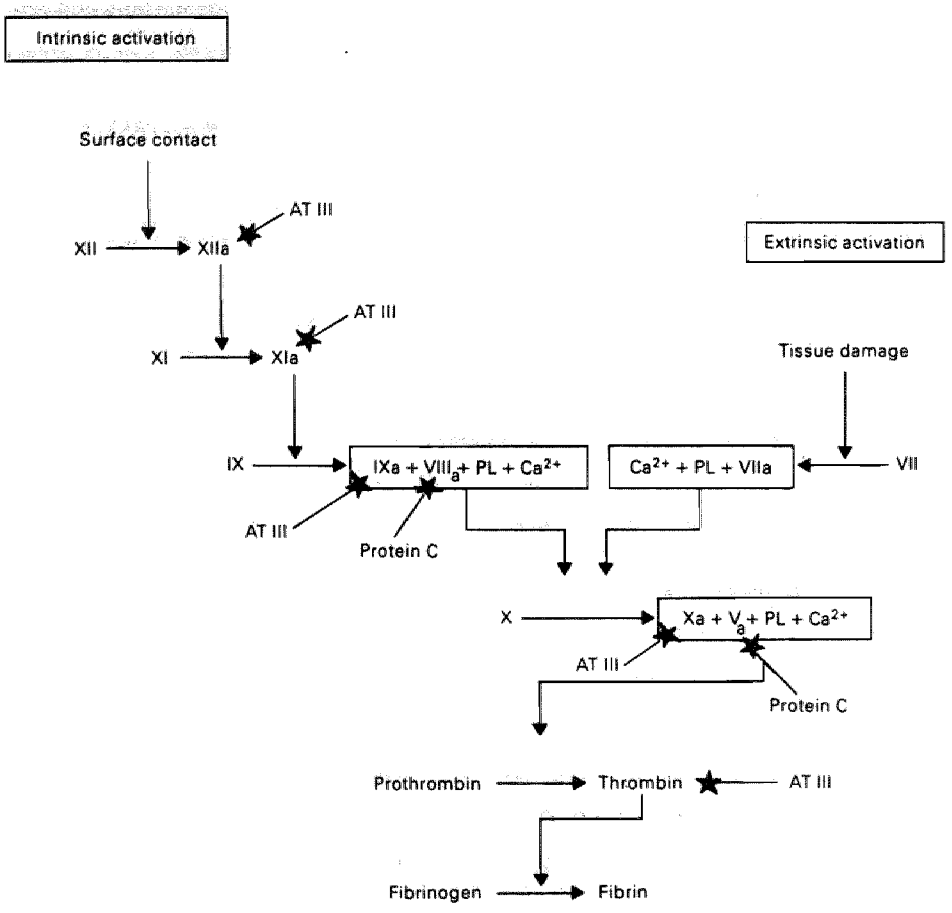

Fig. 2. Sites of attack of natural inhibitors in plasma.

Antithrombin

Antithrombin III (AT-III) neutralizes most of the serine proteases of the coagulation cascade by forming covalent 1:1 molar complexes (35-40). Within the wide spectrum of neutralizing actions of AT-III, the inhibition of thrombin and factor X_a is considered to be the predominant anticoagulant mechanism. The importance of factor X_a inhibition by AT-III has been questioned, however (41). Complex formation between AT-III and coagulation factors normally proceeds slowly but is markedly accelerated by heparin. This constitutes one of the most important effects of heparin administered for anticoagulant therapy.

In addition to the anticoagulant activity of AT-III a small part of antithrombin activity in plasma is accounted for by the heparin cofactor II (HC-II) (42-44). Unlike AT-III, HC-II has a narrow specificity and only neutralizes thrombin. Complex formation between HC-II and thrombin can be accelerated not only by heparin but also by dermatan-sulfate (45,46). Although it has been reported that the hereditary deficiency of HC-II is associated with thrombosis, the relevance of HC-II deficiency as a risk factor for the development of thrombosis is still a matter of discussion (47).

Protein C

Protein C is a vitamin K-dependent protein which possesses a selective anticoagulant activity in the coagulation cascade. The clinical findings that a hereditary deficiency of protein C can lead to repeated thrombotic episodes and the effects observed during acquired deficiencies such as liver disease and during the starting phase of oral anticoagulant therapy suggest that protein C plays an important role in physiological hemostasis (48-52).

Protein C is activated in vivo at the vessel wall by a complex of thrombin and thrombomodulin (51). Activated protein C selectively destroys factor $VIII_a$ and factor V_a in the presence of another vitamin K-dependent protein, protein S, and thus serves to limit propagation and generation of thrombin (52). It has been shown that activated protein C also stimulates the fibrinolytic system through reducing the inhibition of plasminogen

activator (4). Therefore, a reduction of protein C in plasma will result not only in a disturbed regulation of the thrombin formation but also in delayed activation of the fibrinolytic system. It is possible that the combination of these two biologic effects is responsible for the increased thrombotic tendency in congenital deficiency.

ANTICOAGULANTS

Oral anticoagulants

Prothrombin, the factors VII, IX, X and the anticoagulant proteins C and S belong to the vitamin K-dependent proteins. The common characteristic of these proteins is the presence of γ-carboxylated glutamic acid residues in the N-terminal region of the protein chain (53). Vitamin K is essential for the carboxylation of certain glutamic acid residues in these proteins in a posttranslational step during hepatic synthesis (54,55). The γ-carboxylated coagulation factors can bind to negatively charged phospholipid surfaces via Ca^{2+}-bridges. This interaction greatly enhances the efficiency of their participation in the coagulation cascade reactions.

Vitamin K-antagonists such as the coumarin congeners interfere with the carboxylation of the vitamin K-dependent coagulation factors, which results in the synthesis of biologically inactive but immunologically detectable forms (56,57). These inactive forms, called PIVKA (58), reach the blood stream but are unable to bind to negatively charged phospholipids via Ca^{2+}-bridges (59,60). Therefore, the formation and activity of at least three procoagulant complexes, i.e. the factor X converting complexes (IX_a-$VIII_a$-phospholipid and $VII_{(a)}$-Thromboplastin) and the prothrombin converting complex (prothrombinase), will be influenced by oral anticoagulant therapy with vitamin K antagonists. This will, of course, result in a decrease in thrombin generation.

Due to the different half-life times of prothrombin, factor VII, factor IX and factor X, the anticoagulant effect gradually reaches its steady level after initiation of vitamin K antagonist therapy. Treatment with vitamin K antagonist first brings about a reduction of the factor VII activity, later also the effective activity of the factors IX, X and pro-

thrombin decrease (61). For a deep and stable anticoagulant effect the levels of the vitamin K-dependent clotting factors are normally regulated down to about 20 % of normal (61). This level of clotting activity is considered as an effective anticoagulant treatment. However, this estimation of the potential clotting activity is based on the overall clotting test, i.e. prothrombin time. The relative contributions of the individual clotting factors to this hypocoagulability of plasma are not clearly understood. In chapter III of this thesis, a study of the relative importance of various vitamin K-dependent clotting factors on prothrombinase activity in dicoumarol plasma is presented.

Heparin

Heparin is a mucopolysaccharide. It is composed of a monosaccharide chain of variable length containing L-glucourionic acid, L-iduronic acid and D-glucosamine. The iduronic acid and glucosamine are partly sulfated, the latter in addition showing partial acetylation (62). In commercial heparin, the anticoagulant activity is present in fractions with molecular weight between 4,000 - 40,000 daltons and over 20 molecular species differing in charge can be identified in a single preparation by electrophoretic analysis (63). These distinct molecular species are all called heparin because they share a measurable net anticoagulant activity defined by specific in vitro assays. However, they may differ from each other in a variety of other biologic activities, as well as in the mechanism by which they act to prevent thrombosis.

The action of heparin in plasma is to accelerate the formation of complexes between active proteases and antithrombins (antithrombin III and heparin cofactor II). However, the administration of heparin for the treatment of a variety of acute thrombotic disorders is not always efficient, and the dose of heparin that can be administered is limited by the bleeding risk. Moreover, some patients develop thrombocytopenia and thrombotic complications in association with heparin therapy. These clinical findings greatly stimulated the interest in compounds related to heparin which are reported to possess greater antithrombotic effectiveness while producing less bleeding than commercial heparin. Studies on low molecular weight derivatives of commercial heparin, on heparinoids, which are naturally

occuring mucopolysaccharides chemically distinct from heparin, and on synthetic oligosaccharide sequences have received increasing attention in recent years (64-69).

Stichopus japonicus acidic mucopolysaccharide (SJAMP) is a heparin-like compound that can be isolated from the sea cucumber, stichopus japonicus selenka. Structure analysis shows that it contains N-acetylgalactosamine, glucouronic acid, fucose and sulphate in the approximate ratio of 1:1:1:4, respectively (70). The molecular weight of SJAMP is about 30,000 - 50,000 daltons. Some physical properties of SJAMP, like for instance the metachromatic intensity and the electrophoretic behaviour, are similar to those of heparin (70). The action of SJAMP with respect to its anticoagulant activity is not clear, although some studies report that SJAMP acts as an anticoagulant by inhibition of thrombin and that the mode of action of SJAMP is different from that of heparin (71). In chapter IV of this thesis, a study of effect of SJAMP on the generation of thrombin activity in both platelet poor plasma and platelet rich plasma is presented, and a study of the cofactor dependency of the inhibition of thrombin activity by SJAMP is finally presented in chapter V of this thesis.

THE AIM OF THIS THESIS

There is a tendency for modern coagulation biochemistry to become so sophisticated that the medical relevance of the findings is no longer immediately obvious to the medical doctors. It has been the aim of the author being a medical doctor, planning to work in medical surroundings for the rest of his career, to try and establish links between biochemistry and the practice of medicine. Thus, the medical question involved in the first experimental chapter is essentially "Why do hemophiliacs bleed". In the second experimental chapter we try to establish what clotting factor is essential for the efficacy of oral anticoagulant therapy. The rest of the work is devoted to the elucidation of the mode of action of a traditional chinese antithrombotic medicine.

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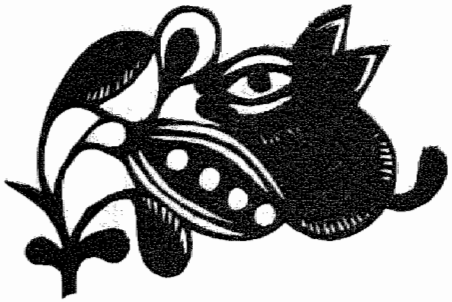

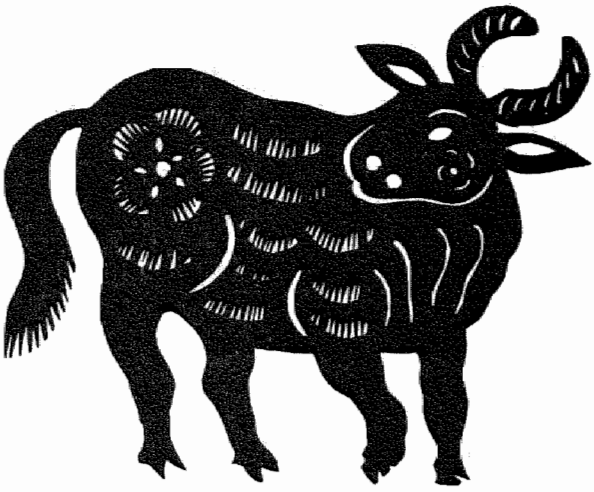

CHAPTER II

THE IMPORTANCE OF FACTOR IX ACTIVATION IN THROMBOPLASTIN-DEPENDENT COAGULATION (THE JOSSO PATHWAY) IN PLASMA

SUMMARY

In this chapter a study of the importance of factor IX activation in thromboplastin-dependent coagulation (the Josso pathway) in plasma is described. Diluted, $CaCl_2$-containing thromboplastin solutions were used to trigger the coagulation in plasma from the congenital factor IX and factor VIII deficient patients in the presence and the absence of added factors IX and VIII and the generation of thrombin activity in these experimental systems was monitored.

When coagulation is triggered with the high thromboplastin concentrations normally used in clinical routine tests, the generation of thrombin activity in congenital factor IX deficient plasma before and after reconstitution with purified factor IX appears independent of the amounts of factor IX (the thrombin generation curves in both reaction mixture are similar). When, however, the coagulation is triggered with low thromboplastin concentrations, a clear dependency of the generation of thrombin activity on the concentration of factor IX becomes evident at factor IX concentrations lower than 30 nM (about 40 % clotting factor activity). Factor VIII seems to be a compulsory cofactor for this factor IX activity because the thrombin generation at optimal factor IX concentration is still dependent upon the amount of factor VIII present.

It can be concluded that the factor IX activation in thromboplastin-dependent coagulation is of physiological importance. This pathway appears increasingly important when low amounts of thromboplastin are present.

INTRODUCTION

Blood coagulation is based on a complex series of enzymatic conversions of serine protease zymogens into active serine proteases (1,2). In the classical view, the activation can be accomplished via two distinct pathways: the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is initiated by the so-called contact activation, which is triggered when blood comes in contact with damaged vascular endothelium or with foreign surfaces such as for instance glass (3,4). The initial steps of this pathway involve the four contact factors, factor XII, prekallikrein, high molecular weight kininogen, factor XI, and the two antihemophiliac factors, the factors IX and VIII. Contact activation results in the conversion of factor X into factor X_a.

The extrinsic pathway is initiated when blood is exposed to tissue thromboplastin, the reaction sequences involves interaction of factor VII and tissue thromboplastin and subsequent activation of factor X (5). Thus both classical pathways converge in the activation of factor X. Later reaction sequences, the conversion of prothrombin to thrombin and that of fibrinogen to fibrin, are common to both pathways.

Evidence for a close interrelationship of the classical coagulation pathways has accumulated, however. Biggs and Nossel found that hemophilia plasma did not generate normal amounts of thrombin activity when plasma coagulation was triggered with a diluted thromboplastin solution (6). Josso and Prou-Wartelle described that factor VII was essential for the procoagulant activity of diluted thromboplastin and postulated the importance of antihemophilic factors in the thromboplastin-dependent pathway (7). Direct evidence for an essential linkage between both classical pathways was obtained by Østerud and Rappaport (8). They clearly showed that a mixture of factor VII and thromboplastin could activate factor IX in partially purified system. This activation step was studied further by several groups and it was clearly shown that factor $VII_{(a)}$ in the presence of thromboplastin can activate both factor IX and factor X (9-15).

Although numerous studies have postulated that the factor IX activation in thromboplastin-dependent coagulation is of important pathway, attention is mostly focussed on the factor X activation. A clear evidence to which the generation of thrombin activity is mediated via thromboplastin-induced

factor IX activation pathway is lacking, however. From a physiological point of view prothrombinase activity and thrombin generation are more important. In this respect we studied the effect of thromboplastin on the generation of thrombin activity in factor IX and factor VIII deficient plasma. The studies described in this chapter were carried out in order to obtain a better impression of the relative importance of the thromboplastin-induced factor IX activation (i.e. the Josso pathway).

MATERIALS AND METHODS

Hemophilia patients

Diagnosis of both hemophilia A and B was based on a positive family history with a recessive sex-linked inheritance, joint and deep muscle bleeding and a prolonged partial thromboplastin time.

The activity of the clotting factors VIII and IX was quantitatively determined by a one-stage method according to the standard procedure of the manufacturer (Behring Institute, West Germany). The factor VIII activity in hemophilia A plasma and the factor IX activity in hemophilia B plasma were < 1% and 1.5% respectively.

Hemophilic plasma

Blood from hemophilia patients (A and B) was collected in trisodium citrate (9 volumes of blood to 1 volume of 0.13 M trisodium citrate) and centrifugated for 15 minutes at 3000 x g at 4 ^{o}C. Plasmas were stored in 1 ml aliquots at -80 ^{o}C.

Thromboplastin

Human brain thromboplastin was prepared by a modification of the method of Owen et al (16). The crude preparation obtained was homogenized in a Potter Elvehjem homogeniser for 3 minutes, centrifugated for 15 minutes at 2000 x g at room temperature. The preparation was stored in 1 ml aliquots at -20 ^{o}C. Prior to use the preparation was thawed, diluted with 0.05 M

Tris-HCl (pH 7.35) containing 0.1 M $CaCl_2$ and prewarmed for 1 hour at 37 °C.

Phospholipid

Phospholipid vesicles containing 25% phosphatidylserine (PS) and 75% phosphatidylcholine (PC) were prepared as described previously (17).

Proteins

Bovine factor IX was prepared as described by Fujikawa et al (18). Bovine factor VIII was prepared according to Vehar and Davie (19) with the modification by van Dieijen et al (20).

Chromogenic substrate

Chromogenic substrate S 2238 was obtained from Kabi Vitrum (Stockholm, Sweden).

Measurement of thrombin generation in plasma

For the measurement of thrombin generation the procedure described by Hemker et al (21) was used. Briefly, 240 µl of plasma and 60 µl of buffer (Tris-HCl, pH 7.35) were incubated for 4 minutes at 37 °C. The thrombin generation was triggered by the addition of 60 µl of thromboplastin solution containing 0.1 M $CaCl_2$. At fixed time intervals, 10 µl aliquots of the incubation mixture were subsampled into tubes containing 465 µl of buffer (0.05 M Tris-HCl, 0.1 M NaCl, 0.5% albumin and 0.02 M EDTA, pH 7.9) and 25 µl of S2238 (4 mM) at 37 °C. The subsampling tubes were incubated for 2 minutes at 37 °C, and then the reaction was stopped by the addition of 300 µl of concentrated acetic acid. The pipettes for sampling and stopping the reaction were connected to an Apple IIe computer, programmed to record the moment of sampling and the moment of stopping the reaction. The optical density was read at 405 nm in a spectrophotometer (LKB-Ultrospec). From the change in optical density and the time interval between subsampling and stopping the Δ O.D./min was automatically calculated.

Measurement of the decay constant of endogenous thrombin

Briefly, 120 µl of plasma and 24 µl of buffer (Tris-HCl, pH 7,35) were incubated for 4 minutes at 37 °C. Thrombin generation in plasma was triggered by the addition of 30 µl of thromboplastin solution. At the moment after the maximal thrombin formation, 6 µl of Soybean trypsin inhibitor solution (SBTI, 10 mg/ml) was added to the reaction mixture. At fixed intervals after addition of SBTI, 10 µl aliquots of the reaction mixture were subsampled into the tubes containing S2238 (4 mM) and EDTA (20 mM) by using the time recording pipette. The determination was the same as that described for the measurement of thrombin generation.

The data obtained from thrombin amidolytic activity at the different time points (C_t) were fitted to the formula $C_t = C_R + C_o e^{-kt}$, which gives the level at the moment of SBTI addition ($C_R + C_o$), the steady end level (C_R) and the decay constant (k). The parameters were determined by means of an ordinary least squares fit of the model to the data. The parameter values that minimize the sum of squared residuals were calculated using the Box-Kanemasu modification of Gauss' method (22). The linear equations were solved by means of Haussholder transformations. The decay constant thus obtained is the sum of the α_2-macroglobulin dependent decay constant (k_2) and the antithrombin III dependent decay constant (k_1) (21).

Estimation of the prothrombin conversion velocity

The prothrombin conversion velocity (prothrombinase, nM min^{-1}) was calculated by a computer using the thrombin generation data and the experimentally determined decay constant of endogenous thrombin. As described above, the decay constant of endogenous thrombin obtained is the sum of k_2 and k_1. The k_2 used is 0.232 $\pm$ 0.004 min^{-1} (n = 25). For technical details see ref. 21.

RESULTS

Generation of thrombin activity in factor IX deficient plasma after triggering coagulation with thromboplastin.

To investigate the effect of factor IX on thrombin generation after triggering coagulation with thromboplastin, congenital factor IX deficient plasma was used. Part of the factor IX deficient plasma was reconstituted with 90 nM factor IX (about 100% clotting activity) and another part was used without the addition of factor IX. When the coagulation was triggered with various concentrations of thromboplastin, the curves of thrombin generation in both plasma preparations shown in figure 1 were obtained.

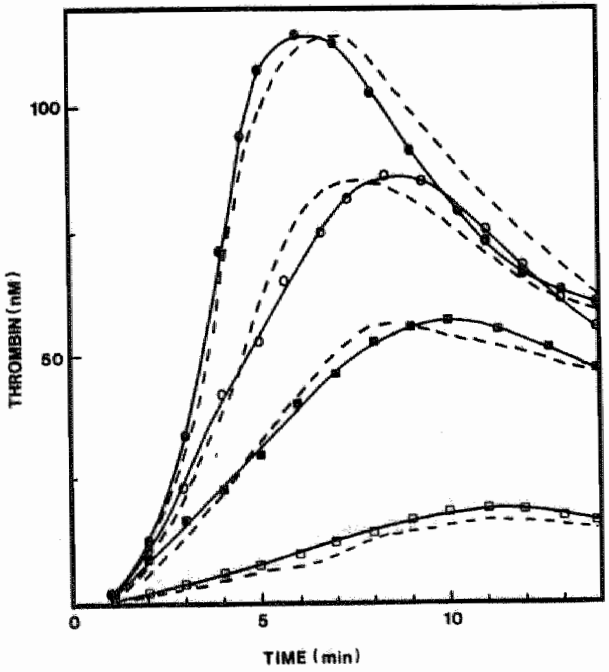

Fig. 1. Generation of thrombin activity in factor IX deficient plasma before and after reconstitution with factor IX (90 nM). Coagulation was triggered with a series of dilutions of thromboplastin. Factor IX deficient plasma: (- - - -); Reconstituted plasma: (——). Final thromboplastin dilution in the reaction mixture: 1/240 (●—●); 1/360 (○—○); 1/480 (■—■); 1/600 (□—□).

It was unexpected that the curves of thrombin generation in the congenital factor IX deficient plasma and in the reconstituted plasma were very similar in the thromboplastin concentration range studied (final thromboplastin dilution in the reaction mixture: 1/240 - 1/600). Figure 1 also gives the impression, however, that the relation between the thromboplastin concentration and the maximal amount of thrombin formed is not linear; thrombin generation seems to be markedly reduced at the highest

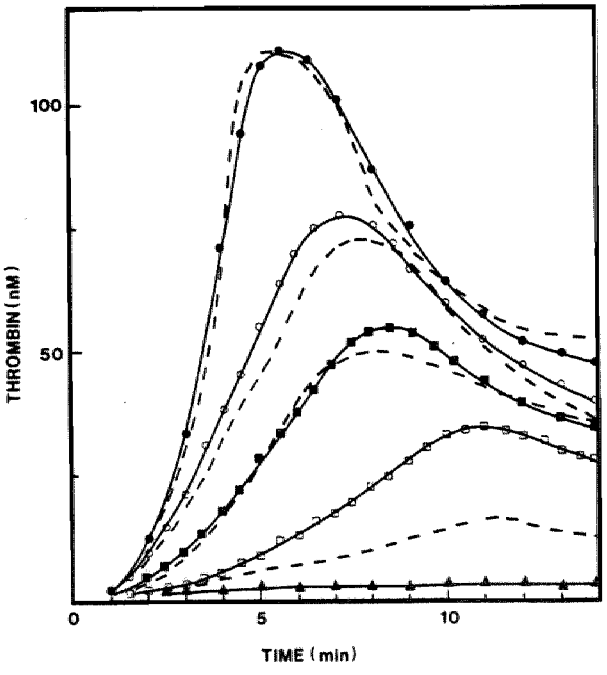

Fig. 2. Generation of thrombin activity in factor IX deficient plasma before and after reconstitution with factor IX (90 nM).
The plasma contained additional phospholipids (1 μM). Coagulation was triggered with a series of dilutions of thromboplastin. Factor IX deficient plasma: (-----); Reconstituted plasma: (———). Final thromboplastin dilution in the reaction mixture: 1/240 (●—●); 1/360 (○—○); 1/480 (■—■); 1/600 (□—□); $CaCl_2$ solution without thromboplastin ( )

thromboplastin dilution. This observation could point to a shortage of phospholipids in the reaction mixture, because at the highest thromboplastin dilutions the available phospholipid/water interface could become the limiting factor in thrombin generation. We therefore repeated the experiments but this time incluced phospholipid vesicles in the reaction mixture (final concentration 1 μM), so that the amount of tissue factor varies but the amount of phospholipid not. When a $CaCl_2$ solution was added to the incubation mixture no thrombin generation was observed. From this observation we conclude that the phospholipid mixture per se does not initiate thrombin formation and the contact activation is neglectable in our system.

Figure 2 shows that in the presence of additional phospholipid vesicles the maximal amount of thrombin formed is greater in the reconstituted plasma than in the congenital factor IX deficient plasma and that the influence of factor IX on thrombin generation is more important at low thromboplastin concentration.

Generation of prothrombinase activity in factor IX deficient plasma after triggering coagulation with thromboplastin.

Information about the prothrombinase activity can be derived from the thrombin generation curves shown in figure 2 by computer processing. To allow computation the decay constant of endogenous thrombin activity must be determined. The decay constant (k) of endogenous thrombin in factor IX deficient plasma was 1,19 min^{-1}. As has been described in the section Materials and Methods, the overall decay is the effect of thrombin inhibition by antithrombin III and by α_2-macroglobulin. Therefore, the decay constant k includes the decay constant of thrombin by antithrombin III (k_1) and the decay constant of thrombin by α_2-macroglobulin (k_2). For the α_2-macroglobulin dependent process the decay constant determined in normal plasma was used ($k_2 = 0.232 \pm 0.004$ min^{-1}; n = 25) (21).

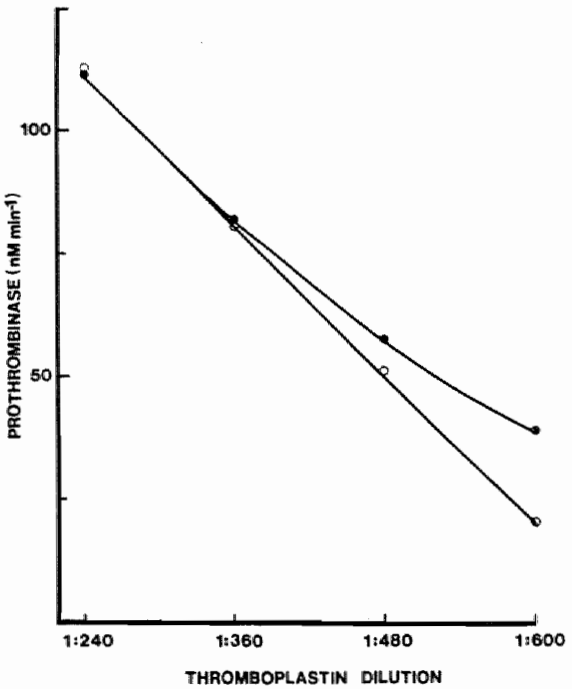

Fig. 3. Maximal prothrombinase activity in factor IX deficient plasma before and after reconstitution with factor IX (90 nM). The plasmas contained additional phospholipids (1 μM). Coagulation was triggered with a series of dilutions of thromboplastin. Factor IX deficient plasma: (○—○); Reconstituted plasma: (●—●).

The prothrombinase data obtained by the computer program are plotted as a function of thromboplastin dilution in figure 3. It can be seen from this figure that at thromboplastin dilutions greater than 1 : 480, the prothrombinase activity in congenital factor IX deficient plasma is lower than that in the reconstituted plasma. There is a clear parallel between the effect of factor IX on thrombin generation (figure 2) and on prothrombinase activity (figure 3).

Factor IX dependency of thrombin activity and prothrombinase activity after triggering coagulation with thromboplastin.

In order to quantify the factor IX dependency of thrombin generation after triggering coagulation with thromboplastin, various amounts of factor IX (15-90 nM) were added to congenital factor IX deficient plasma. After addition of phospholipid vesicles (1 μM) coagulation was triggered with thromboplastin (final dilution 1 : 600). The curves of thrombin generation obtained are shown in figure 4.

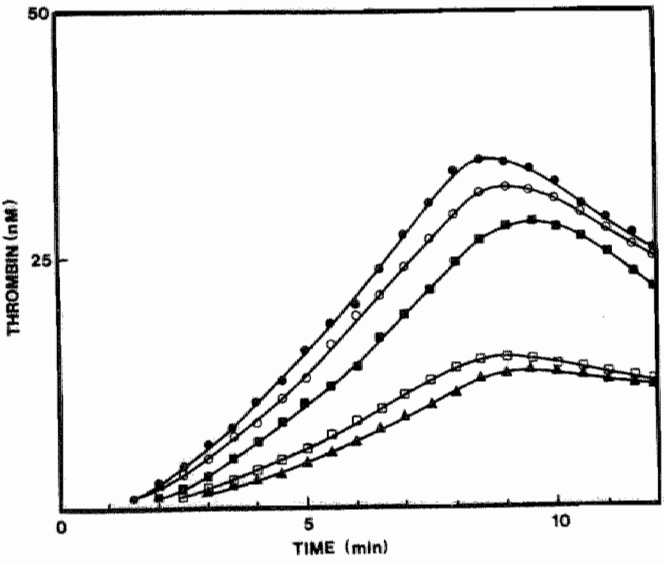

Fig. 4. Influence of factor IX on thrombin generation. The plasmas contained additional phospholipid (1 μM). Coagulation was triggered with thromboplastin (final dilution 1/600). Concentration of factor IX in the reconstituted plasma: 90 nM (●—●); 60 nM (○—○); 30 nM (■—■); 15 nM (□—□); no additional factor IX (▲—▲).

It is clearly seen in this figure that in the concentration range tested factor IX stimulates thrombin generation, but there is no linear relationship between the factor IX concentration and the thrombin formation. At a concentration of 15 nM factor IX the thrombin formation was only slightly increased over the control value (no additional factor IX). A further increase in the factor IX concentration stimulated thrombin generation and the process showed the characterisitics of a saturation phenomenon. When prothrombinase activity was calculated from the thrombin generation curves, the same tendency was observed (figure 5).

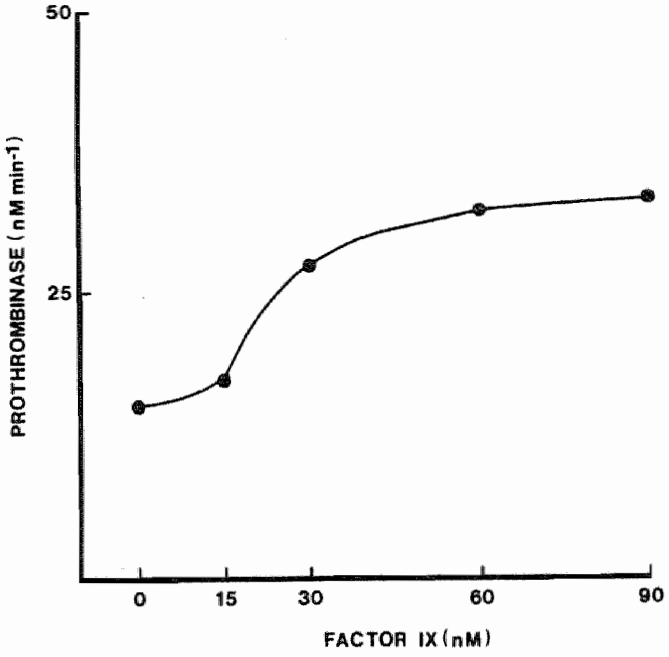

Fig. 5. Influence of factor IX on prothrombinase activity. For experimental details see the legend to figure 4.

Generation of thrombin activity in factor VIII deficient plasma after triggering cogulation with thromboplastin.

As factor $VIII_a$ is involved as a cofactor in the activation of factor X by factor IX_a we also studied the effect of factor VIII on thrombin generation after triggering coagulation by thromboplastin. One part of the congenital factor VIII deficient plasma was reconstituted with 1 U factor VIII (about 100% clotting activity) and another part of congenital factor VIII deficient plasma was used without the addition of factor VIII. After addition of phospholipids (1 μM) to both plasma preparations the coagulation was triggered with thromboplastin (final dilution 1:600). In a control experiment $CaCl_2$ solution (0.1 M) was used instead of the thromboplastin solution. The thrombin generation curves obtained are shown in figure 6. It was evident that the presence of factor VIII clearly stimulates the thrombin generation. The maximal amount of thrombin formed in the reconstituted plasma was about two-fold compared to that formed in the congenital factor VIII deficient plasma.

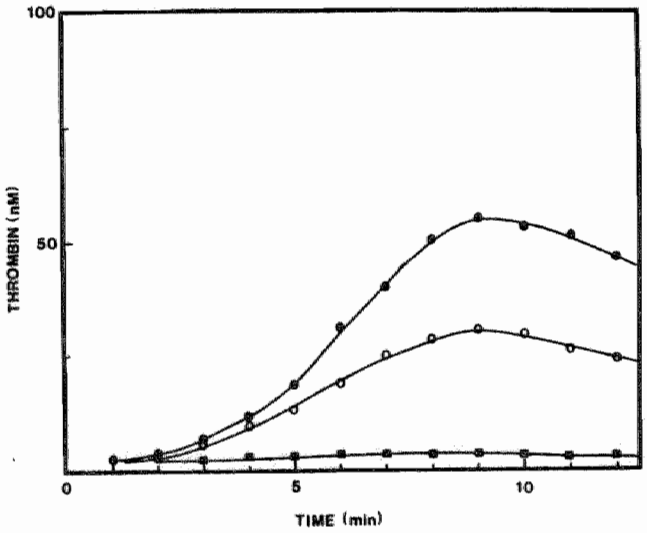

Fig. 6. Generation of thrombin activity in factor VIII deficient plasma before and after reconstitution with factor VIII (1 U/ml). The plasma contained additional phospholipids (1 μM). Coagulation was triggered with thromboplastin (final dilution 1/600). Factor VIII deficient plasma: (○—○); Reconstituted plasma: (●—●); $CaCl_2$ solution without thromboplastin: (■—■).

Generation of prothrombinase activity in factor VIII deficient plasma after triggering coagulation with thromboplastin.

The thrombin generation curves shown in figure 6 were again processed by the computer to obtain prothrombinase activity data. The results of these calculations are shown in figure 7. Addition of factor VIII to the congenital factor VIII deficient plasma caused a two-fold increase in the maximal amount of prothrombinase activity generated. This enhancement in prothrombinase activity upon addition of factor VIII parallelled the enhancement in the process of thrombin generation (see fig. 6).

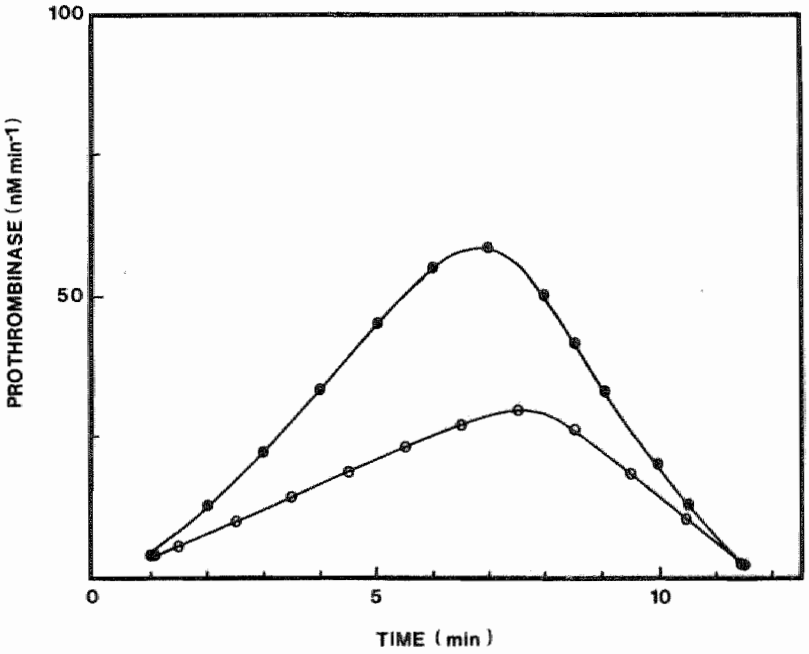

Fig. 7. Prothrombinase activity in factor VIII deficient plasma before and after reconstitution with factor VIII (1U/ml). For experimental details see figure 6.

Factor VIII dependency of thrombin activity and prothrombinase activity after triggering coagulation with thromboplastin.

In order to quantify the factor VIII dependency of thrombin generation after triggering coagulation with thromboplastin, various amounts of factor VIII (0.2 - 1U/ml) were added to congenital factor VIII deficient plasma. Additonal phospholipids (1 µM) were added to the plasma preparation and the coagulation was triggered with thromboplastin (final dilution 1:600). The thrombin generation data and the prothrombinase activity data are shown in the figures 8 and 9, respectively.

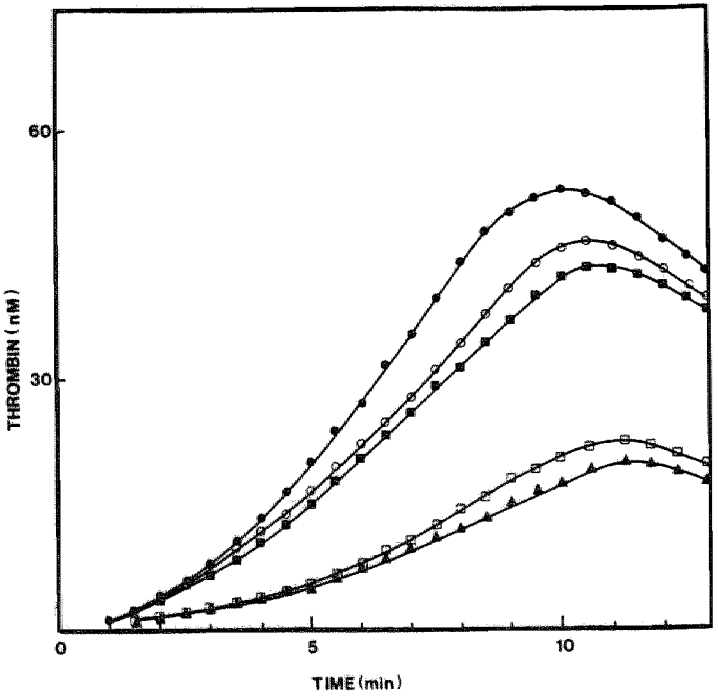

Fig. 8. Influence of factor VIII on thrombin generation. The plasmas contained additional phospholipid (1 µM). Coagulation was triggered with thromboplastin (final dilution 1/600). Concentration of factor VIII in the reconstituted plasma: 1.0 U/ml (●—●); 0.5 U/ml (○—○); 0.3 U/ml (■—■); 0.2 U/ml (□—□); no additional factor VIII (▲—▲).

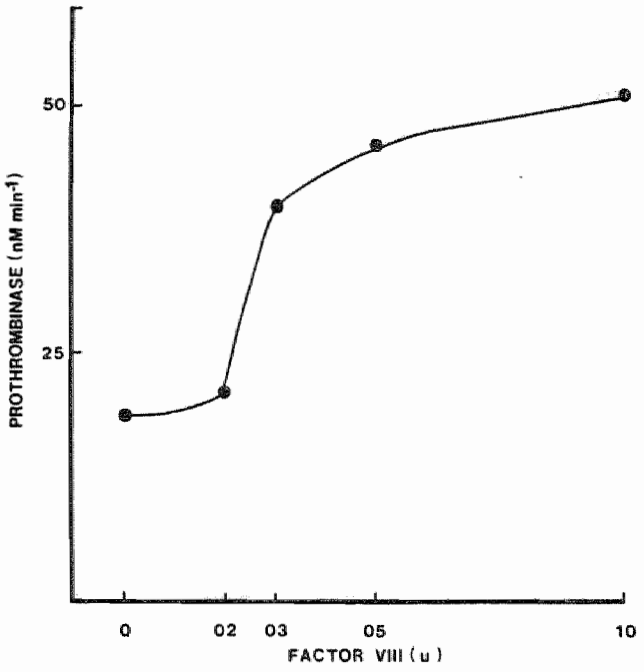

Fig. 9. The influence of factor VIII on prothrombinase activity. For experimental details see the legend to figure 8.

When a low amount of factor VIII (0.2 U/ml) was added, the maximal amount of thrombin generated was only slightly higher than in that in the congenital factor VIII deficient plsma. A further increase in the factor VIII concentration stimulated thrombin generation and again the dependency showed saturation characteristics. The prothrombinase activity data showed the same tendency (figure 9).

DISCUSSION

In this chapter a study of some aspects of the interrelationship between the intrinsic and the extrinsic pathways of coagulation is described. Diluted, $CaCl_2$-containing thromboplastin solutions were used to trigger coagulation in congenital factor IX and factor VIII deficient plasmas to which various amounts of the purified factors were added and the generation of thrombin activity and the prothrombinase activity in these experimental systems were registrated.

When the coagulation of factor IX deficient plasma was triggered with a series of dilutions of thromboplastin, the relation between the maximal amount of thrombin formed and the thromboplastin concentration is not linear. At high thromboplastin dilution (1/600), the generation of thrombin activity in factor IX deficient plasma showed to be markedly reduced. This result is in accordance with the observation of Biggs and Nossel, who reported an abnormal amounts of thrombin in plasma of both hemaophilia A and haemophilia B patients after triggering coagulation with low amounts of thromboplastin (6). In our experimental system, this reduction of thrombin activity was not reversed by the addition of factor IX (90 nM) to the factor IX deficient plasma, although this amount of factor IX is sufficient to restore the clotting activity to normal level. When, however, phospholipid vesicles were added to the factor IX deficient plasma the importance of factor IX in the process of thrombin generation after triggering coagulation with small amounts of thromboplastin became evident. This is indicated that at higher thromboplastin dilution the amount of phospholipids available becomes a rate limiting factor. For this reason additional phospholipids (final concentrations 1μM) were systematically included in the reaction mixture.

It is evident that the influence of factor IX on thrombin generation is more important at higher thromboplastin dilutions. Such an effect is also reflected in the prothrombinase data. These results show that in situations where low amounts of thromboplastin are available the interaction between intrinsic and extrinsic pathway becomes increasingly important.

The effect of factor VIII on thrombin generation after triggering coagulation with small amounts of thromboplastin is shown to similar as seen in factor IX deficient plasma after reconstritution with purified

factor IX. As could be expected, the effect of factor VIII on thrombin generation in congenital factor VIII deficient plasma closely resembles the effect of factor IX on thrombin generation in factor IX deficient plasma. When coagulation is triggered with a small amount of thromboplastin, the presence of factor VIII stimulates thrombin generation and prothrombinase activity; the phenomenon shows saturation characteristics. The role of factor VIII as a cofactor in the factor X activation by factor IX_a has well been studied (23-25). In the absence of factor $VIII_a$ the rate of factor X activation by factor IX_a is extremely slow, even in the presence of a sufficient amount of phospholipids. Factor VIII brings about a 2.10^5-fold increase in the rate of factor X activation by factor IX_a (25).

The dependency of thrombin generation on factor IX and factor VIII after triggering with small amounts of thromboplastin is only evident at factor IX concentrations lower than 30 nM (about 40% of normal clotting activity) or factor VIII concentrations lower than 0.3 U/ml (about 30% of normal clotting activity). These will not be detected with a routine prothrombin time test, because the amount of thromboplastin used in the prothrombin time test is too high. For this reason these tests "bypass" the factor VII/thromboplastin - factor IX pathway and therefore plasma from haemophilia patients will give a normal thrombin generation.

The interaction between the intrinsic and the extrinsic pathways of coagulation might also explain several clinical observations. Some patients with a deficiency of factor VII have a serious hemorrhagic diathesis indeed, whereas patients with deficiencies in the contact activation system have no significant hemorrhagic diathesis, but on the contrary may be associated with thromboembolic episodes. The first patient described with Hageman trait died of pulmonary embolisms (26). Factor XI deficiency which presents as a mild hemorrhage takes an intermediate position.

The results presented in this chapter show the importance of the factors IX and VIII in the thromboplastin-dependent coagulation process in those situations in which trace amounts of thromboplastin are present. This interrelation between the extrinsic and instrinsic pathways could be able to explain properly why hemophilia patients in the deficiencies of the intrinsic pathway are not compensated by the intact extrinsic pathway.

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CHAPTER III

THE RELATIVE IMPORTANCE OF THE VARIOUS VITAMIN K-DEPENDENT CLOTTING FACTORS ON THE PROTHROMBINASE ACTIVITY IN PLASMA OF ORALLY ANTICOAGULATED PATIENTS

SUMMARY

In this chapter, the relative importance of the various vitamin K-dependent clotting factors on the prothrombinase activity in the plasma of orally anticoagulated patients is described.

Addition of purified factors VII, IX or X to plasma from deeply anticoagulated patients (Thrombotest value in the 10-15 % range) did not influence the amount of prothrombinase formation or the amount of thrombin formed. Only the factor II level in the plasma determines the course of thrombin generation. Addition of increasing amounts of purified factor II, VII, IX or X to plasmas deficient in respectively factor II, VII, IX or X showed that the prothrombinase activity linearily increases with the concentration of factor II added and that the concentration below which the factors VII, IX and X start to have an effect on prothrombinase activity were 5 %, 20 % and 40 %, respectively. The half maximal amount of prothrombinase activity was found at about 1% factor VII, 5% factor IX and 10% factor X respectively.

From these observations we conclude that only the changes in factor II level determines the antithrombotic effect of oral anticoagulant therapy. Therefore, it seems likely that, for the control of oral anticoagulant therapy, tests that reflect factor II activity would be suitable.

INTRODUCTION

The coagulation proteins that are synthesized in the hepatic parenchymal cells dependent on vitamin K have the common characteristic of the occurence of γ-carboxylated glutamic acid residues in the N-terminal region of the protein chain. Factors II, VII, IX, X and the anticoagulant proteins C and S belong to this group. Vitamin K stimulates the carboxylation of these protein in a postribosomal step of hepatic synthesis (1). The carboxylated coagulation proteins are capable of binding to a negatively charged phospholipid surfaces via Ca^{2+}-bridges (2). This interaction is essential for their effective participation in the coagulation processes.

Vitamin K antagonists, the coumarin congeners, interfere with the carboxylation of vitamin K-dependent coagulation proteins, and this results in the synthesis of biologically inactive but immunologically detectable forms. These inactive forms termed PIVKA reach the blood stream but are unable to bind to a negatively charged phospholipid via Ca^{2+}-bridges (3). Therefore the formation of at least three multicomponent enzymatic complexes, i.e. the factor X activator formed by thromboplastin and factor VII_a the factor X activator (tenase, FIX_a - $FVIII_a$ - PL) and prothrombin converting activity (prothrombinase, FX_a - FV_a - PL) will be diminished by vitamin K antagonist therapy. This will of course results in a diminution of thrombin generation.

Because the effect of coumarin therapy that interferes with the synthesis of effective clotting factors is not immediate, the preexistant circulating clotting factors have to disappear before the level of clotting factors adapts to the new dimnished level of synthesis. The various factors decline with velocities dependent upon their half-life times which are approximately: factor VII: 6 hours, factor IX: 14 hours, factor X: 45 hours, factor II: 60 hours (4). Shortly after the beginning of anticoagulant therapy the levels of clotting factors will show the following order VII < IX < X < II. Only when factor II, the "slowest" one, has adapted to the prevailing level of synthesis the relative levels will be equal. If the therapy is stopped the velocity of reapperance is again a function of half-life time and the order will be VII > IX > X > II. Because of fluctuations in dosage and (patho)physiological circumstances, stable anticoagulation with all factors at the same level is less common than

would be desirable. Therefore knowledge of the relative importance of the level of each individual clotting factor to the overall anticoagulant (i.e. antithrombosis) effect is of great practical importance.

For deep and stable anticoagulant effect the levels of the vitamin K-dependent clotting factors are normally regulated down to about 20% of normal (5). This level of clotting activity is considered as an effective anticoagulant treatment. However, this estimation of the potential clotting activity is based on an overall clotting test, i.e. prothrombin time. The relative contribution of the individual clotting factors to this "hypocoagulability" of plasma is not clearly understood. In this chapter, we describe the results of a study on the effect of vitamin K-dependent clotting factors on prothrombinase activity in dicoumarol plasma.

MATERIALS AND METHODS

Patients

46 patients with a variety of thrombotic disorders undergoing oral anticoagulation with dicoumarol drugs were selected for this study. All patients were in the optimal therapeutic range as judged by the laboratory control of oral anticoagulant therapy (according to the standard of the Thrombose Stichting Nederland). The thrombotest values were between 10% and 15% of those found in normal plasma.

Plasmas

Blood from patients undergoing oral anticoagulant treatment was collected in trisodium citrate (9 volumes of blood to 1 volume of 0.13 M trisodium citrate) and centrifugated for 15 minutes at 3000 x g at 15 ^{o}C. Plasma samples from the 46 patients were pooled and centrifugated for 1 hour at 23000 xg at 4 ^{o}C. The pooled platelet free plasma was stored at -80 ^{o}C.

Thromboplastin

Human brain thromboplastin was prepared by a modification of the method of Owen (6). The preparation obtained was homogenized in a Potter Elvehjem homogeniser for 3 minutes, centrifugated at 2000 x g for 15 minutes and stored in 1 ml aliquots at -20 °C. Prior to use the preparation was thawed, diluted 1:40 with 0.05 M Tris-HCl (pH 7.35) containing 0.1 M $CaCl_2$ and prewarmed at 37 °C for 1 hour. When normal plasma was incubated with this thromboplastin dilution under the same experimental conditions as used in the thrombin generation experiments the clotting time was 80 seconds.

Cephaloplastin

Cephaloplastin was obtained as a commercial APTT-reagent from Dade (Aguada, Puerto Rico, USA). The commercial reagent was diluted 1:5 with 0.05 M Tris-HCl (pH 7.35) containing 0.1 M $CaCl_2$, prewarmed for 10 minutes at 37 °C and mixed before use.

Proteins

Bovine prothrombin and factor X were prepared as described by Lindhout et al (7). Bovine factor IX was prepared by the method of Fujikawa et al (8). Bovine factor VII was kindly supplied by Dr. C. Reutelingsperger.

Commercial reagents

Human congenital deficient plasmas were obtained from Behring Institute (West Germany). Soybean trypsin inhibitor (SBTI) was obtained from Sigma (St. Louis, USA). The chromogenic substrate S2238 was obtained from Kabi Vitrum (Stockholm, Sweden). All other chemicals were of the highest grade commercially available.

Measurement of clotting factor II, VII, IX and X activities in dicoumarol plasma

The activity levels of the clotting factors II, VII, IX and X in dicoumarol plasma were quantitatively determined by a standard one-stage method according to the procedure of manufacturer (Behring Institute, West Germany).

Measurement of thrombin generation in plasma

For the measurement of thrombin generation the procedure described by Hemker et al (9) was used. Briefly, 240 µl of plasma and 60 µl of buffer (Tris-HCl, pH 7.35) were incubated for 4 minutes at 37 °C. Thrombin generation was triggered by the addition of 60 µl of 0.1 M $CaCl_2$ solution containing a suitable trigger of coagulation. For activation of the extrinsic pathway the trigger consisted of thromboplastin (final dilution 1/240) and for activation of the intrinsic pathway Cephaloplastin (final dilution 1/30) was used. At fixed time intervals, 10 µl aliquots of the incubation mixture were subsampled into a test tube containing 465 ul of buffer (Tris-HCl, 0.1 M NaCl, 0.5% albumin, 0.02 M EDTA, pH 7.9) and 25 µl of S2238 (4 mM) at 37 °C. The subsampling tubes were incubated for 2 minutes at 37 °C, and then the reaction was stopped by the addition of 300 µl of concentrated acetic acid. The pipettes used for sampling and stopping the reaction were connected to an Apple IIe computer, programmed to record the moment of sampling and the moment of stopping the reaction. The optical density was read at 405 nm in a spectrophotometer (LKB-Ultraspec.). From the change in optical density and the time interval between sampling and stopping the Δ O.D./min was automatically calculated.

Measurement of the decay constant of endogenous thrombin

Briefly, 120 µl of plasma and 24 µl of buffer (Tris-HCl, pH 7,35) were incubated for 4 minutes at 37 °C. Thrombin generation was triggered by the addition of 30 µl of thromboplastin. At the moment after a maximal thrombin formation, 6 µl of Soybean Trypsin Inhibitor (SBTI, 10 mg/ml) were added to the incubation mixture. At fixed intervals after the addition of SBTI, 10

µl aliquots of the incubation mixture were subsampled into a test tube containing S2238 (4 mM) and EDTA (20 mM) by using the time recording pipette. The measuring procedure was the same as that described for the measurement of thrombin generation (9).

The data obtained from thrombin amidolytic activity at the different time points (Ct) were fitted to the formula $C_t = C_R + C_o e^{-kt}$, which gives the level at the moment of SBTI addition ($C_o + C_R$), the steady end level (C_R) and the decay constant (k). The parameters were determined by means of an ordinary least squares fit of the model to the data. The parameter values that minimize the sum of squared residuals were calculated using the Box-Kanemasu modification of Gauss' method (10). The linear equations were solved by means of Haussholder transformations. The decay constant thus obtained is the sum of the α_2 macroglobulin dependent decay constant (k_2) and the antithrombin III dependent decay constant (k_1) (9).

Estimation of the prothrombin conversion velocity

The prothrombin conversion velocity, i.e prothrombinase activity (nM min^{-1}) was calculated by a computer, using the thrombin generation and the experimentally determined decay constant of endogenous thrombin. As described above, the decay constant of endogenous thrombin obtained is the sum of k_2 and k_1. The k_2 used is 0.232 ± 0.004 min^{-1} (n = 25). For technical details see ref. 9.

RESULTS

Time course of thrombin generation and prothrombinase formation in normal and dicoumarol plasmas

The levels of factor II, factor VII, factor IX and factor X activity in the pooled dicoumarol plasma are shown in table 1. Figure 1 shows the time course of thrombin generation and prothrombinase formation in normal and dicoumarol plasma after triggering coagulation with thromboplastin. As can be seen, the initial rate of thrombin formation and the maximal amount of thrombin generated were significantly decreased in dicoumarol plasma compared to normal plasma.

TABLE I

Levels of the clotting factor II, VII, IX and X activity in pooled dicoumarol plasma

Factor	Activity %	Approximate Conc. (nM)
II	20	300
VII	10	1
IX	40	30
X	20	35

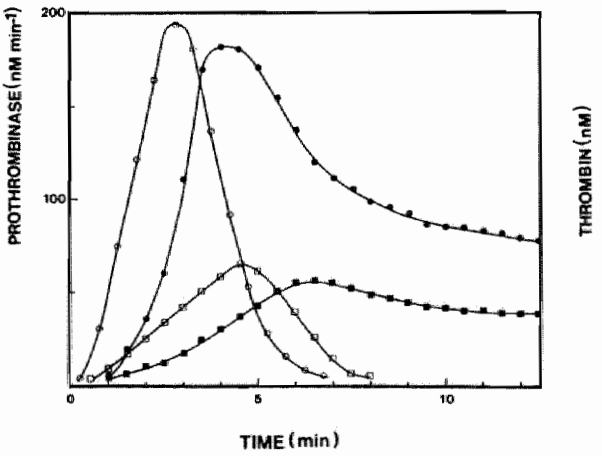

Fig. 1. Time course of the thrombin generation and prothrombinase formation in normal and dicoumarol plasmas after triggering coagulation with thromboplastin (final dilution 1/240).

●——● Thrombin in normal plasma
○——○ Prothrombinase in normal plasma
■——■ Thrombin in dicoumarol plasma
□——□ Prothrombinase in dicoumarol plasma

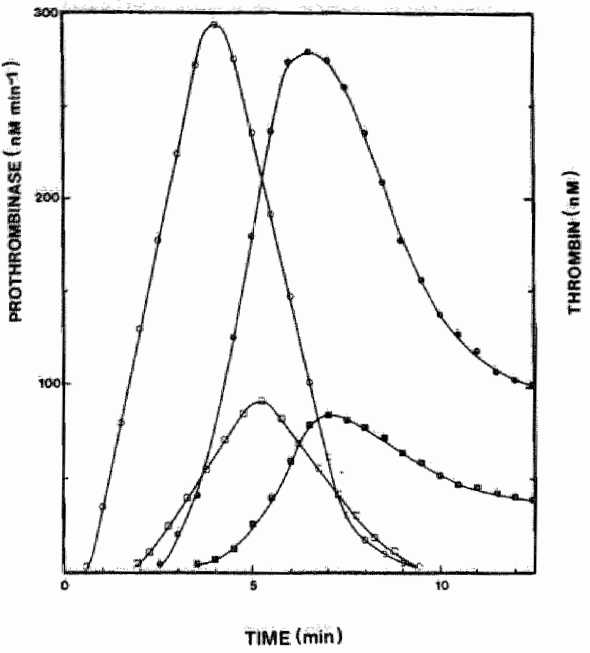

Fig. 2. Time course of the thrombin generation and the prothrombinase formation in normal and dicoumarol plasma after triggering coagulation with Cephaloplastin (final dilution 1/30).

●——● Thrombin in normal plasma
○——○ Prothrombinase in normal plasma
■——■ Thrombin in dicoumarol plasma
□——□ Prothrombinase in dicoumarol plasma

Figure 2 shows the time course of thrombin generation and prothrombinase formation in normal and dicoumarol plasma after triggering coagulation with Cephaloplastin. The decrease in both the thrombin generation and the prothrombinase formation were similar to those obtained in thromboplastin triggered coagulation, but in addition, the lag time in the thrombin generation and prothrombin formation were significantly prolonged in dicoumarol plasma compared to normal plasma.

Effect of the addition of a various amounts of factor II, factor VII, factor IX and factor X to dicoumarol plasmas on thrombin generation and prothrombinase formation.

To determine which one among the four coagulation factors influenced by dicoumarol drugs is mainly responsible for the decreases of thrombin generation and prothrombinase activity, we investigated the effect of the addition of increasing amounts of respectively factor II, factor VII, factor IX or factor X to dicoumarol plasma. When studying the effects of factor II, factor VII and factor X, coagulation was triggered with thromboplastin and when studying the effect of factor IX coagulation was triggered with Cephaloplastin.

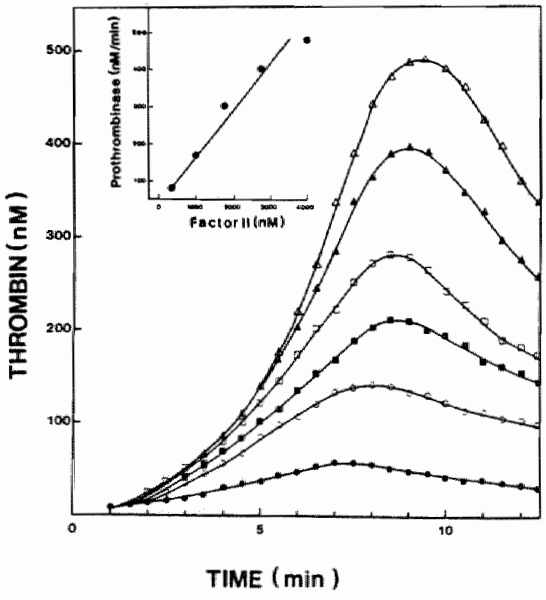

Fig. 3. Effect of the addition of various amounts of factor II to dicoumarol plasma on thrombin generation.
Coagulation was triggered with thromboplastin (final dilution 1/240). The inset represents the maximal prothrombinase activity calculated from the thrombin generation curves.

Figure 3 shows the effect of the addition of increasing amounts of factor II thrombin to dicoumarol plasma on thrombin generation and prothrombinase formation. As can be seen, both thrombin generation and prothrombinase formation linearily increased with the amounts of factor II added. In both cases the increases were directly proportional to the concentration of factor II added to dicoumarol plasma. Theoretically we should expect a saturation phenomenon at high factor II levels. From fig. 3 (inset) it is clear that even 4 μM of factor II (more than a twice the physiological concentration) still is far from the saturating concentration. These observations indicate that the prothrombin converting capacity originally present in dicoumarol plasma is sufficient to catalyze the conversion of much higher amounts of prothrombin than that are present in dicoumarol plasma. Figure 4,5 and 6 show the effects of the addition of factor VII, factor IX and factor X to dicoumarol plasma on thrombin generation and prothrombinase formation. These figures show that neither the thrombin generation nor the prothrombinase formation could be stimulated by the addition of the factors VII, IX and X.

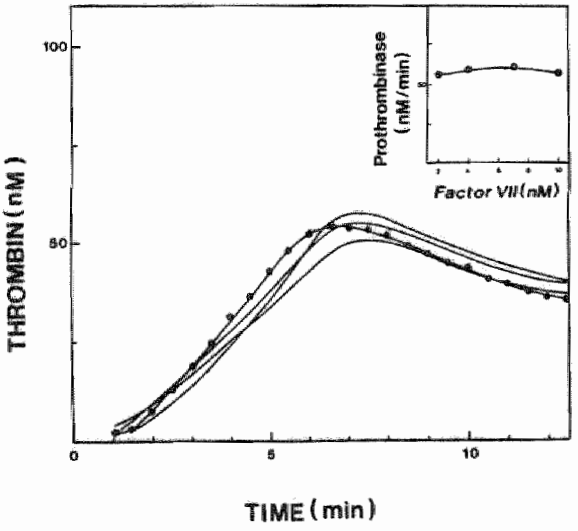

Fig. 4. Effect of the addition of various amounts of factor VII to dicoumarol plasma on thrombin generation.
Coagulation was triggered with thromboplastin (final dilution 1/240). The inset represents the maximal prothrombinase activity calcualted from the thrombin generation curves.

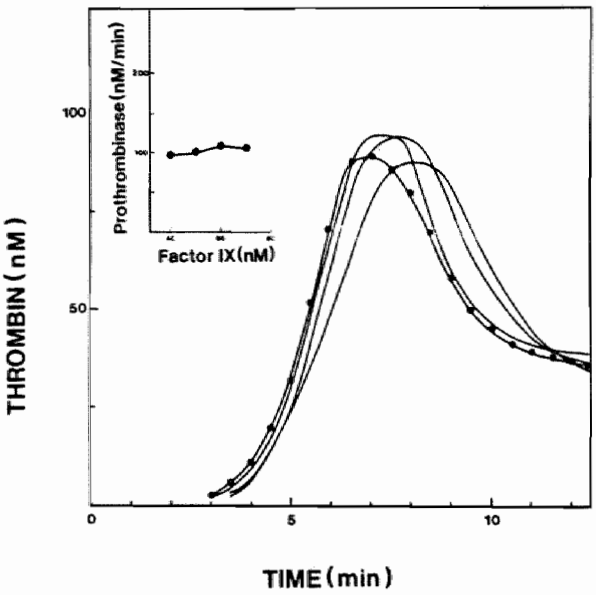

Fig. 5. Effect of the addition of various amounts of factor IX to dicoumarol plasma on thrombin generation. Coagulation was triggered with Cephaloplastin (final dilution 1/30). The inset represents the maximal prothrombinase activity calculated from the thrombin generation curves.

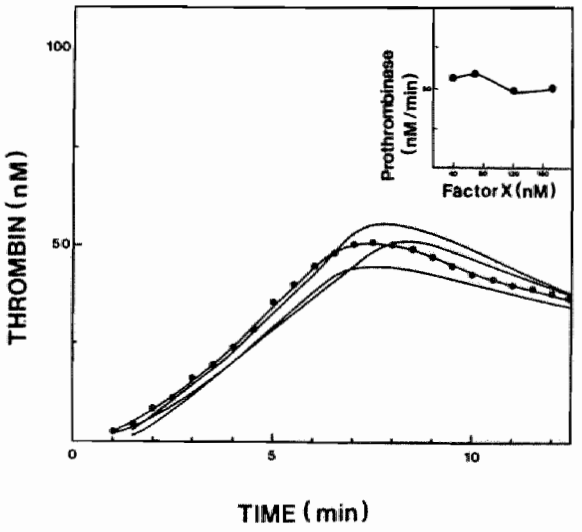

Fig. 6. Effect of the addition of various amounts of factor X to dicoumarol plasma on thrombin generation. Coagulation was triggered with thromboplastin (final dilution 1/240). The inset represents the maximal prothrombinase activity calculated from the thrombin generation curves.

Effects of the addition of various amounts of factor II, factor VII, factor IX and factor X to congenital deficient plasma on thrombin generation and prothrombinase formation.

In order to obtain an impression of the levels below which prothrombin and the factors VII, IX and X start to have an influence on prothrombin conversion, we performed further experiments in which the effect of the addition of a various amounts of the four vitamin K-dependent clotting factors to congenital deficient plasmas was studied. The results of these experiments are summarized in figure 7.

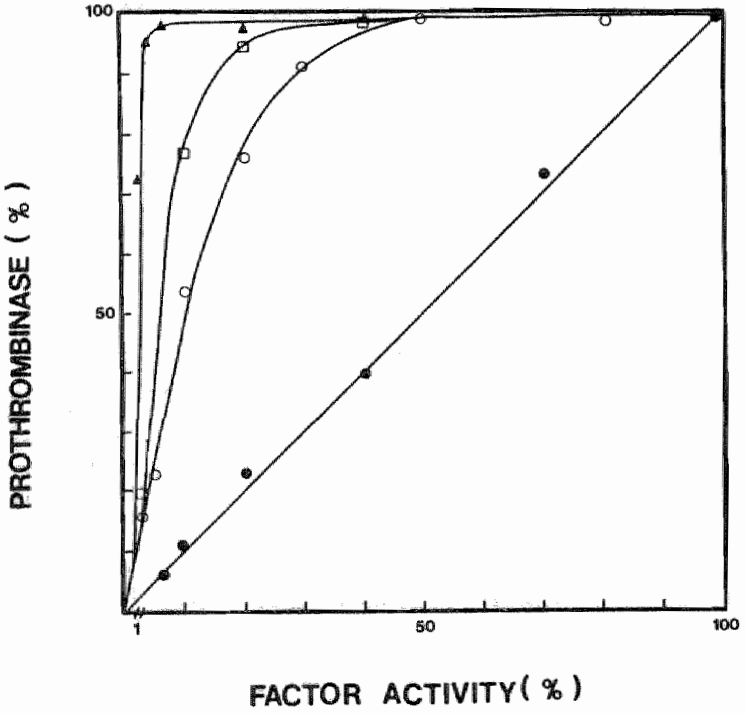

Fig. 7. Effect of the addition of various amounts of factor II (●), factor VII (▲), factor IX (□) and factor X (○) on maximal of prothrombinase activity formed in congenital deficient plasmas.

It can be clearly seen that the prothrombinase formation linearily increases with the amounts of factor II added to factor II deficient plasma. The factors VII, IX and X start to have an effect on prothrombinase activity when the levels of factor VII, IX and X are below 5 %, 20 % and 40 %, respectively. Half maximal prothrombinase activity is found roughly for factor VII at 1 %, for factor IX at 5 % and for factor X at 10 %.

DISCUSSION

The hypocoagulability of dicoumarol plasma is due to the diminution of the effective coagulation activity of the four vitamin K-dependent clotting factors, prothrombin, factor VII, factor IX and factor X. The underlying mechanism is that dicoumarol derivatives inhibit the carboxylation of certain glutamic acid residues during the synthesis of precursors of the vitamin K-dependent clotting factors. The γ-carboxyglutamic acid residues formed during this carboxylation process are involved in the binding of the vitamin K-dependent clotting factors to phospholipid surfaces via Ca^{2+}-bridges (2). A diminution in the concentrations of the effective (i.e. γ-carboxylated) vitamin K-dependent clotting factors, therefore, can be expected to influence the formation of the multicomponent enzymatic complexes involved in the coagulation cascade. As a result of these events the thrombin generation will be reduced (Fig. 1 and Fig. 2).

Due to the different half-life times of the vitamin K-dependent clotting factors, the effect of oral anticoagulant drugs starts with the reduction of the factor VII activity within 24 hours after administration of the drug, the reduction of the factors IX, X and prothrombin follows within 72 hours (4). Since a reduction in the effective concentration of vitamin K-dependent clotting factors is more easily detected in the thromboplastin-dependent global clotting assays than in the contact activation-dependent assay, oral anticoagulant therapy is usually monitored by the prothrombin time type tests. Therapeutic levels are defined by the extent to which the prothrombin time is prolonged. It is good to realize that oral anticoagulant therapy determine the effective concentration of three factors that influence the prothrombin time (prothrombin, factor VII

and factor X) and that one fixed clotting time (e.g. 30 seconds) can represent an infinite variety of combinations of concentrations of these three clotting factors (5). In the initial period of oral anticoagulant therapy a reduction of the factor VII activity may be expected to determine the prothrombin time while after a longer period of time the effective response will probably depend on the reduced factor X and/or prothrombin activity. The exact mechanism of the development of the antithrombotic effect by diminution of clotting factors is unknown. Clinical experience has empirically teached us to keep the plasma thromboplastin time at a certain level in order to provide protection against thrombosis with a minimal risk of haemorrhagic complications (5).

In an attempt to better understand the relative contribution of the different vitamin K-dependent clotting factors to the overall clotting activity, we first measured the effect on thrombin generation of the addition of purified vitamin K-dependent clotting factors to dicoumarol plasma. We found that only the prothrombin level originally present in the dicoumarol plasma determine the course of thrombin generation. The prothrombinase activity formed (about 20% of normal) in dicoumarol plasma was sufficient to catalyze the conversion of a normal amount of prothrombin (or even more) into thrombin (Fig. 3). The addition of the factors VII, IX or X to dicoumarol plasma did not promote thrombin generation; it made no difference whether coagulation was triggered via the extrinsic or via the intrinsic pathway (Fig. 4,5,6). This implicates that, during the initial and the intermediate stages of oral anticoagulant therapy, a prolonged in vitro prothrombin time caused by a reduced activity of the factors VII and X does not adequately reflect the thrombin generation potency of the plasma.

In order to find out below which level(s) the vitamin K-dependent clotting factors start to have an effect on prothrombinase activity, we studied the effect of the addition of the purified vitamin K-dependent clotting factors to congenital deficient plasmas. It was shown that the prothrombinase activity linearily increases with the concentration of prothrombin and that the factors VII, IX and X start to have an effect on the prothrombinase activity when the concentrations were lower than 5 %, 20 % and 40 %, respectively. A half maximal prothrombinase activity is found at about 1% (factor VII), 5% (factor IX) and 10% (factor X) (Fig. 7). It

can be concluded that both the optimal factor X converting activity (tenase) and the optimal prothrombin converting activity (prothrombinase) only require small amounts of the enzymes (factor IX_a and factor X_a). Also, for triggering the thromboplastin-dependent pathway, only a small amount of factor VII is needed.

We conclude that the principal antithrombotic effect of oral anticoagulant therapy is achieved by influencing the effective prothrombin level. We therefore think that it may be interesting to use an assay that monitors the effective prothrombin level for the control of oral anticoagulant therapy.

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CHAPTER IV

SJAMP, A HEPARIN-LIKE MATERIAL FROM STICHOPUS JAPONICUS SELENKA: EFFECT ONTHE GENERATION OF THROMBIN ACTIVITY IN PLATELET POOR AND PLATELET RICH PLASMA

SUMMARY

Stichopus japonicus acidic mucopolysaccharide (SJAMP) is a heparin-like material that can be isolated from stichopus japonicus selenka (sea cucumber). In This chapter the influence of SJAMP on the generation of thrombin activity in platelet poor and platelet rich plasma is described.

The main effect of SJAMP is to inhibit thrombin. This effect of SJAMP is about sixteen-fold weaker than that of 4th international standard heparin on a weight basis. When the experiments are carried out in platelet poor plasma after triggering coagulation with thromboplastin, inhibition of the amount of thrombin formed can be explained by the acceleration of thrombin decay achieved by SJAMP. When coagulation in platelet poor plasma is triggered with Cephaloplastin an additional effect of SJAMP is seen; it increases the lag time in the thrombin generation process. This phenomenon could be explained by inhibition of the feedback activation of factor VIII by thrombin. In these aspects SJAMP acts exactly like standard heparin.

When the effect of SJAMP is studied in platelet rich plasma after triggering coagulation with trace amounts of thromboplastin a slight stimulation of thrombin generation is observed at low SJAMP concentrations, while at higher SJAMP concentrations inhibition of the maximal amount of thrombin formed and an increase in the lag time of the thrombin generation process is observed. This indicates that the inhibitory effect of SJAMP on

thrombin activity, unlike that of heparin, can not be neutralized by platelet factor 4. The increase in lag time of the thrombin generation process may be explained by inhibition of the feedback activation of factor VIII and by inhibition of the thrombin-induced platelet stimulation.

INTRODUCTION

Stichopus japonicus acidic mucopolysaccharide (SJAMP) is a heparin-like material that can be isolated from stichopus japonicus selenka (a sea cucumber).

Structure analysis shows that it contains N-acetylgalactosamine, glucuronic acid, fucose and sulphate in the approximate ratio of 1:1:1:4, respectively (1). The molecular weight of SJAMP is about 30,000 - 50,000 Dalton. Some physical properties of SJAMP, like for instance the metachromatic intensity and the electrophoretic behaviour, are similar to those of heparin (1). The mechanism of action of SJAMP with respect to its anticoagulant activity is still unknown, but remarkable differences between SJAMP and heparin have been described (2) (see below).

A crude preparation of SJAMP was previously used in China as a traditional drug for the treatment of patients with gastric carcinoma. It was generally considered to be a beneficial complementary drug for these patients because it could mitigate the symptoms and reduce the pain. However, administration of a large dose, or of a low dose for a long time, was associated with bleeding symptoms in some patients.

These phenomena have received much attention in the past few years (1-3). It was concluded that the effect of SJAMP on blood coagulation is due to its action on thrombin and that the mode of action of SJAMP is different from that of heparin in that it is independet upon antithrombin III. These conclusions were based on the observation that the presence of SJAMP causes a prolongation of the thrombin time in both normal and artificial antithrombin III-deficient plasmas.

Studies in platelet rich plasma revealed that the inhibitory action of SJAMP towards thrombin was not influenced by a change in the amount of platelet present (2). The authors therefore concluded that SJAMP, in contrast to heparin, could not be neutralized by platelet factor 4 released during activation of the platelets.

It was also observed that SJAMP induces the aggregation of human and rabbit platelets in vitro (2). The platelet aggregation curve induced by SJAMP is similar to that induced by ADP. As SJAMP failed to induce platelet aggregation in platelet rich plasma prepared from Na_2-EDTA treated blood, it was concluded that the induction of platelet aggregation by SJAMP is Ca^{2+}-dependent.

Finally it was observed that administration of SJAMP can cause a reduction in the amount of circulating platelets in the blood of experimental animals (2). This reduction in the amount of circulating platelets can probably be explained by the aggregating effect of SJAMP on blood platelets. This aggregating effect could not be influenced by aspirin and therefore seems to be independent of the arachidonic acid metabolism (2).

In view of the observations cited in this introduction we thought it worthwile to study the influence of SJAMP on the generation of thrombin activity in plasma. In this chapter we report the results of these investigations.

MATERIALS AND METHODS

Platelet free plasma

Blood from healthy donors was collected in trisodium citrate (9 volumes of blood to 1 volume of 0.13 M trisodium citrate) and centrifugated for 15 minutes at 3000 x g at 15 °C. Subsequently the platelet poor plasma obtained was centrifugated for 1 hour at 23000 x g at 4 °C. The platelet free plasma was stored at -80 °C.

Platelet rich plasma

Platelet rich plasma was prepared by centrifugation of freshly drawn citrated blood for 10 minutes at 900 x g at 15 °C and was used within 2 hours.

Thromboplastin

Human brain thromboplastin was prepared by a modification of the method of Owen and Aas (4). The preparation obtained was homogenized in a Potter Elvehjem homogeniser for 3 minutes, centrifugated at 2000 x g for 15 minutes and stored in 1 ml aliquots at -20 °C. Prior to use the preparation was thawed, diluted 1 : 40 with 0.05 M Tris-HCl (pH 7.35) containing 0.1 M $CaCl_2$ and prewarmed at 37 °C for 1 hour. When normal plasma was incubated with this thromboplastin dilution under the same experimental conditions as used in the thrombin generation experiments the clotting time was 80 seconds.

Cephaloplastin

Cephaloplastin was obtained as a commercial APTT-reagent from Dade (Aguada, Puerto Rico, USA). The commercial reagent was diluted 1 : 5 with 0.05 M Tris-HCl (pH 7.35) containing 0.1 M $CaCl_2$, prewarmed for 10 minutes at 37 °C and mixed before use.

Stichopus japonicus acidic mucopolysaccharide (SJAMP)

SJAMP was kindly supplied by Dr. Jia-Zeng Li, Institute of Hematology, Tianjin, China. The compound was isolated and purified in the Biochemical Laboratory of Tianjin Medical and Pharmacological Institute, China.

Heparin

4th international standard heparin was obtained from the National Institute for Biological Standards and Control (NIBSC).

Buffers

Buffer A: 0.05 M Tris-HCl, 0.1 M NaCl (pH 7.35)
Buffer B: 0.05 M Tris-HCl, 0.1 M NaCl, 0.5% albumin, 0.02 M EDTA (pH 7.9)

Commercial reagents

The chromogenic substrate S2238 was obtained from Kabi Vitrum (Stockholm, Sweden). Soybean trypsin inhibitor was obtained from Sigma (St. Louis, USA). All other chemicals were of the highest grade commercially available.

Measurements of thrombin generation in plasma

For the measurement of thrombin generation the procedure described by Hemker et al (5) was used. To 240 µl of a plasma preparation, 60 µl of buffer A containing the compound to be tested was added. The test tube was incubated for 5 minutes at 37 °C. Thrombin generation was triggered by the addition of 60 µl of a 0.1 M $CaCl_2$ solution containing a suitable trigger of coagulation. For activation of the extrinsic pathway the trigger consisted of thromboplastin and for activation of the intrinsic pathway Cephaloplastin was used. At fixed time intervals, 10 µl aliquots of the incubation mixture were subsampled into tubes containing 465 µl of buffer B and 25 ul of S2238 (4mM) at 37 °C. The subsampling tubes were incubated for 2 minutes at 37 °C, and then the reaction was stopped by the addition of 300 µl concentrated acetic acid. The pipettes used for sampling and stopping the reaction were connected to an Apple IIe computer. A program was developed that allowed automatic registration of the moment of subsampling and the moment of stopping the reaction. The optical density was read at 405 nm in a spectrophotometer (LKB-Ultrospec). From the change in optical density and the time interval between subsampling and stopping the Δ O.D./min was automatically calculated.

Measurement of the decay constant of endogenous thrombin

Briefly, 120 µl of plasma and 24 µl of buffer A were incubated for 5 minutes at 37 °C. Thrombin generation was triggered by the addition of 30 µl of thromboplastin solution or Cephaloplastin solution. At the moment after maximal thrombin formation, 6 µl of soybean trypsin inhibitor solution (SBTI, 10 mg/ml) and 10 µl of a solution of the compound to be tested, taken up in the same pipette, were added to the reaction mixture.

At shortest possible intervals after addition of SBTI and the compound to be tested, 10 µl aliquots of the incubation mixture were subsampled into tubes containing S2238 (4mM) and EDTA (20 mM) by using the time recording pipette. The measuring procedure was the same as that described for the measurement of thrombin generation.

The data obtained from thrombin amidolytic activity at the different time points (Ct) were fitted to the formula $C_t = C_R + C_o e^{-kt}$, which gives the level at the moment of SBTI addition ($C_R + C_o$), the steady end level (C_R) and the decay constant (k). The parameters were determined by means of an ordinary least squares fit of the model to the data. The parameter values that minimize the sum of squared residuals were calculated using the Box-Kanemasu modification of Gauss' method (6). The linear equations were solved by means of Haussholder transformations. The decay constant thus obtained is the sum of the α_2-macroglobulin dependent decay constant (k_2) and the antithrombin III dependent decay constant (k_1) (5).

Estimation of the prothrombin conversion velocity

The prothrombin conversion velocity (prothrombinase nM min^{-1}) was calculated by a computer using the thrombin generation data and the experimentally determined decay constant of endogenous thrombin. As described above, the decay constant of endogenous thrombin obtained is the sum of k_2 and k_1. The k_2 used is 0.232 $\pm$ 0,004 min^{-1} (n = 25). For technical details see ref. 5.

RESULTS

Influence of SJAMP on the decay of endogenous thrombin in plasma

Before studying the influence of SJAMP on the decay of endogenous thrombin in plasma we first determined the time dependency of thrombin generation in plasma without the addition SJAMP or heparin (Fig. 1). This enabled us to design the decay-experiments in such a way that the prothrombinase inhibitor SBTI was added just after the maximum in the thrombin activity versus time curve. In the thrombin decay measurements SBTI and

SJAMP were simultaneously added to the incubation mixture five minutes after starting the coagulation. The amount of SBTI used was shown to stop the prothrombinase activity completely and not to influence the thrombin activity (5).

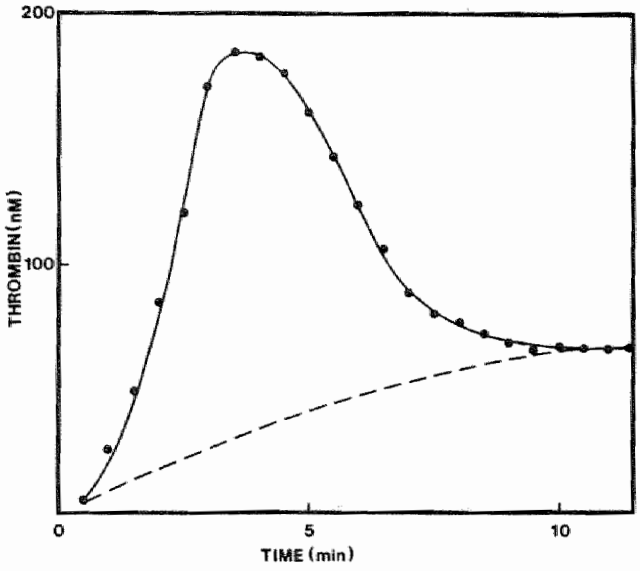

Fig. 1 Amidolytic activity of thrombin in plasma as a function of time. Coagulation was triggered with thromboplastin. The broken line represents the level of the α_2 macroglobulin-thrombin complex. For technical details see the section Materials and Methods.

The effect of SJAMP on the decay of endogenous thrombin was compared to that of the standard heparin under the same experimental conditions. The results are shown in figure 2. It can be seen that for both compounds the decay constant increases practically linearily with the concentration of the polysaccharide. From a comparison of the slopes of the lines it can be concluded that, on a weight basis, the effect of SJAMP on thrombin decay is sixteen-fold weaker than that of heparin.

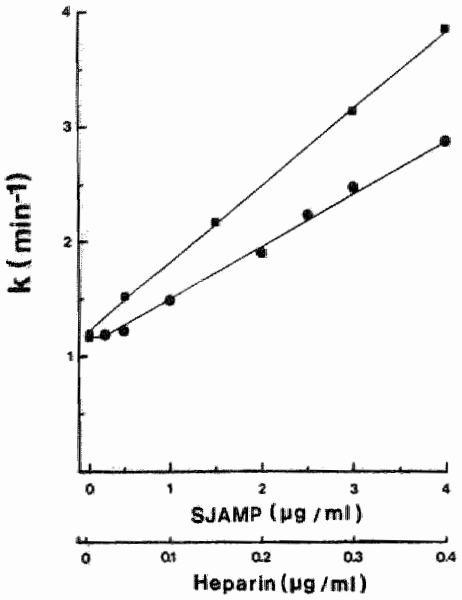

Fig. 2 The influence of SJAMP (●) and heparin (■) on the decay constant of thrombin in plasma.
For each concentration of SJAMP or heparin the experiments were carried out at least three times. For technical details see the section Materials and Methods.

Effect of SJAMP on generation of thrombin activity and prothrombinase activity after triggering coagulation with thromboplastin

To study the effect of SJAMP on thrombin generation and prothrombinase activity in the extrinsic pathway, SJAMP was added to platelet poor plasma and the coagulation was triggered with thromboplastin (final thromboplastin dilution in the reaction mixture was 1 : 240), then thrombin generation was measured in time and prothrombinase activity was calculated. The results are shown in figure 3. It can be seen that in the presence of increasing amounts of SJAMP both the velocity of thrombin formation and the maximal amount of thrombin formed were decreased. In the concentration range tested there was no effect of SJAMP on the initial lag time in time course of thrombin generation.

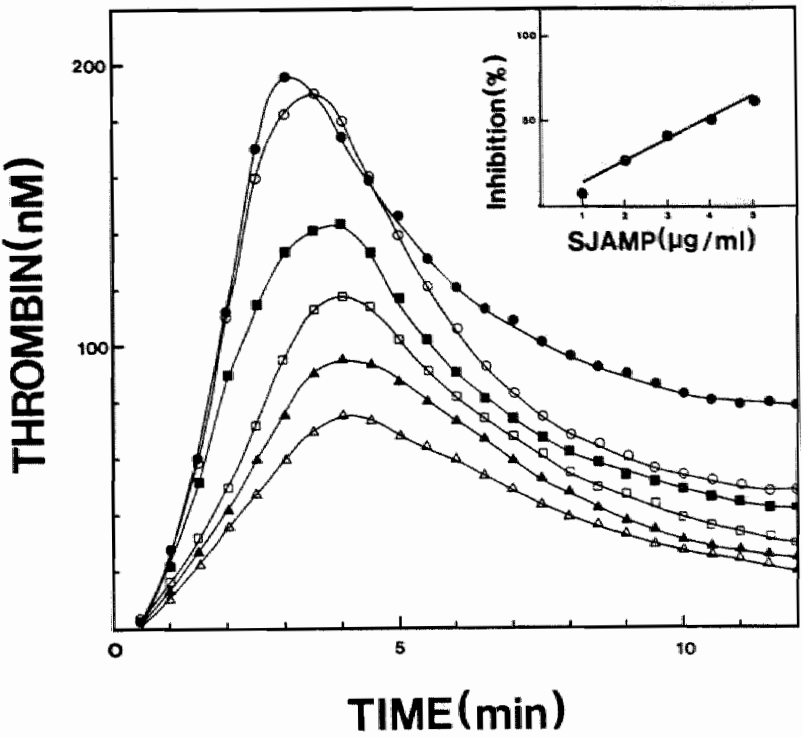

Fig. 3 The influence of SJAMP on the generation of thrombin activity via the extrinsic pathway in platelet poor plasma.
The inset represents the relationship between the maximal amount of thrombin formed and the SJAMP concentration.

●—● Buffer
○—○ SJAMP, 1 μg/ml
■—■ SJAMP, 2 μg/ml
□—□ SJAMP, 3 μg/ml
▲—▲ SJAMP, 4 μg/ml
△—△ SJAMP, 5 μg/ml

When the thrombin generation data were processed by computer to obtain the prothrombinase activity, the curves shown in figure 4 were obtained. It can be seen from figure 4 that SJAMP does not have a significant influence on prothrombinase activity in the concentration ranges tested. From this observation we conclude that SJAMP inhibits the thrombin generation via the extrinsic pathway only by its influence on thrombin activity and not by influencing prothrombinase activity.

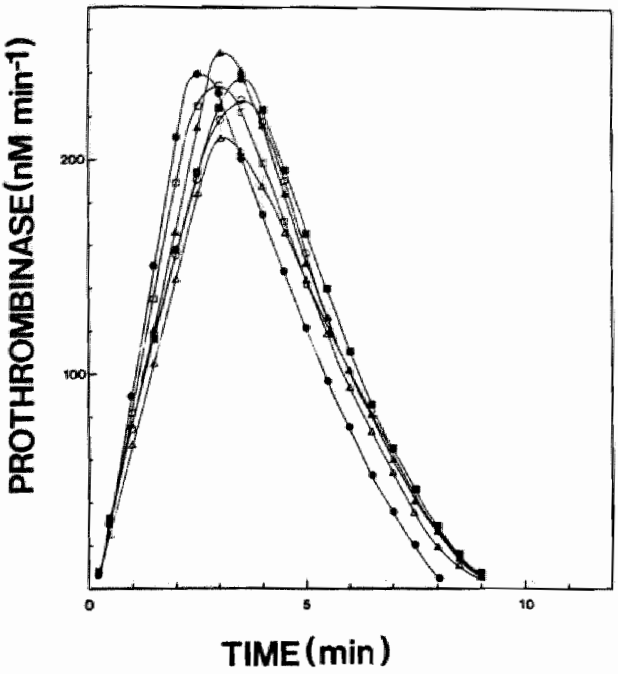

Fig. 4 The influence of SJAMP on the prothrombinase formation via the extrinsic pathway in platelet poor plasma.
The prothrombinase activity was obtained by computer processing of the thrombin generation curves shown in figure 3. For technical details see the section Materials and Methods.

●—● Buffer
○—○ SJAMP, 1 µg/ml
■—■ SJAMP, 2 µg/ml
□—□ SJAMP, 3 µg/ml
▲—▲ SJAMP, 4 µg/ml
△—△ SJAMP, 5 µg/ml

Effect of SJAMP on generation of thrombin activity and prothrombinase activity after triggering the coagulation with Cephaloplastin

In order to study the effect of SJAMP on thrombin generation and prothrombinase activty in the intrinsic coagulation pathway, Cephaloplastin was used to trigger coagulation. The generation of thrombin activity in platelet poor plasma containing various amounts of SJAMP is shown in figure 5.

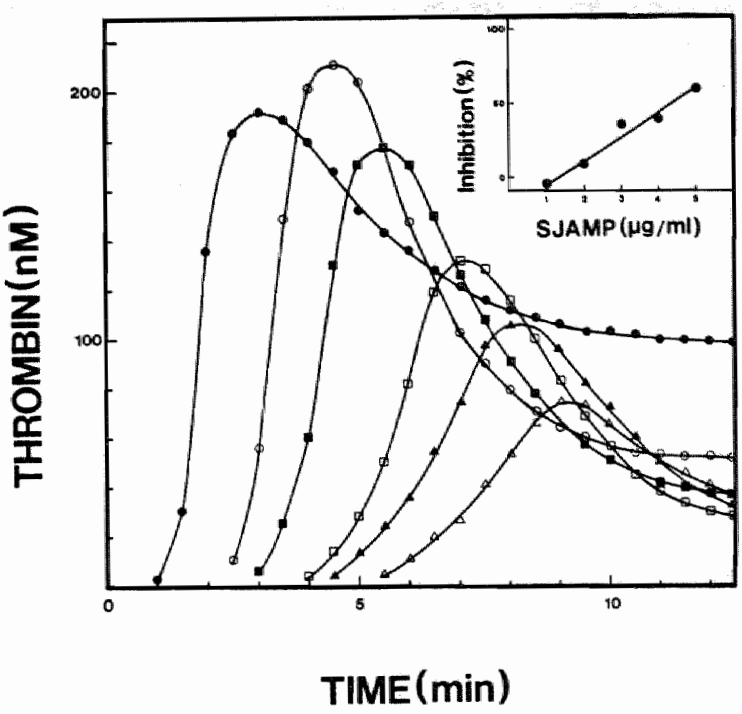

Fig. 5 The influence of SJAMP on the generation of thrombin activity via the intrinsic pathway in platelet poor plasma.
The inset represents the relationship between the maximal amount of thrombin formed and the SJAMP concentration.

●—● Buffer
○—○ SJAMP, 1 μg/ml
■—■ SJAMP, 2 μg/ml
□—□ SJAMP, 3 μg/ml
▲—▲ SJAMP, 4 μg/ml
△—△ SJAMP, 5 μg/ml

It is clear from this figure that, except at the lowest concentration tested (1 μg/ml), SJAMP caused an inhibition of thrombin generation not only reducing the maximal amount of thrombin formed, but also increasing the initial lag time in the time course of thrombin generation and that both effects were dose-dependent. A striking phenomenon was that at a low SJAMP concentration (1 μg/ml) the maximal amount of thrombin generated was slightly increased. However, after maximal thrombin formation, SJAMP accelerated the decay of thrombin activity at all concentrations tested. These results suggest that the predominant effect of SJAMP on thrombin

generation is the inhibition of thrombin activity. When, however, the thrombin generation data were processed by the computer into prothrombinase data (figure 6) it turned out that the prothrombinase activity generated via the intrinsic pathway was stimulated by SJAMP in a dose-dependent way.

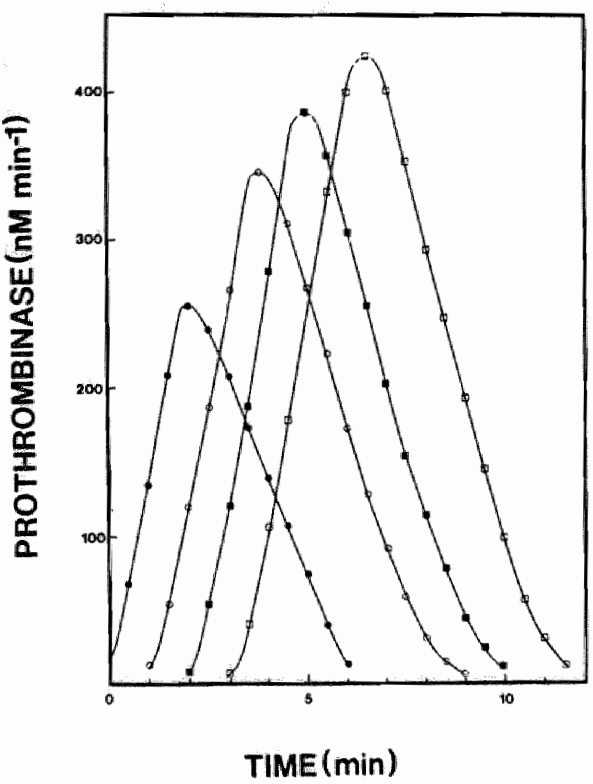

Fig. 6 The influence of SJAMP on the prothrombinase formation via the intrinsic pathway.
The prothrombinase activity was obtained by computer processing of the thrombin generation curves shown in figure 5. For technical details see the section Materials and Methods.

●——● Buffer
○——○ SJAMP, 1 µg/ml
■——■ SJAMP, 2 µg/ml
□——□ SJAMP, 3 µg/ml

Effect of SJAMP on the generation of thrombin activity induced by the combined effect of thromboplastin and thrombocytes

Low concentrations of human thromboplastin that show no effect in platelet poor plasma cause significant thrombin generation in platelet rich plasma (7). Obviously, this effect is due to the cooperative effect of thromboplastin and thrombocytes in thrombin generation. The conditions mimicked in these in vitro experiments probably have a closer resemblance to the in vivo situations than the "classical" in vitro coagulation assays.

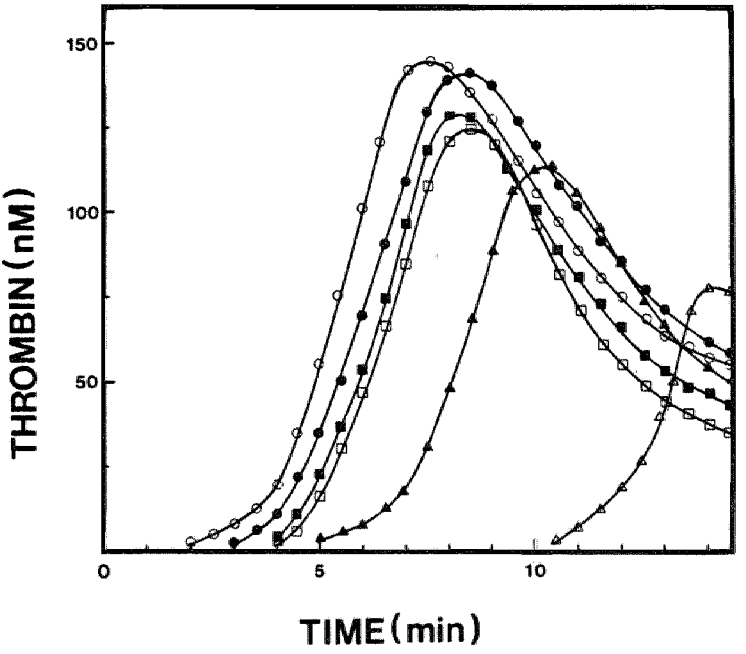

Fig. 7 The influence of SJAMP on the generation of thrombin activity in platelet rich plasma.
The coagulation was triggered with a trace amount of thromboplastin (final concentration 1 : 2400).

●——● Buffer
○——○ SJAMP, 1 µg/ml
■——■ SJAMP, 2 µg/ml
□——□ SJAMP, 3 µg/ml
▲——▲ SJAMP, 4 µg/ml
△——△ SJAMP, 5 µg/ml

In our experiments a thromboplastin solution diluted with a 0.1 M $CaCl_2$ solution (final thromboplastin dilution in the reaction mixture 1:2400) was chosen to trigger thrombin generation in platelet rich plasma (300,000 platelets/µl). The generation of thrombin activity in platelet rich plasma containing various amounts of SJAMP is shown in figure 7. At a low concentration of SJAMP (1 µg/ml) there was a slight stimulation of thrombin generation. While at a higher concentrations of SJAMP there was a clear reduction in the maximal amount of thrombin formed. Once the maximal amount of thrombin formed, however, the decay of thrombin activity was accelerated by SJAMP at all concentrations tested. Furthermore, at concentrations greater than 2 µg/ml SJAMP also increased the initial lag time in the time course of thrombin generation.

DISCUSSION

The administration of heparin for the treatment of a variety of acute thrombotic disorders has been used in clinical practice for about 50 years. The treatment is hampered however by the narrow "therapeutic window" limited by inefficiency on the one side and bleeding on the other. Moreover, some patients develop thrombopeny and thrombotic complications in association with heparin therapy. These clinical findings have greatly stimulated the interest in compounds related to heparin which are reported to possess a greater antithrombotic effectiveness while producing less hemorrhagic complications than commercial heparin and that are therefore supposed to have a wider therapeutic window. Studies on low molecular weight derivatives of commercial heparin, on heparinoids, which are naturally occurring mucopolysaccharides chemically distinct from heparin, and on a synthetic oligosaccharide sequence have received increasing attention in the literature (8-22). This encouraged us to study the effect of SJAMP on the generation of thrombin in plasma.

SJAMP accelerated the decay of endogenous thrombin in platelet poor plasma. The rate constant of thrombin decay increased linearly with the concentration of SJAMP (0-4 µg/ml) added to the plasma (fig. 2). When the effect of SJAMP on the decay of thrombin activity was compared with that of international standard heparin it was observed that, on a weight basis,

the inhibitory effect of SJAMP was about sixteen-fold weaker than that of standard heparin.

The effect of SJAMP on thrombin generation in platelet poor plasma after triggering coagulation with thromboplastin, i.e. coagulation via the extrinsic pathway, can be explained by its inhibitory effect towards thrombin (Fig. 3,4). SJAMP had no influence on the initial lag time of thrombin generation via the extrinsic pathway. We concluded that, in this experimental system, SJAMP mainly influences thrombin generation by its inhibitory effect on thrombin activity. This conclusion is supported by the results presented in figure 4 where the absence of an effect of SJAMP on prothrombinase activity in the extrinsic system is shown.

When the effect of SJAMP on thrombin generation in platelet poor plasma was studied after triggering coagulation with Cephaloplastin, i.e. coagulation via the intrinsic pathway (Fig. 5), SJAMP again accelerated the decay of thrombin activity at all concentrations tested (0-5 μg/ml) but it also caused a significant increase in the initial lag time of the thrombin generation process. At a relatively low concentration (1 μg/ml) SJAMP caused a slight increase in the maximal amount of thrombin activity formed; at higher concentrations the maximal amount of thrombin formed was decreased.

From the results described above we concluded that the influence of SJAMP on thrombin generation via the intrinsic pathway is different from its influence on thrombin generation via the extrinsic pathway. It must be kept in mind, however, that in in vivo situation the intrinsic and extrinsic pathway may be linked (see also chapter 2). As the final step in both the intrinsic and the extrinsic pathway is the activation of prothrombin by the prothrombinase complex, the difference must be caused by the influence of SJAMP on preceeding step(s) of the pathway(s). The effect of SJAMP on the initial lag time of thrombin generation via the intrinsic pathway may be explained by its indirect effect on factor VIII activation. Thrombin activates factor VIII and factor $VIII_a$ is a constituent of the factor X activating complex (tenase complex) in the intrinsic pathway. Therefore, the lag time in the thrombin generation process will depend on the velocity of factor VIII activation by thrombin. This positive feedback reaction of thrombin can be expected to be inhibited by a thrombin scavenger such as SJAMP. To our surprise, SJAMP not only increased the lag

time in the thrombin generation process after triggering coagulation with Cephaloplastin, it also stimulated the formation of prothrombinase activity via the intrinsic pathway in a dose dependent way. This conclusion can be drawn from figure 6 in which the generation of prothrombinase activity at different SJAMP concentrations is given. This stimulatory influence on the prothrombinase activity might explain the slight increase in the maximal amount of thrombin formed seen at low concentrations of SJAMP. At the moment we have no explanation for the enhancing effect of SJAMP on the formation of prothrombinase activity via the intrinsic pathway. It must be stressed, however, that this stimulation of prothrombinase formation by increasing amounts of SJAMP did not result in the formation of higher amounts of thrombin activity (fig. 5); the maxima in the thrombin generation curves decreased with increasing SJAMP concentrations. Therefore we feel that we must conclude that, also in the intrinsic pathway, SJAMP acts as an anticoagulant by inhibiting thrombin activity.

When the effect of SJAMP on thrombin generation was studied in platelet rich plasma after triggering coagulation with trace amounts of thromboplastin, results similar to those obtained in platelet poor plasma after triggering with Cephaloplastin were observed. SJAMP reduces the maximal amount of thrombin formed and increases the initial lag time at concentrations greater than 2 µg/ml. This effect of SJAMP is essentially different from that of conventional heparin. Under the same experimental conditions, heparin only increases the lag time in the process of thrombin generation but does not reduce the maximal amount of thrombin activity that is finally formed (7). This effect of heparin can be explained by its neutralization by platelet factor 4 released from the activated platelets. The thrombin inhibition by SJAMP in platelet rich plasma is not neutralized, in contrast to that of heparin. Increasing amounts of SJAMP cause an increase in the decay of endogenous thrombin activity. This suggests that SJAMP is not neutralized by platelet factor 4, which implicates that after administration in vivo SJAMP might be more efficient than standard heparin.

The generation of thrombin activity in platelet rich plasma after triggering coagulation with small amounts of thromboplastin has been explained by the cooperative effect of thromboplastin and platelets (7). Inhibition of thrombin by the specific thrombin inhibitor hirudin increases the lag time of thrombin generation and small amounts of thrombin decrease it.

Trace amounts of thrombin are supposed to stimulate the platelet "flip-flop", and/or the shedding of phospholipid protein vesicles resulting in the exposure of procoagulant phospholipids which would in turn stimulate thrombin formation (23).

Therefore, we think that the effect of SJAMP on thrombin generation in platelet rich plasma can largely be explained by its inhibitory action towards thrombin, resulting in an inhibition of two positive feedback reactions: factor VIII activation and induction of a procoagulant surface. As can be seen in figure 7 in platelet rich plasma a low concentration of SJAMP (1 µg/ml) not only causes a slight increase in the maximal amount of thrombin formed, as it did in platelet poor plasma after triggering with Cephaloplastin, but it also shortens the initial lag time somewhat. This observation might possibly be explained by a direct interaction of SJAMP with the platelet surface. SJAMP has been reported to induce the aggregation of human and rabbit platelets (2) which might result in the generation of a procoagulant surface. Again, as was described for the effect of SJAMP on the intrinsic pathway of coagulation in platelet poor plasma, at higher concentrations of SJAMP the inhibitory effect of SJAMP towards thrombin becomes more important than its stimulatory effect on thrombin formation and this results in a decrease in the maximal amount of thrombin formed.

In conclusion, SJAMP has shown to accelerate the decay of endogenous thrombin activity and to stimulate the generation of prothrombinase activity via the intrinsic pathway. However, the inhibitory effect towards thrombin activity turns out to be more important than the stimulating effect and this results in a net decrease in the amount of thrombin formed with increasing SJAMP concentrations (0 - 5 µg/ml).

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CHAPTER V

SJAMP, A HEPARIN-LIKE MATERIAL FROM STICHOPUS JAPONICUS SELENKA: COFACTOR DEPENDENCY OF ITS INHIBITORY EFFECT ON THROMBIN ACTIVITY IN PLASMA

SUMMARY

In this chapter the cofactor dependency of thrombin inhibition by SJAMP, a heparin-like acid mucopolysaccharide isolated from stichopus japonicus selenka (a sea cucumber), is described.

In a purified system, as well as in an euglobulin fraction of normal plasma, thrombin inhibition by SJAMP was dependent upon the presence of antithrombin III. In the euglobulin fraction it was observed that, after triggering the coagulation with thromboplastin, the presence of SJAMP causes a shortening of the lag time of the thrombin generation process, both in the absence and in the presence of antithrombin III.

When the effect of SJAMP on thrombin inhibition was studied in an antithrombin III-depleted plasma it was found that in the absence of antithrombin III the inhibition was 20% of that found after reconstitution of the antithrombin III-depleted plasma with antithrombin III. This points to the involvement of other plasma cofactors in the SJAMP-mediated inhibition.

Direct binding studies based on the change in intrinsic fluorescence of antithrombin III upon interaction with SJAMP indicated that SJAMP has a relatively low affinity for antithrombin III; this low affinity is not reflected in its anticoagulant effect. The discrepancy between the SJAMP-antithrombin III binding and the anticoagulant activity of SJAMP may be caused by the interaction of SJAMP with other protein cofactors present in plasma. It is concluded that SJAMP exerts its inhibitory activity not only by antithrombin III, but also by other plasma protein such as heparin cofactor II.

INTRODUCTION

The stichopus japonicus acid mucopolysaccharide (SJAMP) is a heparin-like material isolated from stichopus japonicus selenka (a sea cucumber) (1). The inhibitory action of SJAMP on blood coagulation was described to be due to its action on thrombin and it was supposed that its mode of action was different from that of heparin (2,3). This hypothesis was based on the observation that the presence of SJAMP caused a prolongation of the thrombin time in both normal and antithrombin III deficient plasma. This could not be confirmed by the experiments carried out by our group and described in the previous chapter. We found that in both platelet poor and platelet rich plasma, SJAMP just like heparin, inhibits thrombin activity in an antithrombin III dependent reaction, but it cannot be excluded that SJAMP also can make use of other protein cofactors (e.g. heparin cofactor II). The main difference between SJAMP and heparin was that, on a weight basis, a sixteen-fold higher SJAMP concentration was needed to obtain the same effect.

In this chapter we describe our studies on the cofactor dependency of the inhibition of thrombin by SJAMP. The mode of action was studied in a purified system, in a plasma preparation devoid of all inhibitors (the euglobulin fraction) and in an antithrombin III depleted plasma.

MATERIALS AND METHODS

Normal plasma

Blood from healthy donors was collected in 0.13 M trisodium citrate (9 volumes of blood to 1 volume of anticoagulant) and centrifugated for 15 minutes at 3000 x g at 4 ^{o}C. Subsequently a second centrifugation was performed for 1 hour at 23000 x g at 4 ^{o}C. The platelet free plasma thus obtained was used for the preparation of a euglobulin fraction and an antithrombin III-depleted plasma. The platelet free plasma was stored at -80 ^{o}C until use.

Euglobulin fraction

Defibrinated plasma was used as a starting material for the preparation of the euglobulin fraction. The defibrinated plasma was prepared by mixing 50 volumes of plasma with 1 volume reptilase solution. After clotting for 10 minutes at 37 °C the clotted plasma was kept at 0 °C for another 10 minutes. The fibrin formed was removed by winding it on a small plastic spatula. The euglobulin fraction was prepared from defibrinated plasma by acid precipitation at low ionic strength as described by Josso et al (4). The precipitate was dissolved in Michaelis buffer (pH 7.35) containing 0.02 M trisodium citrate. All preparation steps were carried out in plastic materials.

Antithrombin III depleted plasma

Antithrombin III depleted plasma was prepared by gently stirring 2 ml of human plasma with 0.8 ml of a suspension of anti-antithrombin III-sepharose (gift from Dr. Tran Huu Tri, Basel) for 18 hours at 4 °C. After adsorption the antithrombin activity in the plasma was not influenced by the addition of 1U/ml of heparin.

Purified thrombin

Human thrombin was prepared as previously described by Pletcher and Nelsestuen (5).

Purified antithrombin III

Human antithrombin III was prepared by polyethyleneglycol precipitation of barium citrate-adsorbed plasma, followed by affinity chromatography on heparin-sepharose (6). Finally heparin was removed by ion-exchange chromatography (7). Bovine antithrombin III was prepared as previously described (6).

Thromboplastin

Human brain thromboplastin was prepared by a modification of the method of Owen et al (8). The preparation obtained was homogenized in a Potter Elvehjem homogeniser for 3 minutes, centrifugated at 2000 x g for 5 minutes and stored at -20 °C. Prior to use the preparation was thawed, diluted 1:40 with 0.05 M Tris-HCl (pH 7.35) containing 0.1 M $CaCl_2$ and prewarmed at 37 °C for 1 hour. When normal plasma was clotted with this thromboplastin dilution under the experimental conditions used in this study the clotting time was 80 seconds.

Stichopus japonicus acidic mucopolysaccharide (SJAMP)

SJAMP was kindly supplied by Dr. Jia-Zeng Li, Institute of Hematology, Tianjin, China. The compound was isolated and purified by the Biochemical Laboratory of Tianjin Medical and Pharmacological Institute, China.

Buffers

Buffer A: 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.35
Buffer B: 0.05 M Tris-HCl, 0.1 M NaCl, 0.5% albumin, 0.02 M EDTA (pH 7.9)

Commercial reagents

Chromogenic substrate S2238 for thrombin was obtained from Kabi Vitrum (Stockholm, Sweden). Reptilase was obtained from Laboratories Stago (Asnières, France). 4th International Standard Heparin was obtained from the National Institute for Biological Standards and Control (NIBSC). All other chemicals used were of the highest grade commercially available.

Measurement of thrombin inhibition in a purified system

The influence of SJAMP on the inhibition of thrombin by antithrombin III was studied in a purified system. The initial rate measurements were carried out under pseudo first order conditions. An incubation mixture consisting of antithrombin III (400 nM), SJAMP (range 0.5 - 3 µg/ml) and

buffer A was incubated for 4 minutes at 37 °C. After this incubation purified thrombin (10 nM) was added to the incubation mixture and at fixed time intervals after the addition of thrombin, 15 µl aliquots were subsampled into tubes containing 445 µl of buffer B and 50 ul of S2238 (4 mM). Amidolytic activity was read immediately at 405 nm in a spectrophotometer (LKB Ultrospec II). The thrombin activity was calculated from a reference curve constructed with a series of dilutions of a standard thrombin preparation.

Measurement of thrombin generation in an euglobulin fraction and an antithrombin III depleted plasma

For the measurement of thrombin generation the procedure described by Hemker et al (9) was used. To 240 µl of sample (euglobulin fraction or antithrombin III depleted plasma), 60 µl buffer A containing SJAMP at the concentration to be tested was added and the mixture was incubated for 5 minutes at 37 °C. The generation of thrombin activity was triggered by the addition of 60 µl of the thromboplastin dilution containing 0.1 M $CaCl_2$. At fixed time intervals, 10 µl aliquots of incubation mixture were subsampled into tubes containing 465 µl of buffer B and 25 µl of S2238 (4 mM). The subsampling tubes were incubated for 2 minutes at 37 °C, and then the reaction was stopped by adding 300 µl of concentrated acetic acid. The pipettes for sampling and for stopping the reaction were connected to an Apple IIe computer. A program was developed that allowed automatic registration of the moments of sampling and stopping. The optical density was read at 405 nm in the spectrophotometer. From the change in optical density and the time interval between sampling and stopping the Δ O.D./min was calculated by the computer (10).

Measurement of the binding of SJAMP to antithrombin III

The interaction of SJAMP with antithrombin III was determined by measuring changes in the intrinsic fluorescence of antithrombin III (11). Small aliquots of SJAMP (10 µg/ml) were added in succession to a solution of bovine antithrombin III (2 µM in 0.05 M Tris, 0.12 M NaCl and pH 7.9 room temperature). After each addition the intrinsic fluorescence intensity

of the antithrombin III was determined using an excitation wavelength of 280 nm with a 2 nm slitwidth and an emission wavelength of 340 nm with a 5 nm slitwidth. The increase in fluorescence intensity was plotted against the amount of SJAMP added. The binding constant was estimated from the SJAMP titration data using a non-linear least squares computer fit to the equation:

$$\Delta F = \frac{\Delta F_{max}}{2R}(R + T + K - \sqrt{(R + T + K)^2 - 4\,TR})^{\frac{1}{2}}$$

where, ΔF is the change in fluorescence, R is the total concentration of antithrombim III, T is the total concentration of SJAMP and K is the dissociation constant of the SJAMP-antithrombin III complex.

RESULTS

The inhibitory effect of SJAMP on thrombin activity in a purified system

The inhibition of purified thrombin by antithrombin III in the presence of various amounts of SJAMP was measured under pseudo first order conditions. In figure 1 the rate constant (k) of inhibition of thrombin is plotted against the SJAMP concentration. In the absence of SJAMP the k of thrombin inhibition by purified human antithrombin III was shown to be 0.35 min^{-1}. The rate constant of thrombin inhibition increased linearity with the SJAMP-concentration in the range tested (0.5 µg/ml-3 µg/ml). The inhibition of thrombin activity by SJAMP was completely dependent upon the presence of antithrombin III. No inhibition of thrombin activity was found in the absence of antithrombin III. From these results we conclude that, in the purified system used, the inhibitory action of SJAMP towards thrombin is mediated by antithrombin III. From the straight line of fig. 1 we calculate a second order rate constant of k = 1.4 (min^{-1}/µg/ml).

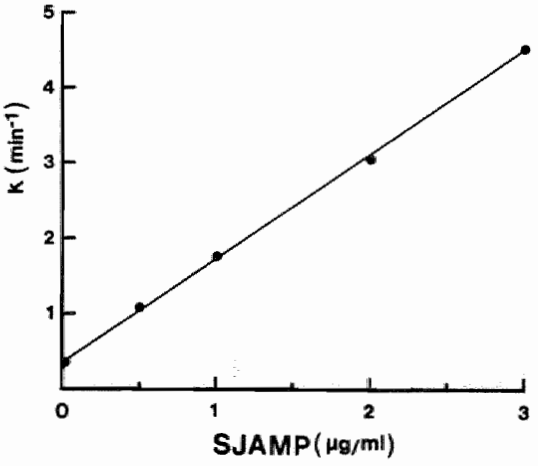

Fig. 1. The rate constant of thrombin inhibition in a purified system as a function of the SJAMP concentration.

The inhibitory effect of SJAMP on thrombin generation in the euglobulin fraction

Following the study of the inhibitory effect of SJAMP towards thrombin in a purified system, the inhibitory effect of SJAMP on thrombin generation in an euglobulin fraction was investigated. To ensure that there was no residual antithrombin activity in the euglobulin fraction, we first determined the effect of standard heparin on the generation of thrombin activity in this experimental system. As shown in Fig. 2, thrombin generation in the euglobulin preparation, as determined by the measurement of amidolytic activity, was not influenced by the presence of heparin. No thrombin inhibition was observed in the euglobulin fraction, indicating that no residual antithrombin III activity was present in this fraction. When the

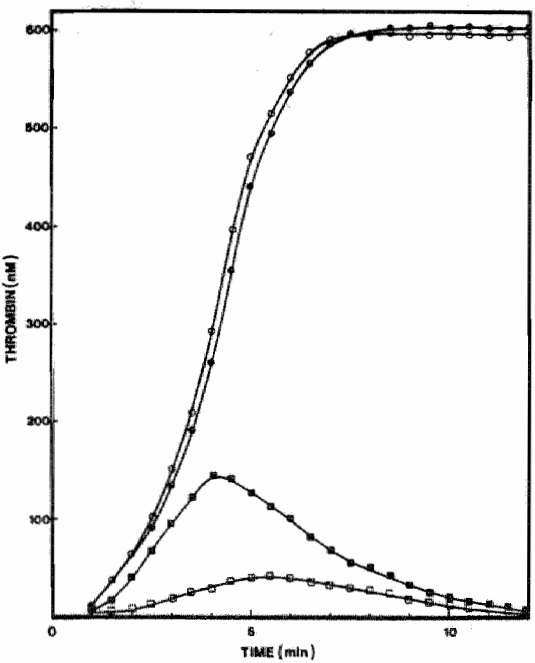

Fig. 2. Effect of heparin on the generation of thrombin activity in an euglobulin preparation before and after reconstitution with antithrombin III.

● buffer ■ AT III (2000 nM)
○ heparin (0.03 U) □ AT III (2000 nM) and heparin (0.03 U)

euglobulin fraction was reconstituted with human antithrombin III, inhibition of thrombin occured both in the presence and in the absence of heparin. As could be expected, the inhibitory action was more pronounced when heparin was present. The amidolytic activity in the reconstituted euglobulin fraction eventually disappeared completely, which means that the euglobulin fraction was also free from α_2-macroglobulin because the α_2-macroglobulin-thrombin complex still displays activity towards chromogenic substrates.

The effect of SJAMP on the generation of thrombin activity in the euglobulin fraction was determined under the same experimental conditions as used for the determination of the heparin effect. The results of these experiments are shown in figure 3. It can be concluded from these figures that the effect of SJAMP has much similarity to the effect of heparin.

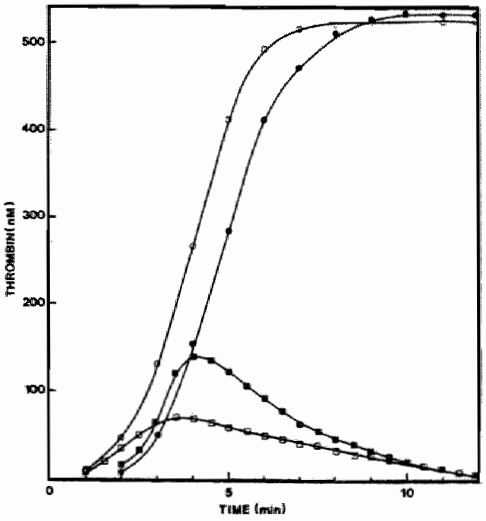

Fig. 3. Effect of SJAMP on the generation of thrombin activity in a euglobulin preparation before and after reconstitution with antithrombin III

● buffer ■ AT III (2000 nM)
○ SJAMP (3µg/ml) □ SJAMP (3µg/ml) and AT III (2000 nM)

However, a difference between SJAMP and heparin can be noted. Whereas heparin has no influence on the lag time of thrombin generation in the euglobulin fraction, SJAMP shortens the lag time of the thrombin burst.

The inhibitory effect of SJAMP on thrombin generation in antithrombin III depleted plasma

The effect of SJAMP on the generation of thrombin activity in antithrombin III-depleted plasma is shown in Fig. 4. As could be expected, the presence of SJAMP caused a strong inhibition of thrombin activity when the antithrombin III depleted plasma was reconstituted with antithrombin III However, the inhibition of thrombin activity was not only seen in the reconstituted plasma, SJAMP also excerted some inhibitory activity in antithrombin III depleted plasma. The data shown in figure 4 indicate that in the plasma reconstituted with antithrombin III also other plasma proteins are involved in thrombin inhibition. About 20% of the inhibitory

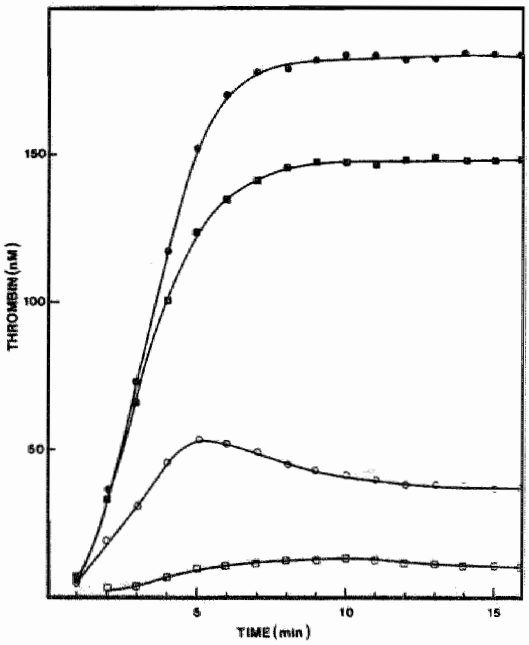

Fig. 4. Effect of SJAMP on thrombin activity in AT III depleted plasma before and after reconstitution with antithrombin III

● Buffer ○ AT III (2000 nM)
■ SJAMP (3 µg/ml) □ SJAMP (3 µg/ml) and AT III (2000 nM)

effect is mediated by these inhibitors. The observation that the amidolytic activity in the antithrombin III-depleted plasma with reconstituted antithrombin III does not go back to the zero level as it did in the reconstituted euglobulin fraction can be explained by the presence of an α_2-macroglobulin-thrombin complex in the former mixture while it is absent from the euglobulin fraction (9).

Binding of SJAMP to antithrombin III

The SJAMP-antithrombin III binding was studied by measuring the changes in the intrinsic fluorescence of antithrombin III upon successive additions of SJAMP. In figure 5 the increase in fluorescence ($F-F_o$) is plotted against the amount of SJAMP added. The dissociation constant (K_d) and the ΔF_{max} of SJAMP were obtained from the data by computer fit procedure as described in the section Materials and Methods. The K_d value was 0.14 mg/ml, which, based on a molecular weight of 30,000 for SJAMP, was 4.6 µM. The ΔF_{max} was only 15 % of that found for the antithrombin III heparin interaction (12).

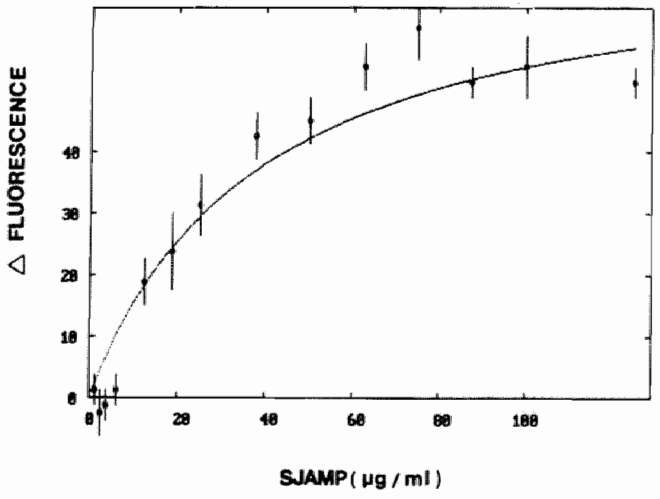

Fig. 5. Measurement of the binding of SJAMP to antithrombin III. See text for further details

DISCUSSION

The enhancing effect of heparin and its analogues on the inhibition of the coagulation serine proteases by plasma inhibitors has been shown to depend largely on the ability of these compounds to bind to the heparin cofactors i.e. antithrombin III and heparin cofactor II (13-18). The inhibition by antithrombin III is considered to be the predominant mechanism for anticoagulation in the case of the heparins themselves. The possibility that part of the inhibitory effect of heparin congeners on coagulation is not mediated by antithrombin III was reported in the literature (19,20).

Previous studies suggested that SJAMP acts as an anticoagulant by inactivation of thrombin but that this inactivation is not dependent on antithrombin III (2,3). Our observations of the effect of SJAMP on thrombin generation in plasma described in the previous chapter are not in agreement with these literature findings, however. We observed that SJAMP accelerates the decay of thrombin activity in plasma in an antithrombin III dependent manner comparable with heparin (see chapter 4). The investigations presented in this chapter also demonstrate that the inhibitory action of SJAMP towards thrombin depends on antithrombin III. This conclusion is based on our observations in a purified system, in an euglobulin fraction which is a plasma fraction devoid of all inhibitors and in an antithrombin III depleted plasma. In the purified system the inhibitory effect of SJAMP was completely antithrombin III dependent (Fig. 1). In the euglobulin fraction, SJAMP will not inhibit thrombin activity when antithrombin III is absent (Fig. 3). From these experiments a direct action of SJAMP on thrombin is excluded. The antithrombin III dependency of the thrombin inhibition by SJAMP is also confirmed by the experiments in antithrombin III depleted plasma (Fig. 4). However, in this system about 20% of the thrombin inhibition by SJAMP must be explained by an antithrombin III independent process. Preliminary experiments (not represented) indicate that this antithrombin III-independent inhibition may be mediated by heparin cofactor II.

It can be noticed that in the euglobulin fraction SJAMP causes a slight shortening of the lag phase in the process of thrombin generation, even in the presence of antithrombin III. A similar observation has been described

in the preceeding chapter, but at the moment we have no explanation for this phenomenon.

Binding of heparin to antithrombin III or heparin cofactor II has been described to be essential for its anticoagulant activity (21-23). It has been postulated that a heparin-induced conformational change in the inhibitor structure accelerates the rate of formation of an inactive complex between antithrombin III and thrombin (24,25). Heparin with low affinity for antithrombin III shows a relatively low efficiency of thrombin inhibition, while heparins with high affinity for antithrombin III are efficient stimulators of this inhibition (26).

To further characterize the interaction between SJAMP and antithrombin III, we determined the binding of SJAMP to antithrombin III by a fluorometric binding assay (Fig. 5). The binding constant (K_D) of SJAMP to antithrombin III was 4.6 μM. In comparison to standard heparin (K_D=0.05 μM) SJAMP has a rather low affinity for antithrombin III (12). However, the second order rate constants for the inhibition of thrombin by SJAMP and heparin are respectively 1.4 $min^{-1}/\mu g/ml$ and 38.7 $min^{-1}/\mu g/ml$ (12). Therefore, the anticoagulant effect of SJAMP is stronger than would be expected from its affinity for antithrombin III. One explanation for this observation could be that the conformational change in antithrombin III induced by SJAMP would produce a more effective antithrombin activity than the conformational change induced by heparin. An alternative, and perhaps more likely, explanation could be the interaction of SJAMP with other plasma inhibitors. This point remains to be investigated.

In conclusion, SJAMP acts as an anticoagulant not only by stimulating the inhibition of thrombin by antithrombin III but it also acts by an effect of other plasma inhibitors such as heparin cofactor II.

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CHAPTER VI

GENERAL DISCUSSION

The aim of the work presented in this thesis was to do biochemical research in the field of blood coagulation that has a direct relation to medical questions.

The studies have thrombin activity in plasma as a central theme. In the studies described in the first two chapters the thrombin generation was studied in plasma systems that were not normal from a physiological point of view. The abnormality was either a congenital deficiency of a single coagulation protein (haemophilia A or B) or a reduction in the effective level of the vitamin K-dependent factors brought about by oral anticoagulation. In this aspect these studies were related to earlier work from clinical laboratories because clinical discovery of a coagulation abnormality very often precedes the discovery of the related clotting protein. Indeed, a large amount of the present knowledge about physiological blood coagulation has been accumulated by the study of patients who lacked a specific clotting protein. The difference with the older studies resides in the fact that, because of the availability of modern measuring techniques and of purified clotting factors, we can now answer questions that were hardly accessible to the clinical investigator of earlier days.

In the second part of the thesis studies on the anticoagulant effect of SJAMP, a heparin-like compound isolated from the stichopus japonicus selenka, (a species of sea cucumber), were described. The aim of these studies was to better understand the inhibition of thrombin activity in plasma by this substance.

Since Biggs and Nossel discovered that haemophiliac plasma do not generate a normal amount of thrombin activity by the thromboplastin-dependent pathway when a dilute thromboplastin is used to trigger coagulation (1) and Josso and Prou-Wartelle found that factor VII is essential for the procoagulant activity of a haemophiliac plasma using a dilute thrombo-

plastin (2), the relationship between the classical intrinsic and extrinsic pathway had been demonstrated but still remained somewhat indistinct for several years. In the past ten years, great biochemical advances have been made in this field. We can now isolate all known coagulation proteins and reconstruct parts of the plasmatic coagulation system with these purified compounds. Based on such a procedure, Østerud and Rapapport directly confirmed that a reaction product of factor VII/thromboplastin could activate the factor IX (3). The physiological importance of this pathway remained to be observed. In vitro evidence accumulated that the rate of factor X activation via a factor VII_a/thromboplastin dependent activation of factor IX is much smaller than that of the direct activation of factor X via a factor VII_a/thromboplastin complex (4-6). This makes it difficult to accept that the thromboplastin-dependent activation of factor IX is of primary importance in vivo.

In chapter II of this thesis, a quantitative study of the contribution of the factors IX and VIII to the process of thrombin generation after triggering coagulation with thromboplastin was presented. We found that, when coagulation is triggered with a concentration of thromboplastin normally used in in vitro tests, thrombin generation is the same in congenital factor IX deficient plasma and in congenital factor IX deficient plasma reconstituted with purified factor IX. Also with factor VIII deficient plasma, thrombin generation in factor VIII deficient plasma is the same as in factor VIII deficient plasma reconstituted with purified factor VIII when coagulation is triggered with a thromboplastin concentration normally used in in vitro tests. When, however, coagulation is triggered with low concentration of thromboplastin, a clear dependency of thrombin generation on the amount of factor IX or factor VIII present becomes evident at a factor IX concentration lower than 30 nM (about 40% clotting activity) and a factor VIII concentration lower than 0.3 U/ml (about 30 % clotting activity), respectively.

These results clearly show that, in situations where only small amounts of thromboplastin are available, the factor IX and VIII play an increasingly important role in plasma coagulation. This interrelation between the two classical pathways could give a proper explanation for the fact that in haemophiliac patients the deficiencies in the intrinsic pathway are not compensated by the intact extrinsic pathway. It is interesting to note in

this respect that haemophiliac patients tend to bleed in thromboplastin poor organs (joints, muscles).

The family of the vitamin K-dependent clotting factor includes prothrombin and the factors VII, IX and X. The role of vitamin K is to promote the carboxylation of a distinct series of glutamic acid residues of the N-terminal end of the polypeptide chain into γ-carboxylated glutamic acid residues. These residues are required for the binding of the clotting factors to negatively charged phospholipid surfaces via Ca^{2+}-bridges. This interaction with phospholipid surfaces is necessary for their participation in the coagulation process (7). Vitamin K antagonists, such as dicoumarol derivatives, interfere with the carboxylation of the vitamin K-dependent clotting factors. Treatment with vitamin K antagonist therefore results in the synthesis of biologically inactive but immunologically detectable forms of the vitamin K-dependent proteins. These inactive forms are called decarboxy factors or PIVKAs (8-12).

Due to the different half-life times of vitamin K-dependent clotting factors, oral anticoagulation starts with the reduction of factor VII activity, later the reduction of the factors IX, X and prothrombin follows (12). Therefore, if an oral anticoagulant is administered, the concentrations of the factor VII, X and prothrombin that together determine the prothrombin time may occur in a large variety of combinations. Any experimentally found prothrombin time may in fact be due to an infinity of different sets of concentrations of the prothrombin and the factors VII and X. From clinical practice it is known that a given value of prothrombin time indicates adequate anticoagulation, we still do not know to what level of which clotting factor is necessary for the effective anticoagulant response. For instance, it is not known whether a reduction of the factor VII activity, and thus a prolonged prothrombin time during the initial period of treatment means an effective antithrombotic response in vivo. It is also possible that an effective in vivo antithrombotic response is only reached after a sufficient reduction of the factor X or the prothrombin activity.

In chapter III of this thesis, a study of the relative importance of vitamin K-dependent clotting factors on prothrombinase activity in dicoumarol plasma was described. We found that the addition of purified factors VII, IX or X to plasma from deeply anticoagulated patients

(thrombotest values in the range 10-15 %) did not influence the rate of prothrombinase formation and the amount of prothrombinase formed. Only the amount of prothrombin originally present in the dicoumarol plasma determined the course of thrombin generation.

To further investigate the level below which the vitamin K-dependent clotting factors start to have an effect on the prothrombinase activity, purified factors VII, IX and X were added in increasing amounts to plasmas deficient in prothrombin and factors VII, IX and X, respectively. It was shown that the concentrations necessary to obtain half maximal prothrombinase formation were < 1% for factor VII, 5% for factor IX and 10% for factor X. The prothrombinase activity showed to increase linearily with increasing amounts of prothrombin added. From this study we conclude that only the changes in the prothrombin level must be held responsible for antithrombotic effect of oral anticoagulant therapy. This also suggests that the estimation of prothrombin activity should be suitable for monitoring oral anticoagulant therapy.

Conventional heparin has been used in clinical practice about 40 years. The treament is not always efficient, however, and the dose of heparin administered is limited by the bleeding risk. Moreover, some patients develop thrombopenia and thrombotic complications in associations with heparin therapy. These clinical findings have greatly stimulated the interest in compunds related to heparin which are reported to posses greater antithrombotic effectiveness while producing less bleeding than conventional heparin and that are therefore supposed to have an increased "therapeutic window" (13-15). SJAMP, a heparin like acidic mucopolysaccaharide isolated from the sea cucumber (stichopus japonicus selenka), has recently been studied as a potential new anticoagulant drug (16). Its action on blood coagulation has been reported to be due to inhibition of thrombin activity, but the precise mechanism of its intervention in coagulation and the cofactor dependency of the inhibitory effect are not yet clear.

In chapter IV of this thesis, a study of the effect of SJAMP on thrombin generation in plasma was described. We found that in platelet poor plasma the main inhibitory effect of SJAMP on thrombin generation after triggering the coagulation via the extrinsic pathway is a reduction of the maximal amount of thrombin formed and an acceleration of thrombin decay.

Both phenomena can be attributed to a SJAMP dependent increase of antithrombin activity. The apparent second order rate constant of SJAMP dependent thrombin decay in plsma is $1.5/min^{-1}/\mu g/ml$. This means that, calculated on a weight basis, the inhibitory effect of SJAMP towards the thrombin activity is about sixteen-fold weaker than that of international standard heparin. Like heparin, SJAMP does not inhibit the prothrombinase activity in the extrinsic pathway in plasma.

When the effect of SJAMP was studied in platelet poor plasma after triggering the coagulation via the intrinsic pathway, an additional effect of SJAMP was observed; SJAMP increased the lag time in the thrombin generation process. This can probably be explained by an inhibition of the feedback activation of factor VIII by thrombin. Previous studies suggested that the lag time in the thrombin generation process reflects the time needed for the activation of factor VIII by (low amounts of) thrombin (17). Yet, SJAMP showed to stimulate the prothrombinase activity in the intrinsic pathway in a dose dependent way. For the moment we have no explanation for this stimulatory effect of SJAMP that anyhow in practice is entirely observed by the antithrombin effect.

The thrombin generation in platelet rich plasma after triggering the coagulation with a trace amount of thromboplastin has been considered to be due to the cooperative effect of thromboplastin and platelets. Obviously, this experimental system has a closer resemblance to in vivo situations than the "classical" in vitro coagulation systems. At sufficiently high concentrations of SJAMP both a reduction of the maximal amount of thrombin formed and an increase in the lag time of the thrombin generation process were observed. From these results we concluded that, in contrast to the inhibitory effect of heparin, the inhibitory effect of SJAMP on thrombin activity is not adequately neutralized by platelet factor 4 released from activated platelets during the hemostatic process (18). Previous studies showed that SJAMP has the ability to induce platelet aggregation (16). A small stimulatory effect of thrombin generation seen low concentrations of SJAMP may be connected with this effect of SJAMP on platelet aggregation. We conclude that the inhibition of thrombin activity by SJAMP is the predominant phenomenon in platelet rich plasma also.

The enhancing effect of heparin and its analogues on the inhibition of coagulation serine proteases by plasma inhibitors has been shown to depend

largely on the ability of these compounds to bind to the heparin cofactors, antithrombin III and heparin cofactor II (19-22). The heparin dependent inhibition of thrombin by antithrombin III is presumably by far the most important mechanism of its anticoagulant effect (19-24). The possibility that part of the inhibitory effect of heparin on coagulation is not mediated by antithrombin III was reported in the literatures (25,26). Previous studies suggested that SJAMP acts as an anticoagulant by inactivation of thrombin but that this inactivation is not dependent on the antithrombin III (16). Our studies presented in chapter IV are not in agreement with these literature findings, however.

In chapter V of this thesis, a study of the antithrombin III dependency of thrombin inhibition by SJAMP was described. We found that, in a purified system and in an inhibitor-depleted plasma system (the euglobulin fraction), inhibition of thrombin activity by SJAMP could only be observed after reconstitution of these systems with purified human antithrombin III. A subsequent study carried out in antithrombin III-depleted plasma indicate that about 20 % of the SJAMP-mediated thrombin inhibition was independent of antithrombin III. This points to the involvement of other plasma inhibitors in the SJAMP-mediated inhibition, probably heparin cofactor II.

Direct binding studies based on the change in intrinsic fluorescence of antithrombin III upon interaction with SJAMP indicated that SJAMP has only a low affinity for antithrombin III; this accounts partly for its relative ineffectiveness as compared to standard heparin on a weight basis. The discrepancy between the SJAMP/antithrombin III binding strength and the anticoagulant activity of SJAMP may also be caused by an interaction of SJAMP with other inhibitors present in plasma. We conclude that SJAMP exerts its inhibitory effect mainly in the antithrombin III-mediated pathway. However, the contribution of other plasma inhibitors such as heparin co-factor II to the thrombin inhibition by SJAMP can not be neglected.

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一般性讨论

本文的目的是从事血液凝固领域中的生物化学研究，所涉及的题目直接关系到临床医学问题，贯穿整个研究的主题是血浆凝血酶活性。在论文的前两章里，作者介绍了先天性单个凝血因子缺陷（A型和B型血友病）和口服抗凝剂引起的维生素K依赖凝血因子有效水平减低时血浆血酶生成的研究，从生理角度看，这些血浆凝固系统都是异常的。在这方面，这些研究与早期临床实验研究有关，因为临床凝血异常的发现经常先于相关凝血蛋白的发现。的确，当今大量有关血液凝血生理学知识大都是通过研究缺乏某种特别凝血蛋白的病人而集累的。现代与早期研究工作区别在于，由于测量和纯化凝血蛋白技术的进步，利用这些手段，现在可以探索那些早期研究者无法涉及的问题。在论文的后两章里，作者介绍了SJAMP（一种从海生黄瓜中提取的肝素样成份）的抗凝作用，目的是更深入的了解这种成份对血浆凝血酶活性的抑制。

自从Biggs和Nossel发现，当用稀释的凝血活酶触发血浆凝固时，血友病血浆不能生成正常量凝血酶（1），而后，Josso和Prou-Wartelle发现，因子Ⅶ是稀释的凝血活酶触发血浆凝固时血友病血浆促凝活性的基础（2），从而提示了经典的内源和外源凝血途径的内在联系，但是这种联系仍不能认识。在过去十年里，由于血液凝固生物化学的巨大发展，现在可以从血浆中分离和提纯所有已知的凝血蛋白并且可以把它们部分重组成血浆凝固系统。基于这种方法，Østerud和Rappaport在部分纯化的实验体系中证实，因子Ⅶ和凝血活酶的反应产物可以激活因子Ⅸ（3），但这条凝血途径的生理意义仍处于探讨阶段。近年来的体外研究显示，经因子Ⅶ——凝血活酶复合物激活因子Ⅸ进而因子Ⅸ激活因子Ⅹ的活化率大大低于因子Ⅶ——凝血活酶复合物直接激活因子Ⅹ（4-6）。因此，因子Ⅸ的凝血活酶依赖活化途径在体内凝血的重要性难以被接受。

在论文的第二章里，作者介绍了因子Ⅸ和因子Ⅷ对外源凝血过程中凝血酶生成的贡献。结果显示，当用于正常体外实验的凝血活酶触发血浆凝固时，因子Ⅸ缺陷血浆和用纯化因子Ⅸ重组的因子Ⅸ缺陷血浆的凝血酶生成率和生成量相同。在同样实验条件下，类似结果也见于纯化因子重组后的因子Ⅷ缺陷血浆。当用低浓度凝血活酶触发血浆凝固时，血浆凝血酶生成呈现出对因子Ⅸ和因子Ⅷ明显的依赖，维持正常凝血酶生成的因子Ⅸ和因子Ⅷ含量分别为30nM（约40%凝固活性）和0.3u/ml（约30%凝固活性）。这些结果清楚地展示，当小量凝血活酶存在时，因子Ⅸ和因子Ⅷ在血浆凝固中起着重要作用，两条经典凝血途径间关系的建立，可以适当地解释，为什么血友病内源凝血途径方面的异常不能够被其完整的外源凝血途径补偿。

维生素K依赖凝血蛋白的家族包括因子Ⅱ、Ⅶ、Ⅸ和Ⅹ。维生素K的作用是促进这些蛋白太链氨基末端上的谷氨酸残基的羧化反应，即使其转变成γ—羧基谷氨酸残基。这些残基对此类凝血蛋白通过钙离子桥结合带阴性电荷的磷脂表面是必需的。换言之，此类凝血因子参与凝血取决于与磷脂表面反应（7）。维生素K拮抗剂，如双香豆素衍生物，干扰维生素K依赖凝血因子的羧化过程，从而导致合成生物学上无活性而免疫学上可检出的形式。这些

无生物活性的凝血因子称为非氧化形式或者ＰＩＶＫＡ（６—12）。

由于维生素Ｋ依赖凝血因子在体内的半衰期不同，口服维生素Ｋ拮抗剂时首先使因子Ⅶ活性减低，接着因子Ⅸ、Ⅹ和Ⅱ活性依次减低（12）。在治疗过程中，决定凝血酶原时间的因子Ⅶ，Ⅹ和Ⅱ活性在不同程度上同时减低，因此，从实验观测到的凝血酶原时间事实上反映着上述三个因子水平处于不同变化的总活性，而个体凝血因子与凝血酶原时间值的关系尚不清楚。例如，是否治疗初期因子Ⅶ活性水平减低代表体内有效抗血栓反应，或者当因子Ⅹ或因子Ⅱ活性水平减低时才能达到。

在论文的第三章里，作者介绍了双香豆素血浆中维生素Ｋ依赖因子在凝血酶原酶活性形成方面的相对重要性的研究。结果显示，于深度口服抗凝剂治疗病人血浆中加入纯化的因子Ⅶ、Ⅸ或Ⅹ并不影响凝血酶原酶的生成率和生成量，唯有存在于血浆中因子Ⅱ活性决定着凝血酶生成过程。进一步研究显示，当于因子Ⅱ、Ⅶ、Ⅸ和Ⅹ缺陷血浆中分别加入纯化的因子Ⅱ、Ⅶ、Ⅸ和Ⅹ时，血浆凝血酶原酶活性的生成率和生成量与加入的因子Ⅱ量呈线性增加，而达到最大量凝血酶原酶活性生成50％所需因子Ⅶ、Ⅸ和Ⅹ的量分别是＜１％，５％和10％。结论认为，口服抗凝剂治疗时，唯有血浆因子Ⅱ活性水平变化反映着体内抗血栓反应。因此，以测量因子Ⅱ活性水平来监测口服抗凝剂治疗是合理的方法。

肝素用于临床实践长达40年之久，治疗不总是那么有效，使用的剂量也受到出血危险的限制。再者，某些病人合并血小板减少症和血栓症也关系到肝素治疗。这些临床所见激励着人们对肝素相关成份的兴趣，据报导，这些肝素相关成份占有较高抗血栓效力同时引起出血的危险性小，从而推测可以增加“治疗窗口”（13—15）。ＳＪＡＭＰ是一种从海生黄瓜（刺参类）提取的肝素样酸性粘多糖，近年来作为一种潜在的抗凝药物研究（16）。据文献报导，ＳＪＡＭＰ对凝血的影响是由于其对凝血酶活性的抑制，但其确切的抗凝机理及其抗凝作用的辅因子依从关系尚不甚清楚。

在论文的第四章里，作者介绍了ＳＪＡＭＰ对血浆凝血酶生成影响的研究。结果显示，在血小板缺少血浆体系中，ＳＪＡＭＰ对外源凝血途径的影响主要是抑制最大量凝血酶生成和加速凝血酶衰变。这两种现象都可归因于ＳＪＡＭＰ依赖的抗凝血酶活性增加。ＳＪＡＭＰ依赖的凝血酶衰变的第二序列比率常数为$1.5/min^{-1}/\mu g/ml$。这表示ＳＪＡＭＰ对凝血酶活性的抑制效力比国际标准肝素低大约16倍。与肝素相同，ＳＪＡＭＰ未显示对血浆外源凝血途径中凝血酶原酶活性有抑制作用。当研究ＳＪＡＭＰ对内源凝血途径的影响时，附带的作用被观察到：ＳＪＡＭＰ增加凝血酶生成过程中的延迟时间。这个现象可解释为由凝血酶诱导的因子Ⅷ反馈活化受到抑制。早期研究提示，凝血酶生成过程中的延迟时间代表着因子Ⅷ被凝血酶激活所需时间（17）。但是，在内源凝血途径中，ＳＪＡＭＰ显示以剂量依赖方式刺激凝血酶原酶活性的生成，对此，尚没有适当的解释。

以微量凝血活酶触发富含血小板血浆的凝血酶生成被认为是凝血活酶和血小板协同作用的结果，这个实验体系显然要比“经典的体外凝血体系”更接近于体内状态。在相对高浓度的ＳＪＡＭＰ存在下，ＳＪＡＭＰ抑制最大量凝血酶的生成和增加凝血酶生成过程中的延迟

时间。结果提示，与肝素相反（18），ＳＪＡＭＰ对凝血酶的抑制作用不易被血小板因子４中和。但是，在相对低浓度的ＳＪＡＭＰ存在下，ＳＪＡＭＰ显示对凝血酶生成有轻度刺激，这种效应可能与ＳＪＡＭＰ诱导血小板聚集作用有关（16）。结论认为，在富含血小板血浆中，ＳＪＡＭＰ的主要作用也是对凝血酶生成的抑制。

肝素及其同系物提高血浆抑制剂对凝血丝氨酸蛋白水解酶的作用，在很大程度上取决于它们与肝素辅因子的结合，即抗凝血酶Ⅲ和肝素辅因子Ⅱ（19—22）。依赖抗凝血酶Ⅲ的凝血酶抑制被认为是肝素最主要的抗凝机制（19—24），但肝素的部分抗凝血作用不依赖抗凝血酶Ⅲ的可能性也见于文献（25、26）。早期研究报导，ＳＪＡＭＰ作为抗凝剂对凝血酶的灭活不依赖抗凝血酶Ⅲ（16）。

在论文的第五章里，作者介绍了ＳＪＡＭＰ对凝血酶抑制作用的辅因子依从关系的研究。结果显示，在纯化的体系和抑制剂去除的血浆体系（优球蛋白成分）中，ＳＪＡＭＰ对凝血酶活性的抑制仅见于这些实验体系用纯化的人抗凝血酶Ⅲ重组后。而在抗凝血酶Ⅲ缺陷血浆体系中的进一步研究显示，大约20％由ＳＪＡＭＰ导介的凝血酶抑制不依赖于抗凝血酶Ⅲ。从而提示，在ＳＪＡＭＰ导介的凝血酶抑制中有其它血浆抑制剂参与，可能是肝素辅因子Ⅱ。

基于抗凝血酶Ⅲ与ＳＪＡＭＰ反应后内源萤光变化的直接结合实验研究提示，ＳＪＡＭＰ对抗凝血酶Ⅲ亲合性较低，部分原因可能解释为ＳＪＡＭＰ与肝素相比抗凝效力较低。ＳＪＡＭＰ抗凝血酶Ⅲ结合强度与ＳＪＡＭＰ实际抗凝活性之间的矛盾涉及ＳＪＡＭＰ与其它浆抑制剂反应。结论认为，ＳＪＡＭＰ对凝血酶的抑制主要通过抗凝血酶Ⅲ依赖途径，但是，不能忽视其它血浆抑制剂（如肝素辅因子Ⅱ）对ＳＪＡＭＰ抑制凝血酶所起的作用。

SUMMARY

The central theme in this thesis is the generation and inhibition of thrombin in plasma. The first part of the thesis presents our studies on thrombin generation in plasma of patients with congenital clotting factor deficiencies and of patients with an acquired reduction in the effective activity of the vitamin K-dependent clotting factors brought about by oral administration of vitamin K antagonists. The second part presents our studies on the influence of a heparin-like material isolated from Stichopus japonicus selenka (a sea cucumber) on thrombin inhibition in plasma.

Chapter I gives a general introduction in the topics that will be discussed in more detail in the following chapters.

In chapter II the importance of factor IX activation during thromboplastin-dependent coagulation in plasma (The Josso Pathway) and thus the question "Why do haemophiliacs bleed?" is investigated. Although numerous studies have postulated that the factor IX activation in thromboplastin-dependent coagulation in plasma is of physiological importance, attention was mostly focussed on factor X activation. Convincing evidence on the generation of thrombin activity via thromboplastin-induced factor IX activation is lacking, however. Therefore, experiments were carried out in plasma of patients with a congenital deficiency of the clotting factors IX and VIII before and after reconstitution of the plasma with purified factor IX and VIII. The generation of thrombin activity in these systems is measured after triggering coagulation with diluted, $CaCl_2$-containing thromboplastin solutions. It is shown that in factor IX deficient plasma the generation of thrombin activity is independent of the amount of factor IX present when the plasma coagulation is triggered with the thromboplastin concentration normally used in routine tests. When, however, plasma coagulation is triggered with low concentrations of thromboplastin, a clear dependency of the generation of thrombin activity on the concentration of factor IX becomes evident at factor IX concentrations lower than 30 nM (about 40% clotting factor activity). Factor VIII seems to be a compulsory cofactor for factor IX activity, because the generation of thrombin

activity at optimal factor IX concentration is still dependent upon the amount of factor VIII present. This factor IX dependency of thromboplastin dependent coagulation probably explains why haemophiliacs bleed.

Chapter III deals with thrombin generation in dicoumarol plasma. The hypocoagulability of dicoumarol plasma is due to the combined diminution of the effective activity of the vitamin K-dependent clotting factors brought about by oral administration of a coumarin congener. The relative contribution of the individual vitamin K-dependent clotting factors to this hypocoagulability of plasma is not yet clearly understood, however. The studies presented show that addition of purified factors VII, IX or X to plasma from deeply anticoagulated patients does not influence the prothrombinase formation and the amount of thrombin formed. Only the prothrombin level in dicoumarol plasma determines the course of thrombin generation. Subsequent experiments, in which increasing amounts of the purified factors II, VII, IX or X are added to plasma deficient in respectively the factors II, VII, IX or X, show that the prothrombinase activity linearily increases with the concentration of factor II and that the concentration below which the factors VII, IX and X start to have an apreciable effect on prothrombinase activity are 5%, 20% and 40%, respectively. The half maximal amount of prothrombinase activity is found at about 1% for factor VII, 5% for factor IX and 10% for factor X. From this study it can be concluded that in dicoumarol plasma only the changes in the prothrombin level determine the antithrombotic effect. Therefore, it seems logical that, for the control of oral anticoagulant therapy, tests that reflect the prothrombin activity would be suitable.

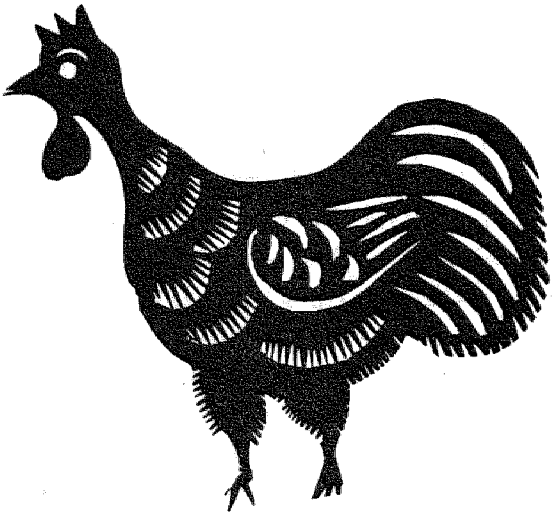
In chapter IV the anticoagulant effect of Stichopus japonicus acid mucopolysaccharide (SJAMP), a heparin-like material isolated from the sea cucumber Stichopus japonicus selenka, is described. SJAMP has recently been studied as a potential anticoagulant drug. The effect of SJAMP on blood coagulation has been reported to be due to inhibition of thrombin activity, but the precise mechanism of its anticoagulant activity and its cofactor dependency were not yet known. The studies presented show that the main effect of SJAMP is the inhibition of thrombin activity in both platelet poor and platelet rich plasmas. On a weight basis the effect of SJAMP towards thrombin is about sixteen-fold weaker than that of the 4th international standard heparin. When experiments are carried out in

platelet poor plasma after triggering coagulation with thromboplastin, SJAMP induces both a reduction of the maximal amount of thrombin formed and an acceleration of thrombin decay. An additional effect of SJAMP is observed when coagulation in platelet poor plasma is triggered with Cephaloplastin. In this system SJAMP increases the lag time in the thrombin generation process. This effect of SJAMP can be explained by an inhibition of the feedback activation of factor VIII by thrombin. When the effect of SJAMP is studied in platelet rich plasma after triggering coagulation with trace amounts of thromboplastin a slight stimulation of the thrombin generation is observed at a low SJAMP concentration while at higher SJAMP concentrations a reduction of the maximal amount of thrombin formed and an increase in the lag time of the thrombin generation process are observed. This indicates that the inhibitory effect of SJAMP on thrombin activity, unlike that of heparin, can not be neutralized by platelet factor 4 released from activated platelets. Also in this experimental system the increase in the lag time of the thrombin generation process may be explained by an inhibition of the feedback activation of factor VIII and of the thrombin-induced platelet stimulation.

Chapter V describes the cofactor dependency of SJAMP. The enhancing effect of heparin and its analogues on the inhibition of the coagulation serine proteases by plasma inhibitors has been shown to depend largely on the ability of these compounds to bind to the heparin cofactors, i.e. antithrombin III and heparin cofactor II. Similarly, the studies presented in this chapter show that in a purified system, as well as in an inhibitor-depleted plasma (euglobulin fraction) the thrombin inhibition by SJAMP is dependent upon the presence of antithrombin III. When, however, the effect of SJAMP on thrombin inhibition is studied in an antithrombin III-depleted plasma, it is shown that in the absence of antithrombin III the thrombin inhibition is about 20% of that found after reconstitution of the plasma with purified antithrombin III. This points to the involvement of other plasma cofactors in the SJAMP-mediated thrombin inhibition.

Direct binding studies, based on the change in the intrinsic fluorescence of antithrombin III upon interaction with SJAMP, indicate that SJAMP has a relatively low affinity for antithrombin III; this low affinity is not reflected in its anticoagulant efficiency, however. The discrepancy between the SJAMP-antithrombin III binding and the anticoagulant efficiency

of SJAMP may be caused by the interaction of SJAMP with other protein co-factors such as heparin cofactor II.



SAMENVATTING

Het centrale thema in dit proefschrift is de vorming en remming van thrombine in plasma. Het eerste deel beschrijft ons onderzoek naar de thrombine generatie in plasma's van patiënten met congenitale stollingsfaktor deficiënties en van patiënten met een verkregen reduktie in de effektive aktiviteit van vitamine K afhankelijke stollingsfaktoren ten gevolge van orale toediening van vitamine K antagonisten. Het tweede deel beschrijft ons onderzoek naar de thrombine remming door een heparineachtig product geïsoleerd uit Stichopus japonicus selenka (een zeekomkommer).
In hoofdstuk I wordt een algemene inleiding gegeven op de onderwerpen die in de volgende hoofdstukken meer in detail zullen worden bediscussieerd.
In hoofdstuk II wordt het belang van faktor IX aktivatie in thromboplastine-afhankelijke stolling in plasma (de Josso pathway) en dus de vraag "Waarom bloeden haemofielen?" onderzocht. In vele onderzoekingen is gepostuleerd dat faktor IX aktivatie in de thromboplastine afhankelijke stolling in plasma fysiologisch belangrijk is maar de aandacht was hierbij voornamelijk gericht op faktor X aktivatie in gezuiverde systemen; een duidelijk bewijs voor de generatie van thrombine aktiviteit via thromboplastine geïnduceerde faktor IX aktivatie in plasma is nog niet beschreven. Daarom werden experimenten opgezet in plasma's van patiënten met congenitale deficiënties van de stollingsfaktoren VIII en IX voor en na reconstructie van de plasmas met de gezuiverde stollingsfaktoren IX en VIII. De generatie van thrombine aktiviteit in deze systemen werd gemeten na initiatie van de stolling met verdunde, $CaCl_2$, bevattende, thromboplastine oplossingen. De experimenten met faktor IX deficiënt plasma laten zien dat, als de stolling wordt geïnitieerd met de thromboplastine concentratie die wordt gebruikt in routine tests, de generatie van de thrombine aktiviteit onafhankelijk van de hoeveelheid faktor IX is. Wanneer echter de stolling wordt geinitieerd met lage thromboplastine concentraties wordt, bij faktor IX concentraties lager dan 30 nM (= ongeveer 40% stollingsfaktor aktiviteit) de generatie van de thrombine aktiviteit afhankelijk van de faktor IX concentratie.
Faktor VIII lijkt een essentiële cofaktor voor faktor IX aktiviteit want de

generatie van thrombine aktiviteit bij een optimale faktor IX concentratie is nog steeds afhankelijk van de aanwezige hoeveelheid faktor VIII. Deze invloed van faktor IX in de thromboplastine afhankelijke stolling kan verklaren waarom haemofielen bloeden.

In hoofdstuk III wordt de thrombine generatie in het plasma van oraal geantistolde patiënten beschreven. De hypocoagulabiliteit van dicoumarol plasma komt tot stand door een gekombineerde afname in de effectieve aktiviteit van de vitamine K afhankelijke stollingsfaktoren onder invloed van orale anticoagulantia. De relatieve bijdrage van de individuele vitamine K afhankelijke stollings faktoren aan de hypocoagulabiliteit van plasma is nog niet geheel opgehelderd. Het onderzoek laat zien dat toevoeging van de gezuiverde stollingsfaktoren VII, IX en X aan plasma van diep geantistolde patiënten geen invloed heeft op de hoeveelheid prothrombinase en de daardoor gevormde hoeveelheid thrombine; alleen de hoeveelheid prothrombine in dicoumarol plasma bepaalt het verloop van de thrombine generatie. Experimenten waarin toenemende hoeveelheden van de gezuiverde faktoren II, VII, IX of X werden toegevoegd aan plasma's deficiënt in respectivelijk de fatoren II, VII, IX of X laten zien dat de prothrombinase aktiviteit liniair toeneemt met de faktor II concentratie en dat de concentraties waar beneden de faktoren VII, IX en X een aanzienlijke invloed op de prothrombinase aktiviteit gaan hebben respectivelijk 5%, 20% en 40% zijn.

De halfmaximale prothrombinase aktiviteit wordt gevonden bij ongeveer 1% faktor VII, 5% faktor IX en 10% faktor X. Uit deze studie kan worden gekonkludeerd dat alleen de verandering in de hoeveelheid prothrombine in dicoumarol plasma verantwoordelijk is voor het antithrombotisch effekt. Daarom lijkt het logisch om voor de kontrole van orale antistollingstherapie een test die de prothrombine aktiviteit meet te gebruiken.

Hoofdstuk IV beschrijft de remming van de thrombine aktiviteit onder invloed van "Stichopus japonicus acid mucopolysaccharide" (SJAMP) een heparineachtig product geïsoleerd uit zeekomkommers. SJAMP is recent naar voren gebracht als een potentieel anticoagulans. Het belangrijkste effekt van SJAMP op de stolling van bloed werd hierbij gesuggeerd als een remming van de bloedplaatjes aktiviteit. Het precieze mechanisme achter de antistollingsaktiviteit en de cofaktorafhanklijkheid waren echter nog niet duidelijk. Ons onderzoek laat zien dat het voornaamste effekt van SJAMP,

zowel in plaatjesarm als in plaatjesrijk plasma, de remming van de thrombine aktiviteit is.
SJAMP heeft, berekend op een gewichtsbasis, een 16 maal geringer effekt op thrombine dan het 4e Internationale standaard heparine. Als de experimenten worden uitgevoerd in plaatjesarm plasma en de stolling wordt geïnitieerd met thromboplastine geeft SJAMP zowel een reductie in de maximale hoeveelheid thrombine die wordt gevormd als een verhoging van de thrombine verdwijnsnelheid. Een additioneel effekt van SJAMP wordt waargenomen na initiatie van de stolling in plaatjesarm plasma met cephaloplastine. SJAMP verlengt in dit systeem de latentie-tijd in het thrombine vormings proces. Dit effekt van SJAMP kan worden verklaard uit een remming van de feedback aktivatie van faktor VIII door thrombine. Wanneer het effekt van SJAMP wordt onderzocht in plaatjesrijk plasma wordt na het initiëren van de stolling met zeer kleine hoeveelheden thromboplastine, geringe stimulatie van de thrombinegeneratie verkregen, terwijl hogere SJAMP concentraties een reductie in de maximale hoeveelheid thrombine en een verlenging in de latentIe-tijd van het thrombine generatie proces bewerkstelligen. Dit laat zien dat het remmende effekt van SJAMP op de thrombine aktiviteit, in tegenstelling tot dat van heparine, niet kan worden geneutraliseerd door plaatjes faktor 4 dat vrijkomt uit geaktiveerde plaatjes. Ook in dit experimentele systeem kan de toename van de latentie-tijd van het thrombine generatie proces worden verklaard door een remming van de feedback aktivatie van faktor VIII en van de door thrombine geïnduceerde plaatjes stimulatie.
Hoofdstuk V beschrijft de cofaktor afhankelijkheid van SJAMP. Het stimulerende effekt van heparine en heparineachtige stoffen op de remming van de stollings serine-proteases door plasma remmers is afhankelijk van het vermogen van deze stoffen aan heparine cofaktoren zoals antithrombine III en heparine cofaktor II te binden. De resultaten in dit hoofdstuk laten zien dat in gezuiverde systemen en ook in remmer gedepleteerd plasma (euglobuline fraktie) de thrombine remming door SJAMP afhankelijk is van de aanwezigheid van antithrombine III. Wanneer echter het effekt van SJAMP op de thrombine remming wordt bepaald in antithrombine III gedepleteerd plamsa blijkt ongeveer 20% van de remming onafhankelijk van antithrombine III. Dit wijst op een bijdrage van andere cofaktoren dan antithrombine III aan het thrombine remmende van SJAMP.

Direkte binding studies, gebaseerd op de verandering in de intrinsieke fluorescentie van antithrombine III bij interaktie met SJAMP, laten zien dat SJAMP een relatief lage affiniteit voor antithrombine III heeft. Deze lage affiniteit wordt echter niet weerspiegeld in de antistollings efficiëntie. Deze discrepantie tussen SJAMP-antithrombine III binding en de antistollings efficiëntie van SJAMP worden mogelijkerwijs veroorzaakt door de interaktie voor SJAMP met andere eiwit cofaktoren zoals heparine cofaktor II.

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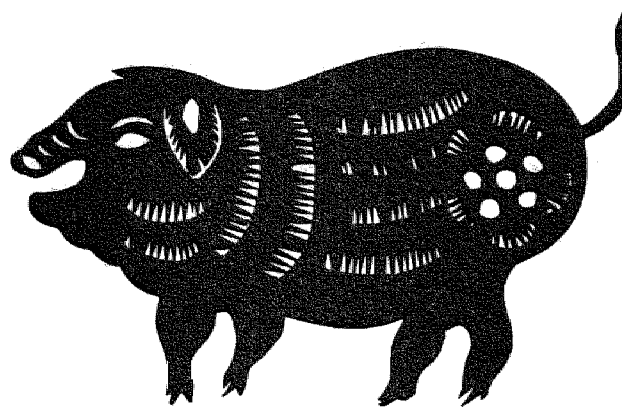

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