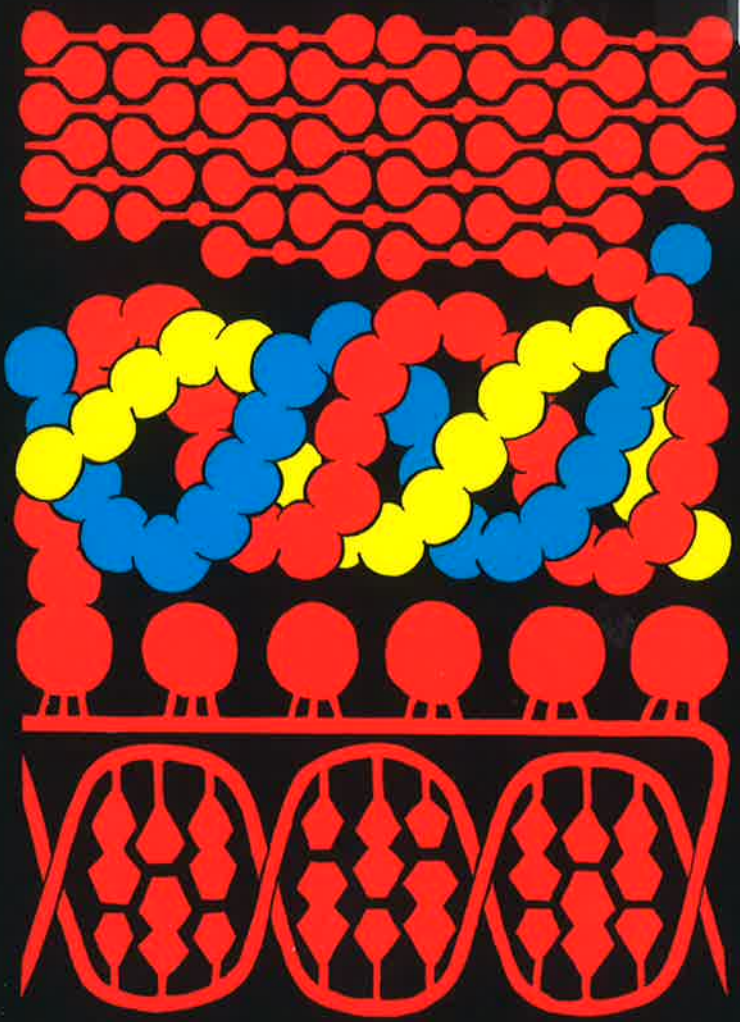
STRUCTURE-FUNCTION ANALYSIS OF HEREDITARY VARIANT HUMAN FIBRINOGENS



Jaap Koopman

STELLINGEN

1. Een specifieke structuur-functie analyse van abnormale fibrinogenen met een mutatie die aanleiding geeft tot een vrij cysteïne residue, wordt bemoeilijkt door de mogelijke vorming van disulfide complexen tussen de abnormale fibrinogeen moleculen en andere plasma eiwitten.
   Dit proefschrift.

2. Bij het beschrijven van de functionele defecten in abnormale fibrinogenen, mag de term "vertraagde fibrine polymerizatie" alleen gebruikt worden als deze functie is bepaald m.b.v. fibrine monomeren waarvan alle fibrinopeptides zijn afgesplitst, of als de fibrinopeptide release normaal is.

3. De conclusie van Siebenlist et al. dat Bβ 1-42 een rol speelt in de binding van trombine aan fibrine via de anion binding site, wordt niet ondersteund door zijn experimentele resultaten.
   Siebenlist et al. (1990) J. Biol. Chem. 265: 18650-18655.

4. De vertraagde afbraak van fibrinogeen Argenteuil en fibrinogeen Poitiers door plasmine, is waarschijnlijk het gevolg van de aanwezigheid van verschillende hoeveelheden trasylol in de gezuiverde fibrinogeen preparaten.
   Gandrille et al. (1988) Br. J. Haematol. 68: 329-337.

5. CNBr fragmenten van fibrinogeen zijn geen goed model voor het stimulerende effect van fibrine op de t-PA geïnduceerde plasminogeen activatie, omdat het stimulerende effect van fibrine afhankelijk is van de fibrine polymerizatie.
   Nieuwenhuizen et al. (1983) Biochim. Biophys. Acta 748: 86-92; Suenson et al. (1986) Biochim. Biophys. Acta 870: 510-519.

6. Het neurale cel adhesie molecuul speelt niet alleen een rol bij de passieve onderlinge binding tussen cellen, maar is ook actief betrokken bij de communicatie tussen cellen.
   Acheson et al. (1991) J. Cell Biol. 114: 143-153.

7. De cyclines die een rol spelen bij de overgang van de G1 naar S fase in de cel cyclus, zijn kandidaat proto-oncogenen.
   Hunter et al. (1991) Cell 66: 1071-1074.

8. Het uitvoeren van wetenschappelijke experimenten is te vergelijken met biljarten, een doordacht concept moet gevolgd worden door een perfecte technische uitvoering.

9. De reclame-slogan "meer bank voor je geld" van de Rabobank kan nu beter worden veranderd in: "meer geld voor je bank".

10. Homeopatische middelen met een verdunningsfactor groter dan 10^{25} kunnen alleen moleculen van het desbetreffende middel bevatten als de dichtheid van de uitgangsmaterie die van een zwart gat benadert.

11. Het herhalen van experimenten beschreven in wetenschappelijke publicaties, zou eenvoudiger worden als deze vergezeld gingen van een video-clip.

12. Hoewel het recht op vrijheid van meningsuiting een belangrijke pilaar is van de democratie, mag dit recht niet ontaarden in vrijheid van leugen.

Leiderdorp, 9 januari 1992 Jaap Koopman

STRUCTURE - FUNCTION ANALYSIS OF HEREDITARY VARIANT HUMAN FIBRINOGENS

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
te verdedigen op woensdag 4 maart 1992
te klokke 15.15 uur

door

Jacob Laurens Koopman

geboren te Sint Maartensdijk in 1959

"ALL IN" BV - KATWIJK

Promotiecommissie:

Promotor:	Prof.dr. R.M. Bertina
Co-promotoren:	Dr. F. Haverkate (IVVO-TNO, Leiden)
	Dr. S.T. Lord (University of North Carolina, Chapel Hill, U.S.A.)
Referent:	Prof.dr. D.A. Lane (University of London, London, U.K.)
Overige leden:	Prof.dr. P. Brakman
	Prof.dr. W. Möller

The study presented in this thesis was performed at the Gaubius Institute TNO, Leiden (Director: Prof.dr. P. Brakman), and was supported by TNO and Nato Grant CRG 860110.
Financial support by the Netherlands Heart Foundation and the Gaubius Laboratory, IVVO-TNO, Leiden, for the publication of this thesis is gratefully acknowledged.

Cover design: Marijn Snoep

ISBN 90-9004889-8

We'll never, ever, reach the moon, at least not the one that we are after.

Leonard Cohen,
Songs of Love and Hate.

Aan Janneke
Voor mijn ouders

Table of contents

CHAPTER 1

GENERAL INTRODUCTION

The balance between coagulation and fibrinolysis

To prevent excessive bleeding in vivo, after damage to the vessel wall, a complex coagulation system is activated. The final phase of in vivo coagulation involves generation of a fibrin clot that provides the major structural support and scaffolding for the cellular and other blood elements that comprise the thrombus. The formation of fibrin is strictly regulated to assure formation of sufficient fibrin to arrest bleeding, while at the same time preventing excessive fibrin formation, which would obstruct the normal blood flow. The thrombus has only a temporary role, and needs to be removed once it has fulfilled its function in e.g. tissue repair. The degradation of the fibrin matrix is initiated by activation of the fibrinolytic system, which is controlled by several regulatory mechanisms to assure efficient and restricted degradation of the fibrin clot. The haemostatic balance theory, first formulated by Astrup in 1958, is the dynamic equilibrium between the reactions that lead to fibrin formation (coagulation) and those that lead to fibrin dissolution (fibrinolysis). Disruption of the equilibrium can lead to a thrombotic tendency (excessive coagulation/insufficient fibrinolysis) or a bleeding tendency (insufficient coagulation/excessive fibrinolysis). The central protein in the haemostatic balance is clearly the fibrin molecule, which is formed from its soluble precursor: fibrinogen. In the following paragraphs the structure of fibrinogen and fibrin, fibrin formation and degradation and the regulatory role of fibrin in haemostasis will be briefly discussed.

Fibrinogen

Human plasma fibrinogen is synthesized and assembled in the liver and secreted into the blood, reaching concentrations of 2 to 4 mg/ml in normal healthy individuals. The fibrinogen molecule is a covalently-linked dimer (Hoeprich and Doolittle, 1983), consisting of three pairs of disulfide-bonded polypeptide chains, designated $A\alpha$, $B\beta$, and γ (Blombäck and Yamashina, 1958; Henschen, 1964; Blombäck, 1973), as illustrated in Fig. 1. The amino acid sequence of all three human fibrinogen chains, has been determined by classic protein chemistry (Doolittle, 1981,1983; Henschen et al., 1983) and derived from mRNA/DNA sequences (Rixon et al., 1983a; Chung et al., 1983; Rixon et

al., 1983b; Kant et al., 1983). The Aα-chain was found to contain 610 amino acid residues based on protein sequence analysis (Doolittle, 1979), while mRNA sequences inferred a total length of 625 amino acids (Kant et al., 1983). This discrepancy is due to the proteolytic removal of the Aα 611-625 peptide during or directly after secretion of the assembled fibrinogen molecule. The computed molecular weight for mature Aα-chain (610 amino acids) is 66,500 Daltons. The Bβ-chain contains 461 amino acids and has a computed molecular weight of 52,000. The γ-chain is present in normal fibrinogen in two different forms (Francis et al., 1980; Wolfenstein-Todel and Mosesson, 1980), due to alternative splicing of the γ-chain gene (Rixon et al., 1983b; Fornace et al., 1984). The most abundant form of the γ-chain (>90%) contains 411 amino acids and has a molecular weight of 47,000, while the other form (<10%) contains 427 amino acids. The Bβ and γ chains contain carbohydrate, each of molecular weight 2500, attached covalently at residues 364 and 52, respectively (Mills and Triantaphyllopoulos, 1969; Blombäck et al., 1973). Thus, the total molecular weight of fibrinogen is approximately 340,000 Dalton, confirming results obtained by physiochemical measurements (Scheraga and Laskowski, 1957). In plasma, different molecular weight forms of fibrinogen have been detected, with molecular weights of 340,000 (HMW fibrinogen), 300,000 (LMW fibrinogen) and 270,000 (LMW' fibrinogen), which are the result of partial degradation from one or both Aα-chains (Mosesson et al., 1974; Lipinska et al., 1974).

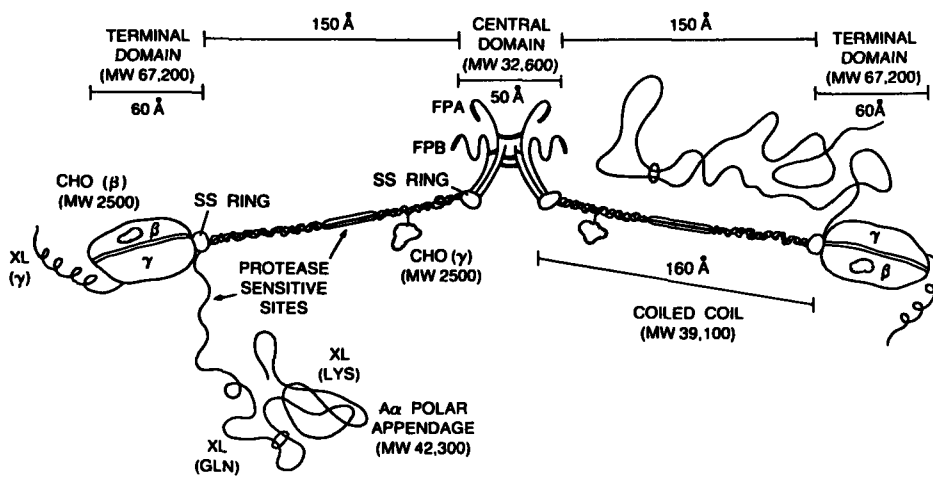


Figure 1. Detailed model of human fibrinogen molecule showing key structural features. Note pseudosymmetry of the halves of the terminal domains contributed by β and γ chains, respectively (reprinted from Doolittle, 1987).

The schematic model of fibrinogen in Fig. 1 (Doolittle, 1987), is based on physiochemical and electron microscopic studies, amino acid sequence information and chemical and

enzymatic fragmentation. Fibrinogen is a tridomainal disulfide-bridged molecule, approximately 45 nm in length, that comprises two symmetrical half-molecules, each consisting of one Aα, Bβ, and γ chain. The central domain (E domain) contains the amino-termini of all six polypeptide chains and joins the two half-molecules by three interchain disulfide bridges. The two distal domains (D domains) are connected to the central domain by a coiled coil region with a length of 112 amino acids. Each end of the coiled coil region is bound by a disulfide ring and the midportion of the coiled coil region is interrupted by a short non-helical stretch that is particularly susceptible to plasmin degradation (Doolittle et al., 1978).

Fibrin formation

The conversion of fibrinogen to fibrin is triggered by the thrombin catalyzed release of small peptides from the amino terminal segment of the Aα and Bβ-chains that are denoted the fibrinopeptides A and B, respectively. FpA release exposes an "A" polymerization site in the amino terminal domain (Kudryk et al., 1974) of the fibrin molecule that subsequently aligns with a complementary "a" site in the carboxyl terminal domains of another molecule (Blombäck et al., 1958; Kudryk et al., 1973; Olexa and Budzynski, 1980; Olexa et al., 1981; Budzynski et al., 1983) to form staggered, overlapping, two-stranded fibrils (Fig. 2). Release of FpB exposes an independent "B" polymerization site in the amino terminal domain (Shainhoff and Dardik, 1979,1983), which interacts with the complementary "b" site. The "b" site is made up of two properly

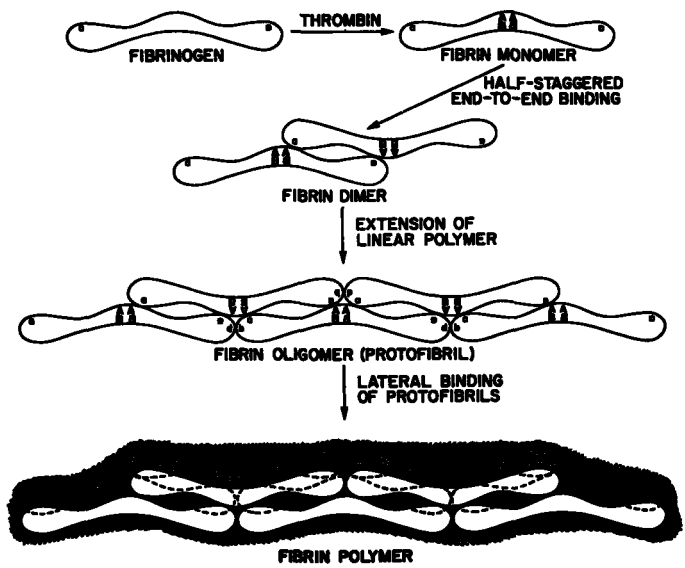


Figure 2. Sequential events in the formation of fibrin (reprinted from Budzynski et al., 1983).

aligned D domains in the fibrin polymer (Shainhoff and Dardik, 1983; Olexa and Budzynski, 1980). The two-stranded fibrils can laterally associate, resulting in increased fiber thickness. The carboxyl terminal part of the α-chain is involved in this lateral association (Hasegawa and Sasaki, 1990) which make up the three-dimensional fibrin matrix. The next step in fibrin formation is the crosslinking of the subunits in the fibrin network by the formation of isopeptide bonds. This reaction is catalyzed by factor XIIIa in the presence of Ca^{2+} ions, and forms a covalent bond between a donor lysine residue and an acceptor glutamine residue from the γ-chains of two adjacent fibrin molecules (Matacic and Loewy, 1968; Pisano et al., 1968)). Slower intermolecular crosslinking among α-chains (McKee et al., 1970; McDonagh et al., 1971; Schwartz et al., 1971) further completes the crosslinking of fibrin.

Fibrinogen and fibrin degradation

Fibrinogen and fibrin can both be degraded by the serine protease plasmin into fibrin or fibrinogen degradation products, designated here as FbDP and Fbgdp, respectively. Fibrinogen contains many plasmin cleavage sites (Henschen, 1983), which are hydrolyzed by plasmin at different rates, resulting in the formation of characteristic fragments during the progress of degradation. First, plasmin cleaves a lysyl-methionine bond only 27 residues from the carboxyl terminus of the α-chain (Cottrell and Doolittle, 1976). The next plasmin cleavage occurs at the sites between amino acids 197-253 in the α-chain, releasing the carboxyl terminal two third of the α-chain (Takagi and Doolittle, 1975b). At about the same time plasmin removes a 42-residue segment from the amino terminus of the β-chain (Takagi and Doolittle, 1975a). Finally the non-helical mid portion of the coiled coil region connecting the amino terminal (E) and carboxyl terminal (D) domains is cleaved and the plasmin degradation products D and E are formed from fibrinogen. The degradation products formed from polymerized and cross-linked fibrin are complexes of fragments similar to D and E linked together by non-covalent bonds due to interaction between the complementary polymerization sites on E and D or due to the covalent isopeptide bonds between two D domains of different fibrin monomer molecules.

Binding of calcium ions to fibrinogen

Human fibrinogen binds three Ca^{2+} ions with high affinity ($K_d \sim 10^{-5}$ M) at pH 7.4 . Two of the high-affinity binding sites are located in the two D domains of the fibrinogen molecule (Lindsey et al., 1978; Nieuwenhuizen et al., 1981). The third high affinity binding site is thought to be located in the amino terminal domain of fibrinogen (Nieuwenhuizen and Haverkate, 1983). The presence of calcium is known to accelerate fibrin formation (Ratnoff and Potts, 1954), by increasing the rate of fibrin polymerization. Different mechanisms have been proposed to explain the accelerating

effect of Ca^{2+} ions on fibrin polymerization (Laudano and Doolittle, 1981; Häberli et al., 1987), indicating that this effect is related to the carboxyl terminal polymerization sites located in the D domains of fibrin monomers. The presence of Ca^{2+} ions protects the γ-chain of fibrinogen against plasmin degradation (Haverkate and Timan, 1977). In the presence of Ca^{2+} ions plasmin degradation results in fragment D_1 with the carboxyl terminus of the γ-chain intact (M_r 38,000) whereas in the absence of Ca^{2+} ions fragment D_3 is formed with the carboxyl terminus of the γ-chain degraded (M_r 26,000) (Haverkate and Timan, 1977). On the other hand, degradation of the carboxyl terminus of the γ-chain results in the loss of the high-affinity calcium binding site in the D domain of fibrinogen (Nieuwenhuizen and Haverkate, 1983). This indicates that the high-affinity calcium binding in the D domain and the protective effect of calcium on plasmin degradation are directly related.

Interaction of fibrinogen with platelets and endothelial cells

The interaction of fibrinogen with platelets and endothelial cells is mediated through the interaction of fibrinogen-specific receptors on the membrane of these cells. Platelets and endothelial cells express homologous but distinct membrane receptors for fibrinogen that are members of a large family of heterodimers commonly referred to as integrins (Hynes, 1987). On platelets the GPIIb-IIIa complex has been shown to be an activation-dependent fibrinogen receptor (Nachman and Leung, 1982). The sites on fibrinogen involved in this interaction are thought to be located in the Aα-chains and the carboxyl terminus of the γ-chains (Hawiger et al., 1982; Kloczewiak et al., 1984). The Aα chain contains two RGD sequences, one located at amino acids 95-97 near the amino terminus and one at residues 572-574 in the carboxyl terminus. The significance of these RGD sites in fibrinogen-platelet interaction is still a matter of controversy. Recently, evidence has been presented that the carboxyl terminal RGD sequence is not involved in platelet interaction (Cheresh et al., 1989; Peerschke, 1987). On endothelial cells the binding of fibrinogen is predominantly mediated by the $\alpha_v\beta_3$ integrin receptor, which interacts with fibrinogen exclusively through the RGD-containing sequence in the carboxyl terminus of the Aα-chain of fibrinogen (Cheresh et al., 1989). This suggests that platelet and endothelial cells interact with distinct sites on fibrinogen, and do not necessarily compete with each other when binding to fibrinogen.

Organisation of the fibrinogen genes

Human fibrinogen is synthesized from three separate mRNA's (Nickerson and Fuller, 1981) that give rise, respectively, to its constituent Aα, Bβ, and γ chains. There is a single copy of each gene located adjacent to one another on the long arm of chromosome 4 (Kant et al., 1985). The Aα and γ chain genes are aligned in the same direction and are transcribed toward the Bβ chain gene; the Bβ gene is transcribed in

the direction of the Aα and γ genes from the opposite DNA strand. The Aα, Bβ and γ genes consist of, respectively, five, eight and ten exons (Crabtree et al., 1985). The complete genomic DNA sequence for all three genes has been elucidated (Chung, 1990).

Normally-occurring fibrinogen variants

Size and charge differences in circulating plasma fibrinogen are mostly related to post-translational modifications. However, there are two types of circulating γ chain variants that arise from separate mRNA's. The two different transcripts are produced by alternative processing of intron IX of the γ gene (Chung and Davie, 1984, Fornace et al., 1984). A number of polymorphic sites have been determined in fibrinogen by both amino acid and DNA sequence analysis (Ebert, 1991). The Aα chain contains three polymorphic sites, respectively at amino acid position 47 (Ser↔Thr), 296 (Thr↔Ala) and 312 (Thr↔Ala). The Bβ chain shows polymorphism at amino acid position 162 (Pro↔Ala), 296 (Asn↔Asp) and 448 (Arg↔Lys). In the γ chain a polymorphism at amino acid position 88 (Ile↔Lys) has been reported.

Regulatory functions of fibrin in haemostasis

In addition to its recognized biological role of providing the structural framework of a thrombus, fibrin displays several properties that give it the ability to regulate its own formation and degradation.

Thrombin interacts with fibrinogen and fibrin through two types of binding sites. The first type of interaction involves the substrate binding site of fibrinogen and the catalytic site of thrombin, which leads to the cleavage of the fibrinopeptides from the Aα and Bβ-chains (Scheraga and Laskowski, 1957; Blombäck and Yamashina, 1958; Blombäck et al., 1978). The second type of binding involves a non-substrate site in fibrin(ogen), located in the amino terminal part of the molecule (Fenton et al., 1988; Vali and Scheraga, 1988; Kaczmarek and McDonagh, 1988), and the anion binding exosite in α-thrombin, which is independent of the catalytic site (Liu et al., 1979; Fenton et al., 1981; Kaminski and McDonagh, 1983; Berliner et al., 1985). Binding of thrombin at the non-substrate site increases the rate of FpA and FpB release from fibrinogen (Fenton et al., 1977; Sonders and Fenton, 1986), resulting in enhanced fibrin formation. However, this type of binding also limits diffusion of thrombin into a forming fibrin clot, as well as the amount of free active thrombin in circulation (Liu et al., 1979), thereby regulating the extent of clot propagation. Another mechanism by which fibrin formation is regulated by fibrin itself, is the stimulatory effect of fibrin polymerization on FXIII activation by α-thrombin (Greenberg and Miraglia, 1985; Greenberg et al., 1985), resulting in a more rapid cross-linking of fibrin. Fibrin degradation is also regulated by the cross-linking of the important plasmin inhibitor α_2-antiplasmin to Aα 303 Lys in fibrin by activated FXIII (Sakata and

Aoki, 1980). The resistance to plasmin degradation of cross-linked whole blood clots is predominantly due to the cross-linking of α_2-antiplasmin to the fibrin clot (Jansen et al., 1987).

Tissue plasminogen activator plays an important role in the regulation of the fibrinolytic system. Activation of plasminogen by t-PA is greatly enhanced in the presence of fibrin (Rånby, 1982; Hoylaerts et al., 1982), and not in the presence of fibrinogen. Structures in the fibrin molecule located between amino acids Aα 148-161 (Nieuwenhuizen et al., 1983; Voskuilen et al., 1987) and γ 311-380 (Yonekawa et al., 1990) are thought to be involved in this acceleratory effect of fibrin. However, the acceleratory effect of fibrin, is also dependent on the fibrin polymerization, as inhibition of fibrin polymerization reduces the acceleratory effect of fibrin to the level observed in the presence of fibrinogen (Suenson and Petersen, 1986; Koopman et al., 1986). These results suggest an important role for fibrin polymerization in the initiation and regulation of fibrinolysis.

AIM AND OUTLINE OF THIS STUDY

The primary goal of this study was to further correlate structure and function in the fibrinogen molecule. The approach chosen involved the structural and functional analysis of variant fibrinogens, detected by prolonged plasma clotting times, in patients with dysfibrinogenemia. In addition to the primary goal, this approach enabled us to investigate the relation between the variant fibrinogens and the clinical symptoms in the patients. In this study, we choose to analyse variant fibrinogens associated with thrombophilia, because of the intriguing discrepancy between the impaired clotting of the variant fibrinogen and the clinical symptoms in the patient. Furthermore, a review of the literature (chapter 2), demonstrated that most variants associated with bleeding, or without clinical symptoms, contained a structural defect located in the amino terminal part of the fibrinogen Aα-chain, affecting the FpA release by thrombin. The structural abnormalities in the variant fibrinogens identified so far, had been determined by protein sequence analysis. In this study, we used the recently-introduced polymerase chain reaction, to determine the mutation in the genomic DNA segments encoding the variant fibrinogen. The advantages of this method, over protein sequence analysis are summarized in chapter 2. When family members of the propositus were available for investigation, the presence of the mutation could be established by Southern blotting or restriction analysis of the amplified genomic DNA. These family studies are compulsory for correlating the structural defect in the variant fibrinogen with the clinical symptoms in the patient. To determine the functional abnormality, the variant fibrinogen was purified from the plasma of the patient and compared with normal fibrinogen. The presence of post-translational modifications introduced by the mutations, was determined

in the plasma or purified protein of the patients. The results from the structural and functional analysis, together with the available family studies, were used to further clarify the structure-function relationship in fibrinogen, and to correlate specific types of variant fibrinogens with thrombophilia.

REFERENCES

Berliner LJ, Sugawara Y, Fenton JW II: Human α-thrombin binding to non-polymerized fibrin-sepharose: evidence for an anionic binding region. Biochemistry 24:7005, 1985

Blombäck B, Yamashina I: On the N-terminal amino acids in fibrinogen and fibrin. Arkiv Kemi 12:299, 1958

Blombäck B: Report of the subcommittee on nomenclature. Thromb Diath Haemorrh (Suppl) 54:425, 1973a

Blombäck B, Grondahl NJ, Hessel B, et al: Primary structure of human fibrinogen and fibrin. II. Structural studies on NH2-terminal part of chain. J Biol Chem 248:5806, 1973b

Blombäck B, Hessel B, Hogg D, Therkildsen L: A two-step fibrinogen-fibrin transition in blood coagulation. Nature 275:501, 1978

Budzynski AZ, Olexa SA, Pandya BV: Fibrin polymerization sites in fibrinogen and fibrin fragments. Ann N Y Acad Sci 408:301, 1983

Cheresh DA, Berliner SA, Vicente V, Rugger ZM: Recognition of distinct adhesive sites on fibrinogen by related integrins on platelets and endothelial cells. Cell 58:945, 1989

Chung DW, Que BGF, Rixon MW, et al: Characterization of complementary deoxyribonucleic and genomic deoxyribonucleic acid for the β chain of human fibrinogen. Biochemistry 22:3244, 1983

Chung DW, Davie EW: γ and γ' chains of human fibrinogen are produced by alternative mRNA processing. Biochemistry 23:4232, 1984

Chung DW: Personal Communication, 1990

Cottrell BA, Doolittle RF: The amino acid sequence of a 27-residue peptide released from the α-chain carboxy-terminus during the plasmic digestion of human fibrinogen. Biochem Biophys Res Comm 71:754, 1976

Crabtree GR, Comeau CM, Fowlkes DM, Fornace AJ, Malley JD, Kant JA: Evolution and structure of the fibrinogen genes. J Mol Biol 185:1, 1985

Doolittle RF, Goldbaum DM, Doolittle LR: Designation of sequences involved in the "coiled coil" interdomainal connector in fibrinogen: Construction of an atomic scale model. J Mol Biol 120:311, 1978

Doolittle RF: The amino acid sequence of the α-chain of human fibrinogen. Nature 280:464, 1979

Doolittle RF: Fibrinogen and fibrin. Ann Rev Biochem 53:195, 1981

Doolittle RF: The structure and evolution of vertebrate fibrinogen. Ann N Y Acad Sci 408:13, 1983

Doolittle RF: Fibrinogen and fibrin, in Bloom A, Thomas DP (eds): Haemostasis and Thrombosis. London, Churchill Livingstone, 1987

Ebert RF (ed): Index of variant human fibrinogens, 1991 edition, CRC Press, Boca Raton

Fenton JW II, Fasco MJ, Stackrow AB, Aronson DL, Young AM, Finlayson JS: Human thrombins. Production, evaluation, and properties of α-thrombin. J Biol Chem 252:3587, 1977

Fenton JW II, Olson TA, Zabinski MP, Wilner GD: Anion-binding exosite of human α-thrombin and fibrin(ogen) recognition. Bochemistry 27:7106, 1988

Fornace AJ Jr, Cummings DE, Corneau CM, Kant JA, Crabtree GR: Structure of the human γ-fibrinogen gene. J Biol Chem 259:12826, 1984

Francis CW, Marder VJ, Martin SE: Demonstration of large molecular weight variant of the γ chain of normal human plasma fibrinogen. J Biol Chem 255:5599, 1980

Greenberg CS, Miraglia CC: The effect of fibrin polymers on thrombin-catalyzed plasma factor XIIIa formation. Blood 66:466, 1985a

Greenberg CS, Miraglia CC, Rickles FR, Shuman MA: Cleavage of blood coagulation factor XIII and fibrinogen by thrombin during in vitro clotting. J Clin Invest 75:1463, 1985b

Häberli A, Straub PW: The influence of calcium ions on fibrin polymerization. Biopolymers 26:27, 1987

Hasegawa N, Sasaki S: Location of the binding site "b" for lateral polymerization of fibrin. Thromb. Res. 57:183, 1990

Haverkate F, Timan G: The protective effect of calcium in the plasmin degradation of fibrinogen and fibrin fragments D. Thromb Res 10:803, 1977

Hawiger J, Timmons S, Kloczewiak M, Strong DD, Doolittle RF: Gamma and alpha chains of human fibrinogen possess sites reactive with human platelet receptors. Proc Natl Acad Sci USA 79:2068, 1982

Henschen A: S-sulfo-derivatives of fibrinogen and fibrin: Preparations and general properties. Arkiv Kemi 22:1, 1964

Henschen A: On the structure of functional sites in fibrinogen. Thromb Res (Suppl V):27, 1983a

Henschen A, Lottspeich F, Kehl M, Southan C: Covalent structure of fibrinogen. Ann N Y Acad Sci 408:28, 1983b

Hoeprich PD, Doolittle RF: Dimeric half-molecules of human fibrinogen are joined through disulfide bonds in an antiparallel orientation. Biochemistry 22:2049, 1983

Hoylaerts M, Rijken DC, Lijnen HR, Collen D: Kinetics of the activation of plasminogen by human tissue plasminogen activator: role of fibrin. J Biol Chem 257:2912, 1982

Hynes RO: Integrins: A family of cell surface receptors. Cell 48:549, 1987

Jansen JWCM, Haverkate F, Koopman J, Nieuwenhuis HK, Kluft C, Boschman ThAC: Influence of factor XIII activity on human whole blood clot lysis in vitro. Thromb Haemostas 57:171, 1987

Kaczmarek E, McDonagh J: Thrombin binding to the Aα-, Bβ-, and γ-chains of fibrinogen and to their remnants contained in fragment E. J Biol Chem 263:13896, 1988

Kaminski M, McDonagh J: Studies on the mechanism of thrombin. Interaction with fibrin. J Biol Chem 258:10530, 1983

Kant JA, Lord ST, Crabtree GR: Partial mRNA sequences for human Aα, Bβ and γ-fibrinogen genes: Evolutionary and functional implications. Proc Natl Acad Sci USA 80:3953, 1983

Kant JA, Fornace AJ, Saxe D, Simon MI, McBride OW, Crabtree GR: Evolution and organization of the fibrinogen locus on chromosome 4: Gene duplication accompanied by transposition and inversion. Proc Natl Acad Sci USA 82:2344, 1985

Kloczewiak M, Timmons S, Lukas TJ, Hawiger J: Platelet receptor recognition site on human fibrinogen: synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the γ chain. Biochemistry 23:1767, 1984

Koopman J, Engesser L, Nieveen M, Haverkate F, Brommer EJP: Fibrin polymerization associated with tissue-type plasminogen activator (t-PA) induced Glu-plasminogen activation, in Muller-Berghaus G, Scheefers-Borchel U, Selmayer E, Henschen A (eds): Fibrinogen and its derivatives. Biochemistry, Physiology and Pathophysiology. Amsterdam, Elsevier, 1986, p 315

Kudryk B, Reuterby J, Blombäck B: Adsorption of plasmic fragment D to thrombin modified fibrinogen-sepharose. Thromb Res 2:297, 1973

Kudryk BJ, Collen D, Woods KR, Blombäck B: Evidence for localization of polymerization sites in fibrinogen. J Biol Chem 249:3322, 1974

Laudano AP, Doolittle RF: Influence of calcium ion on the binding of fibrin amino terminal peptides to fibrinogen. Science 212:457, 1981

Lindsey GG, Brown G, Purves LR: Calcium binding to human fibrinogen - Localization of two calcium specific sites. Thromb Res 13:345, 1978

Lipinska I, Lipinski B, Gurewich V: Fibrinogen heterogeneity in human plasma. Electrophoretic demonstration and characterization of two major fibrinogen components. J Lab Clin Med 84:509, 1974

Liu CY, Nossel HL, Kaplan KL: The binding of thrombin by fibrin. J Biol Chem 254:421, 1979

Matačic S, Loewy AG: The identification of isopeptide crosslinks in insoluble fibrin. Biochem Biophys Res Commun 30:356, 1968

McDonagh RP Jr, McDonagh J, Blombäck M, Blombäck B: Crosslinking of human fibrin: evidence for intermolecular crosslinking involving α-chains. FEBS Lett 14:33, 1971

McKee PA, Mattock P, Hill RL: Subunit structure of human fibrinogen, soluble fibrin, and cross-linked insoluble fibrin. Proc Natl Acad Sci USA 66:738, 1970

Mills DA, Triantaphyllopoulos DC: Distribution of carbohydrate among the polypeptide chains and plasmin digest products of human fibrinogen. Arch Biochem Biophys 135:28, 1969

Mosesson MW, Galanakis DK, Finlayson JS: Comparison of human plasma fibrinogen subfractions and early plasmic fibrinogen derivatives. J Biol Chem 249:4656, 1974

Nachman RL, Leung LLK: Complex formation of platelet membrane glycoproteins IIb and IIIa with fibrinogen. J Clin Invest 69:263, 1982

Nickerson JM, Fuller GM: In vitro synthesis of rat fibrinogen: Identification of pre Aα, pre Bβ, and pre γ polypeptides. Proc Natl Acad Sci USA 78:303, 1981

Nieuwenhuizen W, Van Ruijven-Vermeer IAM, Nooijen WJ, et al: Recalculation of calcium-binding properties of human and rat fibrin(ogen) and their degradation products. Thromb Res 22:653, 1981

Nieuwenhuizen W, Vermond A, Voskuilen M, Traas DW, Verheijen JH: Identification of a site in fibrin(ogen) which is involved in the acceleration of plasminogen activation by tissue-type plasminogen activator. Biochim Biophys Acta 748:86, 1983a

Nieuwenhuizen W, Haverkate F: Calcium binding regions in fibrinogen. Ann N Y Acad Sci 408:92, 1983b

Olexa SA, Budzynski AZ: Evidence for four different polymerization sites involved in human fibrin formation. Proc Natl Acad Sci USA 77:1374, 1980

Olexa SA, Budzynski AZ, Doolittle RF, Cottrell BA, Greene TC: Structure of fragment E species from human cross-linked fibrin. Biochemistry 21:6139, 1981

Peerschke EIB: Irreversible platelet fibrinogen interactions occur independently of fibrinogen alpha chain degradation and are not mediated by intact platelet membrane glycoprotein IIb-IIIa complexes. J Lab Clin Med 111:84, 1987

Pisano JJ, Finlayson JS, Peyton MP: Crosslink in fibrin polymerized by factor XII: ε-(γ-glutamyl0lysine. Science 160:892, 1968

Rånby M: Studies on the kinetics of plasminogen activation by tissue plasminogen activator. Biochim Biophys Acta 704:461, 1982

Ratnoff OD, Potts AM: The accelerating effect of calcium and other cations on the conversion of fibrinogen to fibrin. J Clin Invest 33:206, 1954

Rixon MW, Chan W-Y, Davie EW, Chung DW: Characterization of a complementary deoxynucleic acid coding for the α chain of human fibrinogen. Biochemistry 22:3237, 1983a

Rixon MW, Chung DW, Davie EW: Nucleotide sequence of the gene for the γ chain of human fibrinogen. Biochemistry 24:2077, 1983b

Sakata Y, Aoki N: Cross-linking of α_2-plasmin inhibitor to fibrin by fibrin-stabilizing factor. J Clin Invest 65:290, 1980

Scheraga HA, Laskowski M Jr: The fibrinogen-fibrin conversion. Adv Protein Chem 12:1, 1957

Schwartz ML, Pizzo SV, Hill RL, McKee PA: Effect of fibrin-stabilizing factor on the subunit structure of human fibrin. J Clin Invest 50:1506, 1971

Shainoff JR, Dardik BN: Fibrinopeptide B and aggregation of fibrinogen. Science 204:200, 1979

Shainoff JR, Dardik BN: Fibrinopeptide B in fibrin assembly and metabolism: physiologic significance in delayed release of the peptide. Ann N Y Acad Sci 408:254, 1983

Sonder SA, Fenton JW II: Thrombin specificity with tripeptide chromogenic substrates: Comparison of human and bovine thrombins with and without fibrinogen clotting activities. Clin Chem 32:934, 1986

Suenson E, Petersen LC: Fibrin and plasminogen structures essential to stimulation of plasmin formation by tissue-type plasminogen activator. Biochim Biophys Acta 870:510, 1986

Takagi T, Doolittle RF: Amino acid sequence studies on plasmin derived fragments of human fibrinogen: amino-terminal sequences of intermediate and terminal fragments. Biochemistry 14:940, 1975a

Takagi T, Doolittle RF: The amino acid sequences of those portions of human fibrinogen fragment E which are not included in the amino-terminal disulfide knot. Thromb Res 7:813, 1975b

Vali Z, Scheraga HA: Localization of the binding site on fibrin for the secondary binding site of thrombin. Biochemistry 27:1956, 1988

Voskuilen M, Vermond A, Veeneman GH, et al: Fibrinogen lysine residue Aα 157 plays a crucial role in the fibrin-induced acceleration of plasminogen activation, catalyzed by tissue-type plasminogen activator. J Biol Chem 262:5944, 1987

Wolfenstein-Todel C, Mosesson MW: Human plasma fibrinogen heterogeneity: Evidence for an extended carboxylterminal sequence in a normal γ chain variant (γ'). Proc Natl Acad Sci USA 77:5069, 1980

Yonekawa O, Vermond A, Nieuwenhuizen W: Localization of a new site in fibrin, involved in the acceleration of the tissue-type plasminogen activator (t-PA)-catalysed activation of plasminogen, in Matsuda M, Iwanaga S, Takada A, Henschen A (eds): Fibrinogen, Current Basic and Clinical Aspects (Vol 4). Amsterdam, Elsevier, 1990, p 111

CHAPTER 2

HEREDITARY VARIANTS OF HUMAN FIBRINOGENS

Jaap Koopman[1,2] and Frits Haverkate[1]

1) Gaubius Laboratory IVVO-TNO, Leiden, The Netherlands.
2) Department of Hematology, University Hospital, Leiden, The Netherlands.

INTRODUCTION

Congenital dysfibrinogenemia is a disorder of fibrinogen synthesis in which a structural abnormality results in altered functional characteristics of the protein.

More than 250 variant fibrinogens (for recent catalogue see Ebert, 1991) have been detected in patients with congenital dysfibrinogenemia. Variant fibrinogens are valuable tools for structure-function studies in fibrinogen, when both the structural and functional defects are known. They also provide a basis for correlating the molecular defect with the clinical symptoms of affected individuals. The clinical and laboratory features of only those abnormal fibrinogens from which the molecular defect has been determined, will be discussed in the following paragraphs.

Clinical laboratory diagnosis

The majority of patients with a variant fibrinogen have prolonged thrombin clotting times of plasma and the purified fibrinogen, and demonstrate a discrepancy between the functional and immunological fibrinogen concentration. Exceptions to these characteristics have been reported for fibrinogen Oslo I (Brosstad et al., 1983) which has a shortened thrombin time, and fibrinogens Oslo III (Brosstad et al., 1983) and Tokyo I (Samori et al., 1975) with normal thrombin times. However, in the case of Tokyo I, the evidence that this is truly a structurally-altered fibrinogen is very limited. A prolonged thrombin clotting time is usually accompanied by delayed clotting induced by snake venoms such as Reptilase^R and Arvin^R. Five exceptions to this rule have been reported: fibrinogens Chapel Hill VI, Houston, Naples, Pamplona II, and New Orleans II all show a normal Reptilase^R or Arvin^R clotting time. The variants detected so far have a delayed fibrinopeptide release and/or an impaired fibrin polymerization, which explains the prolonged thrombin clotting times. In addition to defective clotting, a large number of the variants demonstrate defects in functions such as calcium, thrombin, t-PA and plasminogen binding, interaction with platelets and endothelial cells and in vivo catabolism. It is conceivable that variants exist which clot normally, but have a defect in one of these other functions in fibrinogen. However, these abnormalities will not be detected by the assays used until now, because these assays depend on a defect that effects the conversion of fibrinogen to fibrin. As this type of variant fibrinogen is of potential interest, new assays need to be developed which can be performed routinely on plasma samples and can detect abnormalities in fibrinogen or fibrin which are not related to clotting.

Analysis of the molecular defect

Structural identification of variant fibrinogens has typically been performed by amino acid sequence analysis of the purified protein. A major improvement on this approach

was the introduction of HPLC methods (Martinelli and Scheraga, 1979; Koehn and Canfield, 1981; Kehl et al., 1982) which facilitated isolation of polypeptide chains after reduction, CNBr cleavage, and plasmic digestion of fibrinogen. The analysis of the fibrinopeptides A and B particularly was improved, which led to rapid characterization of a large number of variants with a defect in the fibrinopeptide A. If the structural defect is not located in the fibrinopeptides, determination of the abnormality by protein sequence analysis is time-consuming and laborious. This is probably the reason why 45 out of 83 known structural defects are confined to the fibrinopeptides. Four years ago DNA sequence analysis of the genes coding for variant fibrinogens was introduced (Koopman et al., 1988). The development of the polymerase chain reaction (Saiki et al., 1988) makes it possible to select and amplify parts of the genomic DNA (100-5000 bp) within a few hours, using synthetic oligonucleotides and the heat resistant Taq DNA polymerase. The amplified genomic DNA fragment can be subjected to most of the known DNA techniques, such as cloning, sequence analysis, restriction analysis and Southern blotting. The most important advantages of DNA analysis as opposed to protein analysis are:

- DNA sequence analysis is more rapid and easier to perform.
- Homo- or heterozygosity for the defect can be easily determined.
- Once the defect at the DNA level is known, available family members can be screened to determine whether there is a relationship between the presence of the defect and the clinical symptoms in the family.
- All genetic analyses can be performed using the genomic DNA isolated from less than one millilitre of blood.

However, once the defect at the DNA level is known, protein analysis is compulsory to determine the functional abnormality, to detect possible post-translational modification, and to confirm the presence of the variant protein in the plasma of the affected individual.

Structure-function relationships in variant fibrinogens

To extrapolate the information obtained with variant fibrinogens to structure-function relationships in the normal fibrinogen molecule, it is important to know whether the functional abnormalities are primary or secondary to the structural defect (Lane et al., 1983; Lane and Southan, 1987). The importance of this is illustrated by the studies on Chapel Hill II (Carell and McDonagh, 1982) which initially demonstrated impaired fibrin formation, and defective interaction with thrombin, factor XIIIa and plasmin. The structural defect in this variant was found to be a heterozygous Aα Arg^{16} → His substitution (Henschen et al., 1984), demonstrating that the observed functional defects were secondary to the delayed release of FpA. Furthermore, some types of mutations can induce post-translational modification which are not present in the normal molecule.

These post-translational modifications can induce functional abnormalities which are secondary to the observed structural defects in these variants, as will be discussed below. The intriguing question of whether heterozygous abnormal fibrinogens contain homodimers (i.e., both chains in one fibrinogen molecule contain the defect) or heterodimers (i.e., one defective chain and one normal chain in a single fibrinogen molecule) or both, will also be discussed.

VARIANTS WITH A DEFECTIVE RELEASE OF FIBRINOPEPTIDES

The thrombin-catalyzed release of fibrinopeptide A (FpA) and B (FpB) is the first step in the conversion of fibrinogen into the insoluble fibrin matrix. An important domain of fibrinogen which interacts with the catalytic site of thrombin is thought to be located in the Aα-chain between amino acids 1 and 23 (Hogg and Blombäck, 1978; Marsh et al., 1983; Ni et al., 1989a, 1989b; Lord et al., 1990). Forty-eight variants with a mutation in this domain of the Aα-chain have been identified, of which forty-seven show a decreased FpA release (Table 1).

The most amino-terminal mutation in the Aα-chain is found in fibrinogen Lille (Aα $Asp^{7} \rightarrow Asn$), with delayed FpA release and prolonged thrombin and $Reptilase^{R}$ clotting times. In fibrinogen Rouen (Aα $Gly^{12} \rightarrow Val$), the Aα Arg^{16}-Gly^{17} bond in the defective Aα-chain was hydrolyzed much slower by thrombin than in normal Aα-chain, resulting in a decreased release of FpA by thrombin and impaired clotting. Two-dimensional NMR studies, on synthetic oligopeptides demonstrated that Aα Val^{12} disrupts a type-II β turn structure in the oligopeptide, which affects the positioning of the Aα Arg^{16}-Gly^{17} peptide bond in the active centre of thrombin (Ni et al., 1989c).

The most abundant defect found in fibrinogen is the substitution of the arginine at position 16 in the Aα-chain by histidine (Aα $Arg^{16} \rightarrow$ His). This defect was first reported in fibrinogen Petoskey and is currently known in 25 other unrelated abnormal fibrinogens (Table 1). Fibrinogens Bicêtre and Giessen I are homozygous cases of this type of dysfibrinogenemia. Enzyme kinetic studies of heterozygous cases (Higgins et al., 1983, Southan et al., 1983, 1985) have demonstrated that the abnormal AαHis^{16}-Gly^{17} bond is hydrolyzed much more slowly by thrombin than is the normal Arg^{16}-Gly^{17} bond. As expected, the fibrin polymerization is not influenced by this defect, when fibrin monomers are used from which normal FpA and His^{16}FpA are removed (Lane et al., 1983).

Substitution of the Aα Arg^{16} by Cys is also a frequently occurring mutation in abnormal fibrinogens. This defect was first reported in fibrinogen Metz, in which the propositus was homozygous for the defect and subsequently in 13 other abnormal fibrinogens (Table 1). The Aα Cys^{16}-Gly^{17} bond is not hydrolysed by thrombin. However,

if the substituted Cys is s-aminoethylated, the variant FpA, with an abnormal retention time on reverse-phase HPLC, is released. Although fibrinogen Metz and the heterozygous cases either release no FpA or only 1 mol of FpA per mol fibrinogen, respectively, they all released 2 mol of FpB per mol fibrinogen, indicating that removal of FpA is not essential for the release of FpB. However, the reduced FpB release observed in a number of cases with a structural defect in the FpA moiety, suggests that at low thrombin concentrations the FpB release depends on the FpA release. This finding supports the sequential model of fibrinopeptide release by thrombin (Blombäck et al., 1978). At high thrombin concentrations the release of FpB appears to be independent of FpA release (Southan et al., 1983, 1985), indicating that the reduced FpB release at low thrombin concentrations is secondary to the structural defect. Furthermore, the observed impairment of fibrin polymerization in the variants discussed above, is also a secondary functional abnormality, induced by the reduced release of FpA (Lane et al., 1983).

Fibrinogen Detroit was the first variant fibrinogen in which the structural defect was determined (Blombäck et al., 1968). The patient was found to be homozygous for an amino acid substitution in the Aα-chain of fibrinogen, replacing the arginine at position 19 by a serine (Aα Arg^{19} → Ser). The rate of total fibrinopeptide release by thrombin was reduced, and from indirect evidence it was concluded that the FpA release was normal, while the FpB release was delayed. However, a comparative study of fibrinogen Aarhus (Aα Arg^{19} → Gly) and fibrinogen Detroit, showed that the FpA and FpB release of both variants was slower than that of normal fibrinogen (Blombäck et al., 1988). In line with this observation, fibrinogen Mannheim I (Aα Arg^{19} → Gly) containing the same mutation as fibrinogen Aarhus, also demonstrated a delayed release of FpA by thrombin (Dempfle et al., 1990). Also the reputed amino acid substitution Aα Arg^{19} → Asn in fibrinogen Munich I (also known as München) is associated with delayed FpA release (Ebert, 1991). The amino acid substitution in fibrinogen Munich I requires the substitution of two bases in the Aα-chain gene (Kant et al., 1983), which makes gene analysis of this variant highly desirable.

All known variant fibrinogens with a mutation in the FpA segment of the Aα-chain (Table 1), demonstrate an impaired release of FpA, indicating that the integrity of this segment is essential for interaction with the catalytic domain of thrombin. Furthermore, four variants with a substitution of Aα Arg^{19} also demonstrate reduced release of FpA, indicating that this amino acid is part of the substrate binding site for thrombin.

The release of FpB by thrombin from fibrinogen is accomplished by hydrolysis of the Bβ Arg^{14}-Gly^{15} bond. In four variant fibrinogens, single amino acid substitutions, affecting this thrombin cleavage site in the Bβ-chain, have been identified (Table 1). The variant fibrinogens Christchurch II, Seattle I and IJmuiden contained a Bβ Arg^{14} → Cys

substitution. Christchurch II and Seattle I were determined by protein sequence analysis, while IJmuiden was analyzed at the DNA level. All three variants demonstrated a normal FpA release and a reduced FpB release by thrombin, with a maximum FpB release of 1 mol per mol fibrinogen. Fibrinogen Ise was identified as a Bβ $Gly^{15} \rightarrow Cys$ substitution. Measurement of FpB release at low thrombin concentrations demonstrated that half of the FpB peptides were released from fibrinogen Ise.

The results obtained with these four variants clearly show that alteration of the thrombin cleavage site in the Bβ-chain completely prevents or strongly reduces the release of FpB by thrombin, without affecting the release of FpA.

Fibrinogen New York I was identified as a variant with a 64 amino acid deletion in the Bβ-chain from amino acid 9 to 72. The deleted amino acid segment corresponds exactly to the exon II part of the human fibrinogen Bβ gene (Chung et al., 1983), genetic analysis of this variant could reveal whether the deletion is present in the genomic DNA or whether alternative splicing is induced by a more subtle mutation. The release of both FpA and FpB was reduced, with a maximum release of 2 mol FpA, and 1 mol FpB per mol fibrinogen. The thrombin binding to fibrin, fibrin polymerization and acceleration of t-PA induced plasminogen activation in fibrin(ogen) New York I were reported to be impaired, these functional abnormalities will all be addressed in the appropriate paragraphs. Fibrinogen Naples, a homozygous variant with a Bβ $Ala^{68} \rightarrow Thr$ substitution showed a reduced release of FpA and FpB by thrombin, but a normal FpA release by Reptilase^{R}. This indicates that the structural defect in this variant does not affect the FpA cleavage site. The decreased release of FpA in Naples, is probably due to the defective interaction of thrombin by its anionic binding exosite to the variant fibrin(ogen), as will be discussed below.

VARIANTS WITH IMPAIRED FIBRIN POLYMERIZATION

Fibrin monomer polymerization arises from the interaction of polymerization sites on different molecules. Polymerization sites located in the amino terminal part of the molecule are exposed by thrombin cleavage of fibrinopeptides from the Aα and Bβ chains; these sites are designated "A" and "B" respectively (Kudryk et al., 1974). Complementary polymerization sites to the "A" and "B" sites, designated "a" and "b", are located in the carboxyl terminal region of the fibrinogen molecule (Kudryk et al., 1974). Assembly of the mature, three-dimensional fibrin matrix starts with the formation of double-stranded fibrils (Shainhoff and Dardik, 1979, 1983; Olexa and Budzynski, 1980; Olexa et al., 1981; Budzynski et al., 1983) followed by branching and lateral association of these fibrils (Carr et al., 1977; Hantgan and Hermans, 1979; Mosesson, 1990). The initial double stranded fibril formation is predominantly due to interaction between the

amino terminal "A" site and its complementary "a" site in the carboxyl terminal region (Olexa and Budzynski, 1980).

Structural defects in 35 variant fibrinogens with impaired fibrin polymerization have been identified. Eleven of these variants contain structural defects in the amino terminal domain of fibrinogen. Fibrinogen Kyoto II was identified as an abnormal fibrinogen with an Aα Pro^{18} → Leu substitution. Surprisingly, both the FpA and FpB release by thrombin were normal, while the fibrin polymerization was impaired. The variant fibrinogens Aarhus, Mannheim, Detroit and Munich demonstrated a defective fibrin polymerization in addition to the reduced rate of fibrinopeptide release. The impaired fibrin polymerization in these five variants indicates that the Aα Pro^{18} and Arg^{19} are part of the amino terminal polymerization site, exposed after FpA is removed. These results are supported by fibrin polymerization and fibrinogen binding studies with synthetic peptide analogs of the amino acid stretch 17-20 in the α-chain (Laudano et al., 1983).

Surprisingly, the Reptilase^R clotting times in plasma of Christchurch II, Seattle I and IJmuiden, with a Bβ Arg^{14} → Cys substitutions, and Nijmegen with a Bβ Arg^{44} → Cys substitution, were delayed. The fibrin polymerization of purified fibrinogens IJmuiden and Nijmegen after removal of FpA by Reptilase^R was impaired, indicating that the structural defects affected the "A" polymerization site, exposed after removal of FpA. These observations correlate well with the results of Siebenlist et al. (1990), who demonstrated that fibrinogen lacking the Bβ 1-42 segment polymerized much more slowly than did intact fibrinogen after cleavage of FpA by Reptilase^R. However, fibrinogen Ise (Bβ Gly^{15} → Cys) demonstrated a normal fibrin polymerization after removal of FpA by Reptilase^R and an impaired fibrin polymerization after removal of FpA and FpB by thrombin (Yoshida et al., 1991), indicating that the mutation affects the "B" polymerization site, exposed after removal of FpB by thrombin. This suggests that the amino acids Bβ Arg^{14} and Arg^{44} contribute to the "A" polymerization site, whereas Bβ Gly^{15} contributes to the "B" polymerization site. However, the mutations in these five variants create an additional cysteine residue in the variant fibrinogen molecule, which can result in unexpected post-translational modifications, as will be discussed for fibrinogens IJmuiden and Nijmegen. Therefore, the observed polymerization defect in IJmuiden and Nijmegen could be induced by the post-translational modifications and not by the substitution of amino acids crucial for the integrity of the "A" polymerization site. For the same reason, a specific interpretation of the observed impaired fibrin polymerization in fibrinogen New York I (Bβ 9-72 deletion) is virtually impossible.

The "a" and "b" polymerization sites, which are complementary to the "A" and "B" sites, are located in the carboxyl terminal domain of fibrinogen, designated as D domain. Structural defects in this part of the fibrinogen molecule have been identified in 24 variants with impaired fibrin polymerization and a normal release of the fibrinopeptides.

The variants with a γ Arg^{275} → His or a γ Arg^{275} → Cys substitution (Table 1) all

demonstrated a defective fibrin polymerization, after removal of FpA by Reptilase^{R} or FpA and FpB by thrombin. The structural defect in fibrinogen Baltimore I, elucidated by protein sequence analysis and confirmed by gene analysis, was identified as a γ Gly^{292} → Val substitution. Previously reported abnormal release of FpA (Mosesson and Beck, 1969) measured by paper electrophoresis, could not be confirmed by recent HPLC analysis of the fibrinopeptide release (Bantia et al., 1990a), which demonstrated a normal release of both FpA and FpB. The only functional defect reported was impaired fibrin polymerization. Eight other variants with a structural defect in the carboxyl terminus of the γ-chain have been identified (Table 1), all demonstrating an impaired fibrin polymerization. In the variants Vlissingen (γ 319-320 deletion) and Osaka V (γ Arg^{375} → Gly) the impairment of fibrin polymerization was less pronounced or was completely absent at calcium concentrations of 2-5 mM. In addition to the impaired fibrin polymerization, both variants demonstrated a reduced binding of calcium ions, as will be discussed below. Since the presence of calcium is known to increase fibrin polymerization (Ratnoff and Potts, 1954) by a number of mechanisms (Laudano and Doolittle, 1981; Häberli et al., 1987), the loss of the high-affinity calcium binding site in the D domain of fibrinogens Vlissingen and Osaka V, may fully account for the altered fibrin polymerization. However, the impaired fibrin polymerization of fibrinogen Vlissingen in the absence of calcium indicates that the mutation also affects the calcium-independent polymerization. Fibrinogen Asahi (γ Met^{310} → Thr) demonstrated a γ-chain variant with a higher molecular weight, due to an additional glycosylation of the mutated chain, which will be discussed in detail in the paragraph dealing with post-translational modifications.

The five variants with a γ Arg^{275} → Cys substitutions, Baltimore I (γ Gly^{292} → Val), Baltimore III (γ Asn^{308} → Ile), Kyoto I (γ Asn^{308} → Lys), Vlissingen (γ 319-320 deletion), Nagoya (γ Gln^{329} → Arg), Kyoto III (γ Asp^{330} → Tyr), Milan (γ Asp^{330} → Val) and Osaka V (γ Arg^{375} → Gly) demonstrated an altered electrophoretic mobility of the mutant γ-chain on SDS-PAGE according to Laemmli, but a normal mobility on SDS-PAGE according to Weber and Osborn. Such unexpected changes in mobility are thought to be due to changes in hydrophobicity or local conformation of the mutant polypeptide, which affect the electrophoretic mobility of the mutant protein-SDS complex (de Jong et al., 1978). Therefore, relatively large changes in electrophoretic mobility on SDS-PAGE according to Laemmli, of mutant proteins do not necessarily mean that a substantial deletion or insertion has occurred in the mutant protein. Moreover, the Laemmli SDS-PAGE method was proposed as a screening method for the detection of variant fibrinogens (Yoshida et al., 1988b). The data obtained from the functional studies on the 24 variants with a structural defect in the carboxyl terminal domain of fibrinogen, clearly demonstrate the essential role of this segment in fibrin polymerization.

Fibrinogen Pontoise (Bβ Ala^{335} → Thr) is the only variant fibrinogen with a known structural defect in carboxyl terminus of the Bβ-chain. The release of the fibrinopeptides

was normal, whereas the fibrin polymerization was impaired. The mutation in fibrinogen Pontoise gave rise to an increased molecular weight of the mutated chain (Haverkate et al., 1978), due to a post-translational modification related to glycosylation, as will be discussed below. However, this post-translation modification in fibrinogen Pontoise does not allow conclusions to be drawn about the role of the carboxyl-terminal β-chain in fibrin polymerization.

It has been known for many years that fibrin polymers formed from fibrinogen lacking the carboxyl-terminal region of Aα-chains develop less turbid clots (Mosesson et al., 1967), suggesting that fibers in the clot matrix are reduced in thickness. Impaired fibrin polymerization was determined in four variants with structural defects located in the carboxyl terminal region of the α-chain. Fibrinogen Lima was identified as a homozygous variant fibrinogen with a Aα Arg^{141} → Ser substitution. Although the fibrin polymerization was impaired, the plasmin degradation product D_1 derived from fibrinogen Lima, containing the structural defect, inhibited normal fibrin polymerization in a normal fashion. This indicated that the polymerization site in the D domains of fibrinogen Lima, involved in the D:E interaction, leading to double-stranded protofibrils, functioned normally. Initial fibril formation, predominantly due to interaction between the "A" and "a" sites, is followed by branching and lateral association of these fibrils, which is more dependent on the interaction between the "B" and "b" sites (Olexa et al., 1980; Mosesson, 1990). The impaired fibrin polymerization of fibrinogen Lima is therefore most likely to be related to the lateral association of the protofibrils. An Aα Ser^{434} → Asn substitution was identified in the heterozygous fibrinogen Caracas II. Fibrinogen Marburg was homozygous for a single base substitution (A → T), which changes the codon Aα 461 AAA (Lys) to TAA (Stop) resulting in a deletion of amino acids Aα 461 to 610.

Fibrinogen Dusart (Paris V) is a heterozygous variant fibrinogen with an Aα Arg^{554} (CGT) → Cys (TGT) substitution. The fibrin fibers formed from fibrinogen Dusart were much thinner than those from normal fibrin (Koopman et al., 1991e),indicating that the defect impairs the lateral association of fibrin fibers. The functional studies on these four variants suggest an important role for the carboxyl terminal part of the Aα-chain in fibrin polymerization. More specifically, the results obtained with fibrinogens Lima and Dusart suggest that the impaired polymerization is due to a defective lateral association of the double-stranded fibrils. These results are in conjunction with studies which provided direct evidence that this region of the Aα-chain plays a critical role in the process of lateral fibril association, and constitutes all or part of the so-called "b" polymerization site in the fibrin molecule (Hasegawa and Sasaki, 1990). However, all four variants discussed above are subject to post-translational modification, related to glycosylation or formation of disulfide complexes, and this calls for a cautious interpretion of the structure-function relationship in these variants.

VARIANTS WITH A DEFECTIVE INTERACTION OF COAGULATION AND FIBRINOLYTIC ENZYMES

In addition to its role of providing the structural frame work of a thrombus, fibrin displays several properties that enable it to regulate its own formation and degradation. The mechanisms involved in these regulatory functions of fibrin, are based on the interaction of fibrin with different enzymes and inhibitors that play a role in the coagulation and fibrinolytic processes. One of these interactions is the binding of α-thrombin to fibrin by a site independent of the catalytic site (Liu et al., 1979; Fenton II et al., 1981; Kaminski and McDonagh, 1983, 1987; Berliner et al., 1985). The domain in fibrin to which this site binds, is located in the amino-terminal part of the molecule (Fenton II et al., 1988, Vali and Scheraga, 1988, Kaczmarek and McDonagh, 1988).

The fibrin formed from fibrinogen New York I (Bβ 9-72 deletion) demonstrates that the fibrin binding of thrombin was reduced to approximately half that of normal fibrin (Liu et al., 1985). The large deletion in fibrinogen New York I is likely to have multiple effects on the defective fibrinogen and fibrin structure, particularly because Bβ Cys^{65}, which normally forms a disulfide bond with Aα Cys^{36}, is missing. Therefore, a specific interpretation of the structure-function relationships in this variant is troublesome. Another variant with reduced thrombin binding to fibrin is fibrinogen Naples (previously called Milano II, Haverkate et al., 1987), which was identified as a homozygous Bβ 68 Ala(GCT) → Thr(ACT) substitution by analysis of the patients DNA. Binding of active-site inhibited α-thrombin to fibrin formed by $Reptilase^{R}$ from fibrinogen Naples was reduced to less then 10% of normal fibrin. The previously-discussed impaired release of fibrinopeptides by thrombin is most likely to be related to the defective binding of thrombin to fibrin Naples. Without information about the three-dimensional structure of the amino terminal part of fibrin(ogen), it is difficult to determine the exact influence of the Bβ Ala^{68} → Thr substitution on the binding to thrombin. One possible explanation is, that Bβ Ala^{68} participates in a non-polar interaction with thrombin and that this is disrupted by threonine. Alternatively, the slightly larger side chain of threonine could lead to incorrect folding or disulfide bond formation of this part of the fibrinogen molecule, preventing thrombin binding. Whatever the explanation, the functional studies on Naples and New York I indicate that the integrity of the amino-terminal domain of fibrin(ogen) is essential for an effective interaction with thrombin.

After the fibrin polymer has been formed, stabilization of the fibrin occurs by the formation of isopeptide bonds between the γ-chains and α-chains of adjacent fibrin molecules. This calcium dependent reaction is catalyzed by activated factor XIII. Fibrinogen Asahi (γ Met^{310} → Thr) is the only variant in which the mutant γ-chains demonstrate a retarded crosslinking by factor XIIIa (Yamazumi et al., 1989). When fibrinogen was used as a substrate for factor XIII, instead of polymerized fibrin, the

impairment was still present. This indicates that the defective fibrin polymerization, as observed in this variant, could not fully account for the impaired γ-chain crosslinking. These results suggest that the mutation in fibrinogen Asahi inhibits an effective interaction with factor XIIIa. Whether this functional abnormality is due to the extra glycosylation in the mutant γ-chain cannot yet be deduced.

The fibrin clot has only a temporary role, and needs to be removed once it has fulfilled its function. The degradation of the fibrin matrix is initiated by activation of the fibrinolytic system, which is controlled by various regulatory mechanisms to assure efficient and restricted degradation of the fibrin clot. Fibrin participates in these regulatory mechanisms by the binding of key components in the fibrinolytic system such as tissue plasminogen activator (t-PA) and plasminogen. The activation of plasminogen by t-PA is greatly enhanced in the presence of fibrin (Rånby, 1982; Hoylaerts et al., 1982). This is most likely due to ternary complex formation between fibrin, t-PA and plasminogen.

Two variant fibrinogens, of which the derived fibrin demonstrates a reduced binding of t-PA and a decreased stimulatory effect on t-PA induced plasminogen activation, are New York I (Bβ 9-72 deletion) (Liu et al., 1986) and Nijmegen (Bβ Arg^{44} → Cys) (Engesser et al., 1988). Detailed analysis of t-PA binding to fibrin Nijmegen revealed that the kringle 2 mediated binding to fibrin was impaired. A reduced binding of plasminogen (Soria et al., 1983) was detected in fibrin formed from fibrinogen Dusart (Aα Arg^{554} → Cys) resulting in a decreased stimulatory effect on t-PA induced plasminogen activation (Lijnen et al., 1984). Fibrinogens Mannheim I (Aα Arg^{19} → Gly) and IJmuiden (Bβ Arg^{14} → Cys) also demonstrated a decreased t-PA induced plasminogen activation in the presence of the variant fibrins as compared with normal fibrin (Heene et al., 1989; Koopman et al., 1986). These functional abnormalities could be directly related to the structural defect, or to the impairment of fibrin polymerization observed in all five variants. The latter explanation is supported by reports demonstrating that inhibition of fibrin polymerization reduces the stimulatory effect of fibrin on plasminogen activation (Suenson et al., 1986; Koopman et al., 1986; Soria et al., 1986). Whether the fibrin polymerization is essential to the binding of t-PA or plasminogen, or to the formation of the ternary complex, remains to de determined. Furthermore, the variants Dusart, Nijmegen and IJmuiden contain post-translational modifications which could alter the binding and stimulatory capacity of the fibrin.

Increased sensitivity to plasmin degradation was reported for fibrinogen Kyoto I with a γ Asn^{308} → Lys substitution. The mutation resulted in accelerated cleavage of the γ Lys^{356}-Ala^{357} and γ Lys^{302}-Phe^{303} bonds by plasmin, in addition to the generation of a new plasmin cleavage site between γ Lys^{308} and Gly^{309} in the presence of EDTA.

VARIANTS DEMONSTRATING A DEFECTIVE INTERACTION WITH CALCIUM IONS

Human fibrinogen binds three calcium ions per molecule with high affinity at pH 7.5. Each D domain contains one high affinity binding site for calcium ions (Lindsey et al., 1978; Nieuwenhuizen et al., 1979, 1981). Plasmin degradation of the carboxyl-terminal region of the γ-chain results in the loss of calcium binding (Nieuwenhuizen and Haverkate, 1983). On the other hand, binding of calcium ions at this site protects the carboxyl-terminal γ-chain from plasmin degradation (Haverkate and Timan, 1977). Plasmin degradation of normal fibrinogen in the presence of calcium ions results in the formation of fragment D_1, with the carboxyl terminal γ-chain intact, in the absence of calcium ions fragment D_3 is formed with a degraded carboxyl-terminal γ-chain (Haverkate and Timan, 1977).

In the variants Vlissingen (γ 319-320 deletion) and Osaka V (γ Arg^{375} → Gly) a reduced number of high-affinity calcium binding sites was determined (Koopman et al., 1991b; Yoshida and Matsuda, 1990). The number of calcium binding sites in both variants was reduced to two, whereas normal fibrinogen contains three, which is consistent with the heterozygous nature of these two variants. The protective effect of calcium ions on the plasmin degradation of the carboxyl-terminal region of the γ-chain was only partially present in fibrinogens Vlissingen and Osaka V. These results indicate that the structural defects in fibrinogen Vlissingen and Osaka V perturbes the high-affinity calcium binding site in the D domains of fibrinogen, resulting in a decreased protection of the γ 302-303 peptide bond against plasmin degradation. Fibrinogen Haifa (γ Arg^{275} → Cys) also demonstrated a reduced protective effect of calcium against plasmin degradation of the carboxyl-terminal γ-chain (Soria et al., 1987b), although the number of high-affinity calcium binding sites was normal. However, fibrinogen Saga, containing the same mutation as fibrinogen Haifa, showed a normal protective effect of calcium on plasmin degradation (Yamazumi et al., 1988). No explanation has yet been found for the discrepancy between the results obtained with fibrinogen Haifa, and those obtained with fibrinogens Vlissingen, Osaka V and Saga, although reappraisal of the plasmin digest on fibrinogen Haifa seems necessary.

VARIANTS WITH A DEFECTIVE INTERACTION WITH CELLS

In addition to being a precursor of fibrin, fibrinogen also binds to several cell types, of which platelets and endothelial cells are the best studied. This binding is mediated through the interaction of a fibrinogen-specific receptor on the membrane of these cells (Hynes, 1987) with distinct sites on the fibrinogen molecule.

A diminished platelet aggregation support was reported for fibrinogen Stony Brook I (Aα Arg^{16} → Cys), which was thought to be due to the inaccessibility of the binding sites for platelet fibrinogen receptors on the abnormal fibrinogen. The binding of endothelial cells to immobilized fibrinogen Marburg (Aα Lys^{461} → Stop) was almost completely abolished as compared with normal fibrinogen. This is in agreement with studies that have demonstrated that the RGD containing site, Aα 572-574, which is lacking in the homozygous fibrinogen Marburg, is essential for endothelial cell binding (Cheresh et al., 1989). Recently, it has been shown, that the carboxyl-terminal RGD sequence in the Aα-chain is not involved in platelet binding to fibrinogen (Peerschke, 1988; Cheresh et al., 1989). This suggests that platelets and endothelial cells interact with distinct sites on fibrinogen, and do not necessarily compete with each other when binding to fibrinogen.

VARIANTS WITH POST-TRANSLATIONAL MODIFICATIONS

Size and charge differences in normal fibrinogen are mostly related to post-translational modification such as glycosylation, phosphorylation or proteolytic degradation. A number of the variant fibrinogens discussed above, demonstrate post-translational modifications which are not present in normal fibrinogen, and are induced by the structural defect in these variants. Fibrinogen Asahi was the first variant fibrinogen in which an abnormal post-translational modification was identified (Yamazumi et al., 1989). In addition to the amino acid substitution (γ Met^{310} → Thr), N-glycosylation at γ Asn^{308} was also detected. This extra-glycosylation is due to a newly formed consensus sequence for N-glycosylation (Asn-X-Thr) at the mutation site. The oligosaccharides attached to γ Asn^{308} were identical to those attached to normal glycosylation sites in the Bβ and γ-chain of fibrinogen. The same type of abnormal glycosylation was found in fibrinogen Pontoise (Bβ Ala^{335} → Thr) where the oligosaccharide was attached to Bβ Asn^{333}. Fibrinogen Caracas II (Aα Ser^{434} → Asn) also contained an extra oligosaccharide at the Asn residue introduced by the mutation. The mutation in fibrinogen Lima (Aα Arg^{141} → Ser) gave rise to a new N-glycosylation site at Aα Asn 139. This Asn-X-Ser type sequence is unique for fibrinogen, as the consensus sequences for N-glycosylation in normal and abnormal fibrinogens were all of the Asn-X-Thr type.

Another type of post-translational modification involves the formation of disulfide-linked complexes between the variant molecule and other protein(s). This phenomena was first described for the anti-thrombin III variant Northwick Park with an Arg^{393} → Cys mutation (Erdjument et al., 1987, 1988), in which the Cys^{393}, introduced by the mutation, formed a disulfide bond with the free sulfydryl group in albumin (Dugaiczyk et al., 1982). These type of modifications have been detected in fibrinogens IJmuiden (Bβ Arg^{14} → Cys), Nijmegen (Bβ Arg^{44} → Cys) and Dusart (Aα Arg^{554} → Cys) and are due to the

introduction of an additional cysteine residue in the variant fibrinogen. The complex between the variant fibrinogen and albumin was predominant in both purified fibrinogen and plasma. Quantitative analysis of these complexes in plasma of fibrinogens IJmuiden and Nijmegen demonstrated that 20% and 13%, respectively, of the total plasma fibrinogen was disulfide-linked to albumin. In addition to the albumin-linked complexes, high molecular weight complexes, probably consisting of disulfide-linked fibrinogen dimers, were also present, at levels ranging from 5 to 7% of the total plasma fibrinogen concentration. Furthermore, both variants were found to contain significant amounts of free sulfydryl groups. Disulfide-linked fibrinogen-albumin complexes were also detected in the plasma of fibrinogen Marburg (Aα Lys^{461} → Stop). The deletion of the Aα 461-610 segment disrupts normal disulfide bond formation because this segment contains Aα Cys^{472}, which normally forms an intra-chain disulfide bond with Aα Cys^{442} (Henschen et al., 1983). Fibrinogen Osaka II (γ Arg^{275} → Cys) was linked to a free cysteine molecule as evidenced by fast atom bombardment mass spectrometry (Terukina et al., 1988).

The post-translational modifications related to glycosylation and disulfide complex formation with other proteins, can influence the functions of the domain in which the structural defect is located. However, functions residing in domains apart from the mutated domain, can also be affected, severely complicating structure-function analysis of these variant fibrinogens. On the other hand, variants with post-translational modifications usually demonstrate an altered electrophoretic mobility on SDS-PAGE gels, which can facilitate the detection of variant fibrinogens with a normal plasma clotting time. Because of the dimeric structure of fibrinogen, heterozygous variants can contain homodimer molecules, with both chains mutated in one fibrinogen molecule, or heterodimers, containing one mutated and one normal chain or both.

Fibrinogen Louisville (Aα Arg^{16} → His) was found to contain only homodimeric species (Galanakis et al., 1983). However, studies on fibrinogen Birmingham, with the same mutation as Louisville, revealed the presence of heterodimeric molecules (Siebenlist et al., 1988). Data reported on fibrinogen Kawaguchi and Osaka I (Aα Arg^{16} → Cys) demonstrate the existence of homodimers in these variants (Miyata et al., 1987), due to an extra interchain disulfide bond between the two mutated Aα-chains. Similarly, fibrinogen Seattle I (Bβ Arg^{14} → Cys) did not contain heterodimeric molecules (Pirkle et al., 1987). In fibrinogen IJmuiden (Bβ Arg^{14} → Cys) evidence for an extra interchain disulfide bond between two mutated Bβ-chain was found (Koopman et al., 1991c), suggesting the presents of homodimers. However, the fibrinogen-albumin complexes in this variant consisted of one fibrinogen molecule linked to one albumin molecule, indicating that only one additional cysteine residue was present in these variant species, suggesting heterodimers. From the data summarized above, it is clear that the occurrence of homo- or heterodimers depends on the type of defect and the consequent synthesis and assembly of the fibrinogen molecule.

Table 1

Name	Structural defect	Functional defect(s)	Clinical symptoms	References
Lille	Aα Asp^{7}→ Asn	FpA release	Asymptomatic	Denninger et al., 1978, Morris et al., 1981
Rouen	Aα Gly^{12}→ Val	FpA release	Bleeding	Kehl et al., 1983
Amsterdam I	Aα Arg^{16}→ Cys	FpA release	Asymptomatic	Janssens & Vreeken, 1971; Henschen et al., 1984
Bergamo I	Aα Arg^{16}→ Cys	FpA release	Asymptomatic	Reber et al., 1985
Geneva	Aα Arg^{16}→ Cys	FpA release	Asymptomatic	Furlan et al., 1990
Hershey II	Aα Arg^{16}→ Cys[2)]	FpA release	Thrombosis	Galanakis et al., 1990a
Hamburg II	Aα Arg^{16}→ Cys	FpA release	Asymptomatic	Wenzel et al., 1990
Hamburg III	Aα Arg^{16}→ Cys	FpA release	Bleeding	Wenzel et al., 1990
Kawaguchi	Aα Arg^{16}→ Cys	FpA release	Asymptomatic	Matsuda et al., 1985b; 1986
Ledyard	Aα Arg^{16}→ Cys	FpA release	Bleeding	Lee et al., 1990
Leogan	Aα Arg^{16}→ Cys[2)]	FpA release	Asymptomatic	Galanakis et al., 1990a
Metz	Aα Arg^{16}→ Cys	FpA release	Bleeding	Soria et al., 1982
Osaka I	Aα Arg^{16}→ Cys	FpA release	Asymptomatic	Matsuda et al., 1986
Schwarzach	Aα Arg^{16}→ Cys	FpA release	Asymptomatic	Henschen et al., 1983
Stoney Brook I	Aα Arg^{16}→ Cys	FpA release, platelet aggregation	Asymptomatic	Galanakis et al., 1989
Torino	Aα Arg^{16}→ Cys	FpA release	Asymptomatic	Reber et al., 1987
Zürich I	Aα Arg^{16}→ Cys	FpA release	Asymptomatic	Von Felten et al., 1969; Southan et al., 1982
Amiens I	Aα Arg^{16}→ His	FpA release	Not reported	Soria et al., 1987a
Amiens II	Aα Arg^{16}→ His	FpA release	Not reported	Soria et al., 1987a
Barcelona II	Aα Arg^{16}→ His	FpA release	Bleeding	Borrell et al., 1990
Bergamo III	Aα Arg^{16}→ His	FpA release	Asymptomatic	Reber et al., 1987
Bern II	Aα Arg^{16}→ His	FpA release	Asymptomatic	Rupp et al., 1983
Bicêtre	Aα Arg^{16}→ His[1)]	FpA release	Not reported	Henschen et al., 1983
Birmingham	Aα Arg^{16}→ His	FpA release	Bleeding	Siebenlist et al., 1988
Chapel Hill II	Aα Arg^{16}→ His	FpA release	Bleeding	Carell & McDonagh, 1982; Henschen et al., 1984
Clermont-Ferrand	Aα Arg^{16}→ His	FpA release	Not reported	Henschen et al., 1984
Giessen I	Aα Arg^{16}→ His[1)]	FpA release	Bleeding	Alving & Henschen, 1987
Kendal	Aα Arg^{16}→ His	FpA release	Not reported	Lane et al., 1987
Leitchfield	Aα Arg^{16}→ His	Not analyzed	Not reported	Henschen et al., 1984
Long Beach	Aα Arg^{16}→ His	Not analyzed	Not reported	Pirkle et al., 1987a
Louisville	Aα Arg^{16}→ His	FpA release	Bleeding	Galanakis et al., 1983
Manchester	Aα Arg^{16}→ His[2)]	FpA release	Asymptomatic	Lane et al., 1980; Southan et al., 1983; Koopman et al., 1988
Milan VI	Aα Arg^{16}→ His	Not analyzed	Bleeding	Bögli et al., 1990
New Albany	Aα Arg^{16}→ His	FpA release	Asymptomatic	Henschen et al., 1983
Paris VI	Aα Arg^{16}→ His	Not analyzed	Not analyzed	Southan et al., 1987
Petoskey	Aα Arg^{16}→ His	FpA release	Asymptomatic	Higgins & Shafer, 1981
Sapporo	Aα Arg^{16}→ His	FpA release	Asymptomatic	Asakura et al., 1989
Seattle II	Aα Arg^{16}→ His	FpA release	Asymptomatic	Ebert et al., 1986
Sheffield	Aα Arg^{16}→ His	Not analyzed	Bleeding	Southan et al., 1987
Stony Brook II	Aα Arg^{16}→ His	FpA release	Bleeding	Galanakis et al., 1990b
Sydney I	Aα Arg^{16}→ His	FpA release	Asymptomatic	Lane et al., 1982; Southan et al., 1985
Sydney II	Aα Arg^{16}→ His	FpA release	Not reported	Henschen et al., 1983; Southan et al., 1985
White Marsh	Aα Arg^{16}→ His	FpA release	Not reported	Qureshi et al., 1983; Carr & Qureshi, 1987
Kyoto II	Aα Pro^{18}→ Leu	Fibrin polymerization	Not reported	Matsuda et al., 1990
Munich I	Aα Arg^{19}→ Asn	FpA release, fibrin polymerization	Bleeding	Southan et al., 1982; Ebert, 1991
Aarhus	Aα Arg^{19}→ Gly[1)]	FpA release, fibrin polymerization	Not related to haemostasis	Blombäck et al., 1988
Mannheim I	Aα Arg^{19}→ Gly	FpA release, fibrin polymerization	Bleeding	Dempfle & Henschen, 1990
Detroit	Aα Arg^{19}→ Ser[1)]	FpA release, fibrin polymerization	Bleeding	Blombäck et al., 1968; 1988

Table 1 (continued)

Name	Structural defect	Functional defect(s)	Clinical symptoms	References
Lima	Aα Arg^{141}→ Ser[1)]	Fibrin polymerization	Bleeding	Matsuda et al., 1991
Caracas II	Aα Ser^{434}→ Asn	Fibrin polymerization	Asymptomatic	Maekawa et al., 1991
Marburg	Aα Lys^{461}→ Stop[1,3)]	Fibrin polymerization, binding to endothelial cells	Bleeding and Thrombosis	Fuchs et al., 1977; Koopman et al., 1991a
Dusart (Dusard, (Paris V)	Aα Arg^{554}→ Cys[3)]	Fibrin polymerization, plasminogen binding, plasminogen activation	Thrombosis	Soria et al., 1983; Lijnen et al., 1984; Koopman et al., 1991e
New York I	Bβ (9-72) deletion	FpA and FpB release, fibrin polymerization, thrombin binding, plasminogen activation	Thrombosis	Liu et al., 1985; 1979
Christchurch II	Bβ Arg^{14}→ Cys	FpB release, fibrin polymerization	Bleeding	Kaudewitz et al., 1986
IJmuiden	Bβ Arg^{14}→ Cys[3)]	FpB release, fibrin polymerization	Thrombosis	Koopman et al., 1991c
Seattle I	Bβ Arg^{14}→ Cys	FpB release, fibrin polymerization	Asymptomatic	Pirkle et al., 1987b
Ise	Bβ Gly^{15}→ Cys	FpB release, fibrin polymerization	Asymptomatic	Yoshida et al., 1991
Nijmegen	Bβ Arg^{44}→ Cys[3)]	Fibrin polymerization, t-PA binding, plasminogen activation	Thrombosis	Engesser et al., 1988; Koopman et al., 1991c
Naples (Milan II)	Bβ Ala^{68}→ Thr[1,3)]	FpA and FpB release, thrombin binding	Thrombosis	Quattrone et al., 1979; Haverkate et al., 1986; Koopman et al., 1991d
Pontoise	Bβ Ala^{335}→ Thr	Fibrin polymerization	Not reported	Kaudewitz et al., 1986
Baltimore IV	γ Arg^{275}→ Cys	Fibrin polymerization	Asymptomatic	Schmelzer et al., 1989
Morioka	γ Arg^{275}→ Cys	Fibrin polymerization	Asymptomatic	Terukina et al., 1987
Osaka II	γ Arg^{275}→ Cys[2)]	Fibrin polymerization	Not reported	Terukina et al., 1988
Tochigi	γ Arg^{275}→ Cys	Fibrin polymerization	Asymptomatic	Yoshida et al., 1988
Tokyo II	γ Arg^{275}→ Cys	Fibrin polymerization	Asymptomatic	Matsuda et al., 1983; 1985a
Bergamo II	γ Arg^{275}→ His	Fibrin polymerization	Thrombosis	Reber et al., 1986a
Essen	γ Arg^{275}→ His	Fibrin polymerization	Asymptomatic	Reber et al., 1986a
Haifa	γ Arg^{275}→ His	Fibrin polymerization, Ca^{2+} effect on plasmin degradation	Thrombosis	Soria et al., 1987b; Kehl et al., 1984
Perugia	γ Arg^{275}→ His	Fibrin polymerization	Asymptomatic	Reber et al., 1986a
Saga	γ Arg^{275}→ His	Fibrin polymerization	Bleeding	Yamazumi et al., 1988
Baltimore I	γ Gly^{292}→ Val[2)]	Fibrin polymerization	Thrombosis	Bantia et al., 1990a
Baltimore III	γ Asn^{308}→ Ile	Fibrin polymerization	Asymptomatic	Bantia et al., 1990b
Kyoto I	γ Asn^{308}→ Lys[2)]	Fibrin polymerization, plasmin cleavage	Asymptomatic	Yoshida et al., 1988b
Asahi	γ Met^{310}→ Thr[2)]	Fibrin polymerization, crosslinking by F XIII	Bleeding	Yamazumi et al., 1989; 1990; Mimuro et al., 1990
Vlissingen	γ (319-320) deletion[3)]	Fibrin polymerization, Ca^{2+} binding	Thrombosis	Koopman et al., 1991
Nagoya	γ Gln^{329}→ Arg	Fibrin polymerization	Asymptomatic	Miyata et al., 1989
Kyoto III	γ Asp^{330}→ Tyr[2)]	Fibrin polymerization	Asymptomatic	Terukina et al., 1989
Milan I	γ Asp^{330}→ Val	Fibrin polymerization	Asymptomatic	Reber et al., 1986b
Osaka V	γ Arg^{375}→ Gly	Fibrin polymerization, Ca^{2+} binding	Asymptomatic	Yoshida & Matsuda, 1990

1) The propositus was homozygous
2) The structural defect was determined by protein sequence analysis and gene analysis
3) The mutation was determined solely by gene analysis

Recently, the mutations in fibrinogens Bicêtre II (γ Asn^{308}→ Lys), Caracas V (Aα Ser^{532}→ Cys), Frankfurt IV (γ 319-320 deletion), Giessen IV (γ Asp^{318}→ Gly) and Bologna (γ Arg^{275}→ Cys) were identified using gene analysis by Dr. J. Grimbergen from our laboratory, in cooperation with Dr. P. Reitsma (University Hospital, Leiden, The Netherlands).

CLINICAL MANIFESTATIONS

Abnormal fibrinogens are discovered in clinics by means of laboratory tests reflecting an abnormal, usually impaired clotting function of fibrinogen. A tendency to bleeding seems to be a logical consequence of this functional abnormality, and bleeding, mostly of a mild form, is found in 25% of the cases of dysfibrinogenemia (Ebert, 1991). Most of the cases were found by chance, and are asymptomatic. In the last 15 years, the number of cases of dysfibrinogenemia associated with thrombosis has grown and now comprises approximately 20% of the total number. Thrombophilia seems to be significantly associated with dysfibrinogenemia, even when eliminating other risk factors for thrombosis, such as advanced age, cancer and other diseases (Haverkate and Samama, 1991). The occurrence of bleeding or thrombosis, and in a few cases of both symptoms, in dysfibrinogenemia may reflect the essential role of fibrinogen and fibrin in the haemostatic balance. A crucial question is: which structural defect causes which malfunction of the fibrinogen molecule leading to the clinical symptoms? Some difficulties may arise in making or interpreting these associations such as:

- the same defect occurring in a number of propositi may show different clinical symptoms or no symptoms at all;
- in families with thrombophilia, thrombosis is often fatal, so that affected persons are not available for investigation.

We will discuss here in more detail the association between clinical symptoms, functional abnormality and structural defect of the fibrinogen molecule, in those cases of dysfibrinogenemia in which the structural defect has been elucidated.

The majority of the bleeding cases (Table 1) seems to be caused by a defective release of FpA, which in turn is caused by structural modifications in the amino-terminal site of the Aα-chain. They hamper the interaction with thrombin or affect the "A" polymerization site exposed after removal of FpA. These cases are Rouen (Aα Gly^{12} → Val), 11 cases with a substitution of Aα Arg^{16} and 3 cases with a mutation at Aα Arg^{19} (Table 1). Three of these cases i.e. Metz, Giessen and Detroit appear to be homozygous for the defect. However, the bleeding tendency does not seem to be more serious than in the heterozygous cases, suggesting a clinically-dominant disease. In the remaining group, bleeding has been reported in only a few cases, such as Lima (homozygous), Saga, Christchurch II and Asahi. We conclude that a defect in the amino-terminal part of the Aα-chain of fibrinogen increases the risk of bleeding, rather than thrombosis.

Thrombophilia is mainly seen in abnormal fibrinogens with a defect in the carboxyl-terminal part of the Aα- and γ-chains, and also in the amino-terminal part of the Bβ-chain (Table 1). Fibrinogen Hershey II (Aα Arg^{16} → Cys) seems to be an exception. However, this patient had arterial thrombosis at the age of 68, and the association with dysfibrinogenemia is, therefore, not convincing.

Defects in the amino-terminal part of the Bβ-chain

Fibrinogen New York I with a deletion (9-72) in the amino-terminal part of the Bβ-chain is associated with recurrent venous thrombosis. One of the functional defects in the fibrinogen molecule appears to be a defective binding of thrombin by its anionic binding site to fibrin prepared from fibrinogen New York I. In an attempt to explain the thrombophilia, Liu et al. (1979) suggested that defective thrombin binding may lead to an excess of thrombin in the circulation causing excessive coagulation and platelet aggregation. Other abnormal fibrinogens associated with thrombosis are characterized by abnormal thrombin binding such as Fibrinogen Malmö (Soria et al., 1985), and Pamplona II (Fernández et al., 1986), of which the structural abnormality has not yet been determined. A well-documented family study is available for Fibrinogen Naples, also characterized by a defective thrombin binding to its fibrin. Three family members with a homozygous substitution of Bβ Ala^{68} → Thr, suffered from serious arterial or venous thrombosis. Three heterozygous family members had no clinical symptoms. The abnormality in this case is clinically recessive. The clinical symptoms related to these variants strongly suggest that a defective thrombin binding to fibrin increases the risk of arterial or venous thrombosis.

Another explanation for the risk of thrombosis in dysfibrinogenemia could be a decreased stimulatory effect of fibrin on t-PA induced plasminogen activation. Such decreased fibrinolytic potential was demonstrated in fibrinogen New York (Bβ 9-72 deletion) and Nijmegen (Bβ Arg^{44} → Cys) both with episodes of venous thrombosis. Moreover, in Fibrinogen Nijmegen, the kringle 2 mediated t-PA binding to fibrin was reduced.

Fibrinogen IJmuiden (Bβ Arg^{14} → Cys) with both venous and arterial thrombosis at young age, has the same mutation as fibrinogen Christchurch II with a bleeding disorder and as Seattle I with no clinical symptoms. More cases with this mutation are required to draw definite conclusions on its association with thrombosis or bleeding.

Defects in the carboxyl-terminal part of the Aα-chain

The third group are variants with mutations in the carboxyl terminus of the Aα-chain. The homozygous fibrinogen Lima (Aα Arg^{141} → Ser) is associated with bleeding, most likely related to the observed impaired fibrin polymerization. However, no other variants with this type of mutation are known and no information on family members of the propositus is available. Fibrinogen Dusart (Aα Arg^{554} → Cys) is convincingly associated with severe recurrent thrombosis in all affected family members, with the exception of those who were treated prophylactically with oral anticoagulants. The thrombotic events are related to an impaired fibrinolysis of the thrombus formed from fibrinogen Dusart, due to the decreased binding of plasminogen and the reduced stimulatory effect of fibrin Dusart on t-PA induced plasminogen activation. Fibrinogen Marburg (Aα 461-610

deletion) homozygous propositus is associated with both bleeding and thrombosis. The bleeding tendency could easily be explained by the low plasma fibrinogen concentration in the homozygous proband and the impaired fibrin polymerization. The thrombotic tendency could be related to an inefficient initiation and stimulation of fibrinolysis due to the impaired fibrin polymerization as discussed for fibrinogen Dusart. The absence of clinical symptoms in the heterozygous family members, is probably due to the low concentration of variant fibrinogen (10-20%) as compared with the concentration of normal fibrinogen (80-90%) in the plasma of these individuals. As in fibrinogen Naples, the abnormality seems to be clinically recessive.

Defects in the carboxyl-terminal part of the γ-chain

Bleeding was reported in two cases, mild bleeding in fibrinogen Saga, and sporadic severe bleeding in fibrinogen Asahi. Four cases show thrombophilia, and 12 are asymptomatic (Table 1). To interpret the association between the defects and the clinical symptoms is not easy. The thrombotic tendency may be due to an impairment in fibrinolysis, as the stimulatory capacity of fibrin on t-PA induced plasminogen activation is reduced by the inhibition of fibrin polymerization (Suenson and Petersen, 1986; Koopman et al., 1986). Furthermore, this region of the γ-chain contains a site found to stimulate plasminogen activation by t-PA (Yonekawa et al., 1990).

More insight is required into the function of the carboxyl-terminal part of the γ-chain to explain the association between defects in this part of fibrinogen and the clinical symptoms.

CONCLUDING REMARKS

In the last five years the number of variant fibrinogens in which the structural defect has been elucidated has tripled. An interesting development has been the detailed investigation of variants with mutations outside the region directly controlling the release of FpA. This new information has contributed significantly to the structure-function analysis of fibrinogen and fibrin. On the other hand, a number of variants have been shown to contain post-translational modifications, a finding which complicates structure-function analysis, and emphasizes the necessity to complement functional studies with a structural analysis of the variant fibrinogen. In approximately 200 variants the molecular abnormalities have not yet been determined, although a number of these show interesting functional characteristics (Ebert, 1991). The introduction of gene analysis, using the polymerase chain reaction, could accelerate the structural elucidation of these variants and facilitate family studies to correlate the defect with the clinical symptoms observed in the patients. These family studies could clarify the sometimes uncertain

relationship between the structural- and functional abnormalities on the one hand, and the clinical manifestations on the other. The clinical symptoms associated with dysfibrinogenemia include both bleeding and thrombosis, indicating the central role of fibrinogen and fibrin in the haemostatic balance (Astrup, 1958). The data gathered up till now, suggest a relationship between defects in the amino-terminal region of the Aα-chain, affecting FpA release, and an increased risk of bleeding. Thrombophilia is predominantly observed in cases with a structural defect in the carboxyl-terminal part of the Aα- and γ-chains, and the amino-terminal part of the Bβ-chain. The functional abnormalities in these variants suggest that the thrombin binding to fibrin, and the stimulatory effect of fibrin on fibrinolysis, are important in vivo mechanisms for limiting thrombin activity and inducing thrombolysis.

In this review we have focused on hereditary variant fibrinogens with known structural and functional defects, because of the rapid expansion this field has shown over the last five years. We expect this expansion to increase in the near future, because of the new techniques that have become available and because of the emerging clinical significance. Therefore, exciting times lie ahead for the researchers working on hereditary variant fibrinogens, and operating on the borderline between biochemistry, genetics and clinical research.

REFERENCES

Alving B M, Henschen A H 1987 Fibrinogen Giessen I: a congenital homozygously expressed dysfibrinogenemia with Aα 16 Arg → His substitution. American Journal of Hematology 25: 479-482

Asakura S, Terukina S, Yamazumi K et al 1989 Fibrinogen Sapporo: dysfibrinogenemia characterized by the replacement of Aα arginine-16 by histidine resulting in the delayed release of fibrinopeptide A by thrombin. Nippon Ketsueki Gakkai Zasshi 52: 1094-1104

Astrup T 1958 The haemostatic balance. Thrombosis et Diathesis Haemorrhagica 2: 347-357

Bantia S, Mane S M, Bell W R, Dang C V 1990a Fibrinogen Baltimore I: polymerization defect associated with a γ^{292}Gly→ Val (GGC → GTC) mutation. Blood 76: 2279-2283

Bantia S, Bell W R, Dang C V 1990b Polymerization defect of fibrinogen Baltimore III due to a γAsn^{308}→ Ile mutation. Blood 75: 1659-1663

Berliner L J, Sugawara Y, Fenton J W II 1985. Human α-thrombin binding to non-polymerized fibrin-sepharose: evidence for an anionic binding region. Biochemistry 24: 7005-7009

Blombäck M, Blombäck B, Mammen E F, Prasad A S 1968 Fibrinogen Detroit -- a molecular defect in the N-terminal disulphide knot of human fibrinogen. Nature 218: 134-137

Blombäck B, Hessel B, Fields R, Procyk R 1988 Fibrinogen Aarhus: an abnormal fibrinogen with Aα 19 Arg → Gly substitution. In: Mosesson M W, Amrani D L, Siebenlist K R, DiOrio J P (eds) Fibrinogen 3. Biochemistry, biological functions, gene regulation and expression, Elsevier, Amsterdam, p 263-266

Bögli C, Cofrancesco E, Cortellaro M et al 1990 Fibrinogen Milano VI: a heterozygous dysfibrinogenemia (Aα 16 Arg → His) with bleeding tendency. European Journal of Haematology 45: 26-30

Borrell M, Vila L, Solá J, Coll I, Fontcuberta J 1990 Fibrinogen Barcelona II: a new case of Aα 16 Arg → His substitution. Haemostasis 20: 1-7

Brosstad F, Teige B, Olaisen B et al 1983 Two-dimensional electrophoretic characterization (iso-dalt) of fibrinogen and fibrin subunit chains from four different genetic dysfibrinogen variants. In: Haverkate F, Henschen A, Nieuwenhuizen W, Straub P W (eds) Fibrinogen - Structure, functional aspects, metabolism, Walter de Gruyter, Berlin, p 145-154

Budzynski A Z, Olexa S A, Pandya B V 1983 Fibrin polymerization sites in fibrinogen and fibrin fragments. Annuals of the New York Academy of Sciences 408: 301-314

Carr M E Jr, Shen LL, Hermans J 1977 Mass-length ratio of fibrin fibers from gel permeation and light scattering. Biopolymers 16: 1-15

Carr M E Jr, Qureshi G D 1987 The impact of delayed fibrinopeptide-A release on fibrin structure. Journal of Biological Chemistry 262: 15568-15574

Carrell N, McDonagh J 1982 Fibrinogen Chapel Hill II: defective in reactions with thrombin, factor XIIIa and plasmin. British Journal of Haematology 52: 35-47

Cheresh D A, Berliner S A, Vicente V, Ruggeri Z M 1989 Recognition of distinct adhesive sites on fibrinogen by related integrins on platelets and endothelial cells. Cell 58: 945-953

Chung C W, Que B G, Rixon M W, Mace Jr M, Davie E W 1983 Characterization of complementary deoxyribonucleic acid and genomic deoxyribonucleic acid for the β chain of human fibrinogen. Biochemistry 22: 3244-3250

Dempfle C E H, Henschen A 1990 Fibrinogen Mannheim I - identification of an Aα19 Arg → Gly substitution in dysfibrinogenaemia associated with bleeding tendency. In: Matsuda M, Iwanaga S, Takada A, Henschen A (eds) Fibrinogen 4. Current basic and clinical aspects, Elsevier Science Publishers B.V., Amsterdam, p 159-166

Denninger M H, Finlayson J S, Reamer L A, Parquet-Gernez A, Goudemand M, Ménaché D 1978 Congenital dysfibrinogenemia: fibrinogen Lille. Thrombosis Research 13: 453-466

Dugaiczyk A, Law S W, Dennison O E 1982 Nucleotide sequence and the encoded amino acids of human serum albumin mRNA. Proceedings of the National Academy of Sciences USA 79: 71-75

Ebert R F, Schreiber W E, Bell W R 1986 Fibrinogen Seattle II: congenital dysfibrinogenemia with an Arg (Aα 16) → His substitution. Thrombosis Research 43: 7-13

Ebert R F, ed 1991 Index of variant human fibrinogens, 1991 edition, CRC Press, Boca Raton

Engesser L, Koopman J, De Munk G, Haverkate F, Nováková I, Verheijen J H, Briët E, Brommer E J P 1988 Fibrinogen Nijmegen: congenital dysfibrinogenemia associated with impaired t-PA mediated plasminogen activation and decreased binding of t-PA. Thrombosis and Haemostasis 60: 113-120

Erdjument H, Lane D A, Ireland H et al 1987 Formation of a covalent disulfide-linked antithrombin-albumin complex by an antithrombin variant, antithrombin "Northwick Park". Journal of Biological Chemistry 262: 13381-13384

Erdjument H, Lane D A, Panico M, Dimarzo V, Morris H R 1988 Single amino acid substitution in the reactive site of antithrombin leading to thrombosis. Journal of Biological Chemistry 263: 5589-5593

Von Felten A, Frick P G, Straub P W 1969 Studies on fibrin monomer aggregation in congenital dysfibrinogenemia: separation of a pathological from a normal fibrin fraction. British Journal of Haematology 16: 353-361

Fenton II J W, Zabinski M P, Hsieh K, Wilner G D 1981 Thrombin noncovalent-protein binding and fibrin(ogen) recognition. Thrombosis and Haemostasis 46: 177

Fenton II J W, Olsen T A, Zabinski M P, Wilner G D 1988 Anion-binding exosite of human α-thrombin and fibrin(ogen) recognition. Biochemistry 27: 7106-7112

Fernández J, Páramo J A, Cuesta B, Aranda B, Rocha E 1986 Fibrinogen Pamplona II. A new congenital dysfibrinogenemia with abnormal fibrin-enhanced plasminogen activation and defective binding of thrombin to fibrin. In: Müller-Berghaus G, Scheefers-Borchel U, Selmayr E, Henschen A (eds) Fibrinogen and its derivatives. Biochemistry, Physiology and Pathophysiology, Elsevier, Amsterdam, p 25-30

Fuchs G, Egbring R, Havemann K 1977 Fibrinogen Marburg: a new genetic variant of fibrinogen. Blut 34: 107-118

Furlan M, Bögli C, Hofer A, Bouvier C A, De Moerloose P 1990 Fibrinogen Geneva, a new case of Aα 16 Arg → Cys dysfibrinogenaemia. Blood Coagulation and Fibrinolysis 1: 139-144

Galanakis D K, Henschen A, Keeling M, Kehl M, Dismore R, Peerschke E I 1983 Fibrinogen Louisville: an Aα16 Arg → His defect that forms no hybrid molecules in heterozygous individuals and inhibits aggregation of normal fibrin monomers. Annuals of the New York Academy of Sciences, p 644-648

Galanakis D K, Henschen A, Peerschke E I B, Kehl M 1989 Fibrinogen Stony Brook, a heterozygous Aα16 Arg → Cys dysfibrinogenemia. Evaluation of diminished platelet aggregation support and of enhanced inhibition of fibrin assembly. Journal of Clinical Investigation 84: 295-304

Galanakis D K, Henschen A, Schubach W, Lord S, Al-Mondhiry H 1990a Determination of abnormal structure and heterozygosity by amplification of genomic DNA using the polymerase chain reaction and by amino acid sequence analysis. In: Matsuda M, Iwanaga S, Takada A, Henschen A (eds) Fibrinogen 4. Current basic and clinical aspects, Elsevier Science Publishers B.V., *Amsterdam*, p 173-178

Galanakis D K, Hultin M 1990b Fibrinogen Stony Brook II: partial characterization of a heterozygously transmitted peptide A anomaly. Blood Coagulation and Fibrinolysis 1: 567-570

Häberli A, Straub P W, Dietler G, Känzig W 1987 The influence of calcium ions on fibrin polymerization. Biopolymers 26: 27-43

Hantgan R R, Hermans J 1979 Assembly of fibrin: a light scattering study. Journal of Biological Chemistry 254: 11272-11281

Hasegawa N, Sasaki S 1990 Location of the binding site "b" for lateral polymerization of fibrin. Thrombosis Research 57: 183-195

Haverkate F, Timan G 1977 Protective effect of calcium on the plasmin degradation of fibrinogen and fibrin fragments D. Thrombosis Research 10: 803-812

Haverkate F, Timan G, Soria J, Soria C, Samama M M 1978 Studies on the structure of the fragment D moiety of abnormal fibrinogens: influence of calcium ions. Thrombosis Research 13: 689-692

Haverkate F, Koopman J, Kluft C, D'Angelo A, Cattaneo M, Mannucci P M 1986 Fibrinogen Milano II: a congenital dysfibrinogenaemia associated with juvenile arterial and venous thrombosis. Thrombosis and Haemostasis 55: 131-135

Haverkate F, Koopman J, Kluft C, D'Angelo A, Cattaneo M, Mannucci P M 1987 Fibrinogens "Milano II" - and "Naples". Thrombosis and Haemostasis 57: 375

Haverkate F, Samama M M 1991 Manuscript in preparation.

Heene D L, Dempfle C E, Borck P, Kirschstein W, Harenberg J, Brossmer R 1989 Dysfibrinogenaemia with impaired release of fibrinopeptide A and t-PA-induced plasminogen activation: Fibrinogen Mannheim I. Thrombosis and Haemostasis 62: 75 (abstract)

Henschen A, Kehl M, Deutsch E 1983 Novel structure elucidation strategy for genetically abnormal fibrinogens with incomplete fibrinopeptide release as applied to fibrinogen Schwarzach. Hoppe Seylers Zeitschrift Physiologische Chemie 364: 1747-1751

Henschen A, Kehl M, Southan C 1984 Genetically abnormal fibrinogens - strategies for structure elucidation, including fibrinopeptide analysis. In: Beck E A, Furlan M (eds) Variants of human fibrinogen. Hans Huber, Berne, p 273-320

Higgins D L, Shafer J A 1981 Fibrinogen Petoskey, a dysfibrinogenemia characterized by replacement of Arg-Aα 16 by a histidyl residue. Journal of Biological Chemistry 256: 12013-12017

Higgins D L, Lewis S D, Shafer J A 1983 Steady state kinetic parameters for the thrombin-catalysed conversion of human fibrinogen to fibrin. Journal of Biological Chemistry 258: 9276-9282

Hogg D H, Blombäck B 1978 The mechanism of the fibrinogen-thrombin reaction. Thrombosis Research 12: 953-964

Hoylaerts M, Rijken D C, Lijnen H R, Collen D 1982 Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. Journal of Biological Chemistry 257: 2912-2919

Hynes R O 1987 Integrins: a family of cell surface receptors. Cell 48: 549-554

Janssen C L, Vreeken J 1971 Fibrinogen Amsterdam, another hereditary abnormality of fibrinogen. British Journal of Haematology 20: 287-298

De Jong W W, Zweers A, Cohen L H 1978 Influence of single amino acid substitutions on electrophoretic mobility of sodium dodecyl sulphate-protein complexes. Biochemical and Biophysical Research Communications 82: 532-539.

Kaczmarek E, McDonagh J 1988 Thrombin binding to the Aα-, Bβ- and γ-chains of fibrinogen and to their remnants contained in fragment E. Journal of Biological Chemistry 263: 13896-13900

Kaminski M, McDonagh J 1983 Studies on the mechanism of thrombin. Journal of Biological Chemistry 258: 10530-10535

Kaminksi M, McDonagh J 1987 Inhibited thrombins. Interactions with fibrinogen and fibrin. Biochemical Journal 242: 881-887

Kant J A, Lord S T, Crabtree G R 1983 Partial mRNA sequences from human Aα, Bβ, and γ fibrinogen chains: evolutionary and functional implications. Proceedings of the National Academy of Sciences USA 80: 3953-39957

Kaudewitz H, Henschen A, Soria J, Soria C 1986 Fibrinogen Pontoise - A genetically abnormal fibrinogen with defective fibrin polymerization but normal fibrinopeptide release. In: Lane D A, Henschen A, Jasani M K (eds) Fibrinogen, Fibrin Formation and Fibrinolysis, Walter de Gruyter, Berlin, p 91-96

Kehl M, Lottspeich F, Henschen A 1982 High-performance liquid chromatography of proteins as applied to fibrinogen chains. Hoppe Seylers Zeitschrift Physiologische Chemie 363: 1501-1505

Kehl M, Lottspeich F, Henschen A 1983 Genetically abnormal fibrinogens releasing abnormal fibrinopeptides as characterized by high performance liquid chromatography. In: Haverkate F, Henschen A, Nieuwenhuizen W, Straub P (eds) Fibrinogen: Structure, functional aspects, metabolism. Walter de Gruyter, Berlin, p 183-193

Kehl M, Henschen A, Tavory S, Rimon A, Soria J, Soria C 1984 The structural error in fibrinogen Haifa, a genetically abnormal fibrinogen with a polymerisation defect. Proceedings of VIII International Congress on Thrombosis, Istanbul, 82 (abstract)

Koehn J A, Canfield R E 1981 Purification of human fibrinopeptides by high performance liquid chromatography. Analytical Biochemistry 116: 349-356

Koopman J, Engesser L, Nieveen M, Haverkate F, Brommer E J P 1986 Fibrin polymerization associated with tissue-type plasminogen activator (t-PA) induced glu-plasminogen activation. In: Müller-Berghaus G, Scheefers-Borchel U, Selmayr E, Henschen A (eds) Fibrinogen and its derivatives, Excerpta Medica, Amsterdam, p 315-318

Koopman J, Haverkate F, Lane D A, Lord S T 1988 Sequence analysis of the fibrinogen Manchester Aα chain gene after amplification of genomic DNA using the polymerase chain reaction (PCR). In: Mosesson M W, Amrani D L, Siebenlist K R, DiOrio J P (eds) Fibrinogen 3. Biochemistry, biological functions, gene regulation and expression, Elsevier Science Publishers B.V., Amsterdam, p 275-278

Koopman J, Grimbergen J, Haverkate F, Lord S T, Egbring R 1991a Fibrinogen Marburg: a homozygous case of dysfibrinogenemia, lacking Aα 461-610 (Lys 461 AAA → TAA STOP). Thrombosis and Haemostasis 65: 822 (abstract)

Koopman J, Haverkate F, Briët E, Lord S T 1991b A congenitally abnormal fibrinogen (Vlissingen) with a six-base deletion in the γ-chain gene, causing defective calcium binding and impaired fibrin polymerization. Journal of Biological Chemistry 265: 13456-13461

Koopman J, Haverkate F, Grimbergen J et al 1991c Formation of fibrinogen-albumin and fibrinogen-fibrinogen complexes in abnormal fibrinogens by disulphide bridges. Fibrinogen IJmuiden (Bβ Arg 14 → Cys) and Nijmegen (Bβ Arg 44 → Cys). Thrombosis and Haemostasis 65: 822 (abstract)

Koopman J, Haverkate F, Lord S T, Grimbergen J, Mannucci P 1991d The molecular basis of fibrinogen Naples associated with defective α-thrombin binding and congenital thrombophilia: homozygous substitution of Bβ 68 Ala → Thr. Thrombosis and Haemostasis 65: 809 (abstract)

Koopman J, Haverkate F, Grimbergen J et al. 1991e Submitted for publication.

Kudryk B J, Collen D, Woods K R, Blombäck B 1974 Evidence for localization of polymerization sites in fibrinogen. Journal of Biological Chemistry 249: 3322-3325

Lane D A, Van Ross M, Kakkar V V et al 1980 An abnormal fibrinogen with delayed fibrinopeptide A release. British Journal of Haematology 46: 89-98

Lane D A, Ireland H, Thompson E, Frost T J, Cuddigan B 1982 Two more abnormal fibrinogens (London III and Sydney) with impaired fibrinopeptide release. Thrombosis Research 28: 821-824

Lane D A, Southan C, Ireland H, Thompson E, Kehl M, Henschen A 1983 Delayed release of an abnormal fibrinopeptide A from fibrinogen Manchester: effect of the Aα16Arg → His substitution upon fibrin monomer polymerisation and the immunological crossreactivity of the peptide. British Journal of Haematology 53: 587-597

Lane D A, Thompson E, Southan C 1987 Rapid screening method of fibrinopeptide analysis applied to congenital dysfibrinogenaemia: summary of 23 cases. In: Lowe G D O, Douglas J T, Forbes C D, Henschen A (eds) Fibrinogen 2. Biochemistry, physiology and clinical relevance, Elsevier, Amsterdam, p 71-74

Lane D A, Southan C 1987 Inherited abnormalities of fibrinogen synthesis and structure. In: Bloom A, Thomas D (eds) Haemostasis and Thrombosis, Churchill Livingstone, London, p 442-451

Laudano A P, Doolittle R F 1981 Influence of calcium ion on the binding of fibrin amino terminal peptides to fibrinogen. Science 212: 457-459

Laudano A P, Cottrell B, Doolittle R F 1983 Synthetic peptides modelled on fibrin polymerisation sites. Annals of the New York Academy of Sciences 408: 315-329

Lee M H, Kaczmarek E, McDonagh J 1990 Fibrinogen Ledyard (Aα 16 Arg → Cys): biochemical and physiological characterization. Circulation (Supplement III) 82: III-368, abstract

Lijnen H R, Soria J, Soria C, Collen D, Caen J P 1984 Dysfibrinogenemia (fibrinogen Dusard) associated with impaired fibrin-enhanced plasminogen activation. Thrombosis and Haemostasis 51: 108-109

Lindsey G G, Brown G, Purves L R 1978 Calcium binding to human fibrinogen - Localization of two calcium specific sites. Thrombosis Research 13: 345-350

Liu C Y, Nossel H L, Kaplan K L 1979 The binding of thrombin by fibrin. Journal of Biological Chemistry 254: 10421-10425

Liu C Y, Koehn J A, Morgan F J 1985 Characterization of fibrinogen New York I. A dysfunctional fibrinogen with a deletion of Bβ(9-72) corresponding exactly to exon II of the gene. Journal of Biological Chemistry 260: 4390-4396

Liu C Y, Wallén P, Handley D A 1986 Fibrinogen New York I: The structural, functional, and genetic defects and a hypothesis of the role of fibrin in the regulation of coagulation and fibrinolysis. In: Lane D A, Henschen A, Jasani M K (eds). Fibrinogen, Fibrin Formation and Fibrinolysis, Walter de Gruyter, Berlin, p 79-90

Lord S T, Byrd P A, Hede K L, Wei C, Colby T J 1990 Analysis of fibrinogen Aα-fusion proteins: mutants which inhibit thrombin equivalently are not equally good substrates. Journal of Biological Chemistry 265: 838-843

Maekawa H, Yamazumi K, Muramatsu S et al 1991 An Aα Ser-434 to N-glycosylated Asn substitution in a dysfibrinogen, fibrinogen Caracas II, characterized by impaired fibrin gel formation. Thrombosis and Haemostasis 65: 821 (abstract)

Marsh H C, Meinwald Y C, Thannhauser T W, Scheraga H A 1983 Mechanism of action of thrombin on fibrinogen. Kinetic evidence for involvement of aspartic acid at position P_{10}. Biochemistry 22: 4170-4174

Martinelli R A, Scheraga H A 1979 Assay of bovine fibrinopeptides by high performance liquid chromatography. Analytical Biochemistry 96: 246-249

Matsuda M, Baba M, Morimoto K, Nakamikawa C 1983 "Fibrinogen Tokyo II: an abnormal fibrinogen with an impaired polymerization site on the aligned DD domain of fibrin molecules. Journal of Clinical Investigation 72: 1034-1041

Matsuda M, Nakamikawa C, Baba M, Morimoto K 1985a Fibrinogen Tokyo II: an abnormal fibrinogen with an impaired polymerization site on the aligned DD domain of fibrin molecules. In: Henschen A, Hessel B, McDonagh J, Saldeen T (eds) Fibrinogen - structural-variants and interactions, Walter de Gruyter, Berlin, p 213-222

Matsuda M, Saeki E, Kasamatsu A, Nakamikawa C, Manabe S, Samejima Y 1985b Fibrinogen Kawaguchi: an abnormal fibrinogen characterized by defective release of fibrinopeptide A. Thrombosis Research 37: 379-390

Matsuda M, Terukina S, Miyata T, Iwanaga S 1986 Defective release of fibrinopeptide A due to substitution of Aα arginine-16 by cysteine in two abnormal fibrinogens Kawaguchi and Osaka. In: Fibrinogen and its derivatives; biochemistry, physiology, and pathophysiology, Elsevier, Amsterdam, p 37-40

Matsuda M, Yoshida N, Terukina S, Yamazumi K, Maekawa H 1990 Molecular abnormalities of fibrinogen - The present status of structure elucidation. In: Matsuda M, Iwanaga S, Takada A, Henschen A (eds) Fibrinogen 4. Current basic and clinical aspects, Elsevier, Amsterdam, p 139-152

Matsuda M, Maekawa H, Yamazumi K et al. 1991 An Aα Arg-141 to Ser substitution accompanied by an extra oligo-saccharide at Aα Asn-139 in a homozygous dysfibrinogen, fibrinogen Lima, characterized by impaired fibrin polymerization. Thrombosis and Haemostasis 65: 822 (abstract)

Mimuro J, Yamazumi K, Terukina S, Matsuda M 1990 A T → C exchange in exon VIII of the fibrinogen γ chain gene defined by the polymerase chain reaction for fibrinogen Asahi with a γ Met-310 to Thr substitution and N-glycosylated Asn-308. Xth Fibrinogen Workshop, Rouen (abstract)

Miyata T, Terukina S, Matsuda M, et al 1987 Fibrinogens Kawaguchi and Osaka: an amino acid substitution of Aα arginine-16 to cysteine which forms an extra interchain disulfide bridge between the two Aα chains. Journal of Biochemistry (Tokyo) 102: 93-101

Miyata T, Furukawa K, Iwanaga S, Takamatsu J, Saito H 1989 Fibrinogen Nagoya, a replacement of glutamine-329 by arginine in the γ-chain that impairs the polymerization of fibrin monomer. Journal of Biochemistry (Tokyo) 105: 10-14

Morris S, Denninger M H, Finlayson J S, Ménaché D 1981 Fibrinogen Lille: Aα(7) ASP → ASN. Thrombosis and Haemostasis 46: 104 (abstract)

Mosesson M W, Alkjaersig N, Sweet B, Sherry S 1967 Human fibrinogen of relatively high solubility. Comparative biophysical, biochemical, and biological studies with fibrinogen of lower solubility. Biochemistry 6: 3279-3287

Mosesson M W, Beck E A 1969 Chromatographic, ultracentrifugal and related studies on fibrinogen "Baltimore". Journal of Clinical Investigation 48: 1656-1662

Mosesson M W 1990 Fibrin polymerization and its regulatory role in hemostasis. Journal of Laboratory and Clinical Medicine 116: 8-17

Ni F, Konishi Y, Frazier R B, Scheraga H A, Lord S T 1989a High-resolution NMR studies of fibrinogen-like peptides in solution: interaction of thrombin with residues 1-23 of the Aα chain of human fibrinogen. Biochemistry 28: 3082-3094

Ni F, Meinwald Y C, Vásquez M, Scheraga H A 1989b High-resolution NMR studies of fibrinogen-like peptides in solution: structure of a thrombin-bound peptide corresponding to residues 7-16 of the Aα chain of human fibrinogen. Biochemistry 28: 3094-3105

Ni F, Konishi Y, Bullock L D, Rivetna M N, Scheraga H A 1989c High-resolution NMR studies of fibrinogen-like peptides in solution: structural basis for the bleeding disorder caused by a single mutation of Gly(12) to Val(12) in the Aα chain of human fibrinogen Rouen. Biochemistry 28: 3106-3119

Nieuwenhuizen W, Vermond A, Nooijen W J, Haverkate F 1979 Calcium-binding properties of human fibrin(ogen) and degradation products. FEBS Letters 98: 257-259

Nieuwenhuizen W, Ruijven-Vermeer I A M, Nooijen W J, Vermond A, Haverkate F 1981 Recalculation of calcium-binding properties of human and rat fibrin(ogen) and their degradation products. Thrombosis Research 22: 653-657

Nieuwenhuizen W, Haverkate F 1983 Calcium binding regions in fibrinogen. Annals of the New York Academy of Sciences 408: 92-96

Olexa S A, Budzynski A Z 1980 Evidence for four different polymerization sites involved in human fibrin formation. Proceedings of the National Academy of Sciences USA 77: 1374-1378

Olexa S A, Budzynski A Z, Doolittle R F, Cottrell B A, Greene T C 1981 Structure of fragment E species from human cross-linked fibrin. Biochemistry 21: 6139-6145

Peerschke E I B 1988 Irreversible platelet fibrinogen interactions occur independently of fibrinogen alpha chain degradation and are not mediated by intact platelet membrane glycoprotein IIb-IIIa complexes. Journal of Laboratory and Clinical Medicine 111: 84-92

Pirkle H, Theodor I, Vukasin P, et al 1987a Fibrinogen *Long Beach*. Thrombosis and Haemostasis 58: 148 (abstract)

Pirkle H, Kaudewitz H, Henschen A, Theodor I, Simmons G 1987b Substitution of Bβ 14 arginine by cyst(e)ine in fibrinogen Seattle I. In: Lowe G D O, Douglas J T, Forbes C D, Henschen A (eds). Fibrinogen 2. Biochemistry, Physiology and Clinical Relevance, Elsevier, Amsterdam, p 49-52

Quattrone A, Colucci M, Donati M B, et al 1979 Cerebral thrombosis in two siblings with dysfibrinogenemia. Neuroscience Letters 3, suppl 1: 54-62

Qureshi G D, Evans H J, Vennart R M, Magnant J P, Sabau J M, Willoughby J B, Koehn J A 1983 Fibrinogen White Marsh - A new human variant with α-chain defect. Clinical Research 31: 321A (abstract)

Rånby M 1982 Studies on the kinetics of plasminogen activation by tissue plasminogen activator. Biochimica Biophysica Acta 704: 461-469

Ratnoff D O, Potts A M 1954 The accelerating effect of calcium and other cations on the conversion of fibrinogen to fibrin. Journal of Clinical Investigation 33: 206-210

Reber P, Furlan M, Beck E A, Finazzi G, Buelli M, Barbui T 1985 Fibrinogen Bergamo I (Aα 16Arg → Cys): susceptibility towards thrombin following aminoethylation, methylation or carboxamidomethylation of cysteine residues. Thrombosis and Haemostasis 54: 390-393

Reber P, Furlan M, Henschen A, et al 1986a Three abnormal fibrinogen variants with the same amino acid substitution (γ 275 Arg → His): fibrinogens Bergamo II, Essen and Perugia. Thrombosis and Haemostasis 56: 401-406

Reber P, Furlan M, Rupp C, et al 1986b Characterization of fibrinogen Milano I: amino acid exchange γ 330 Asp → Val impairs fibrin polymerization. Blood 67: 1751-1756

Reber P, Furlan M, Beck E A, Barbui T 1987 Fibrinogen Bergamo III and fibrinogen Torino: two further variants with hereditary molecular defects in fibrinopeptide A. Thrombosis Research 46: 163-167

Rupp C, Sievi R, Furlan M, Beck E A 1983 Fibrinogen Bern II: fibrinogenervariante mit dem aminosäurenaustausch Arginin → Histidin in position 16 der A kette. Schweizerische Medizinische Wochenschrift 113: 1460-1462

Saiki R K, Gelfand D H, Stoffel S et al 1988 Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 39: 487-491

Samori T, Yatabe M, Ukita M et al 1975 A new type of congenital dysfibrinogenemia (Fibrinogen Tokyo) with defective stabilization of fibrin polymers. Thrombosis et Diathesis Haemorrhagica 34: 329 (abstract)

Schmelzer C H, Ebert R F, Bell W R 1988 A polymorphism at $B\beta_{448}$of fibrinogen identified during structural studies of fibrinogen Baltimore II. Thrombosis Research 52: 173-177

Schmelzer C H, Ebert R F, Bell W R 1989 Fibrinogen Baltimore IV: congenital dysfibrinogenemia with a γ(275) (Arg → Cys) substitution. Thrombosis Research 56: 307-316

Shainoff J R, Dardik B N 1979 Fibrinopeptide B and aggregation of fibrinogen. Science 204: 200-202

Shainoff J R, Dardik B N 1983 Fibrinopeptide B in fibrin assembly and metabolism: physiologic significance in delayed release of the peptide. Annals of the New York Academy of Science 408: 254-267

Siebenlist K R, Prchal J T, Mosesson M W 1988 Fibrinogen Birmingham: a heterozygous dysfibrinogenemia (Aα 16 Arg → His) containing heterodimeric molecules. Blood 71: 613-618

Soria J, Soria C, Samama M, Henschen A, Southan C 1982 Detection of fibrinogen abnormality in dysfibrinogenemia: special report on fibrinogen Metz characterized by an amino acid substitution located at the peptide bond cleaved by thrombin. In: Henschen A, Graeff H, Lottspeich F (eds) Fibrinogen - Recent biochemical and medical aspects, Walter de Gruyter, Berlin, p 129-144

Soria J, Soria C, Caen P 1983 A new type of congenital dysfibrinogenaemia with defective fibrin lysis-Dusard syndrome: possible relation to thrombosis. British Journal of Haematology 53: 575-586

Soria J, Soria C, Hedner U, Nilsson I M, Bergqvist D, Samama M 1985 Episodes of increased fibronectin level observed in a patient suffering from recurrent thrombosis related to congenital hypodysfibrinogenemia (fibrinogen Malmö). Br J Haematol 61: 727-738

Soria J, Soria C, Bertrand O et al 1986 Role of fibrin polymerization in fibrinolysis: comparison between polymerisable and unpolymerisable fibrins in the potentiating effect of plasminogen activation by tissue-type plasminogen activator. In: Lane D, Henschen A, Jasani M K (eds) Fibrinogen-fibrin formation and fibrinolysis Vol 4, Walter de Gruyter, Berlin, p 153-158

Soria J, Soria C, Bertrand O 1987a Molecular basis for the Aα chain substitutions in dysfibrinogenemia with a description of two new cases of the disorder: Fibrinogen Amiens I and fibrinogen Amiens II. In: Lowe G D O, Douglas J T, Forbes C D, Henschen A (eds) Fibrinogen 2. Biochemistry, physiology and clinical relevance, Elsevier, Amsterdam, p 57-62

Soria J, Soria C, Samama M et al 1987b Fibrinogen Haifa: fibrinogen variant with absence of protective effect of calcium on plasmin degradation of γ-chains. Thrombosis and Haemostasis 57: 310-313

Southan C, Henschen A, Lottspeich F 1982 The search for molecular defects in abnormal fibrinogens. In: Henschen A, Graeff H, Lottspeich F (eds) Fibrinogen: Recent biochemical and medical aspects. Walter de Gruyter, Berlin, p 153-166

Southan C, Kehl M, Henschen A, Lane D A 1983 Fibrinogen Manchester: identification of an abnormal fibrinopeptide A with a C-terminal arginine → histidine substitution. British Journal of Haematology 54: 143-151

Southan C, Lane D A, Bode W, Henschen A 1985 Thrombin-induced fibrinopeptide release from a fibrinogen variant (fibrinogen Sydney I) with an Aα Arg-16 → His substitution. European Journal of Biochemistry 147: 593-600

Southan C, Thompson E, Lane D A 1987 Direct analysis of plasma fibrinogen-derived fibrinopeptides by high-performance liquid chromatography: investigation of nine congenital fibrinogen abnormalities. British Journal of Haematology 65: 469-473

Suenson E, Petersen L C 1986 Fibrin and plasminogen structures essential to stimulation of plasmin formation by tissue-type plasminogen activator. Biochimica et Biophysica Acta 870: 510-519

Terukina S, Matsuda M, Yoshida N, Yamazumi K, Takeda Y, Takano T 1987 Two abnormal fibrinogens designated as Osaka II and Morioka with a hitherto unidentified amino acid substitution; γ Arg-275 by Cys. Thrombosis and Haemostasis 58: 515 (abstract)

Terukina S, Matsuda M, Hirata H, et al 1988 Substitution of γ Arg-275 by Cys in an abnormal fibrinogen, "fibrinogen Osaka II:" evidence for a unique solitary cysteine structure at the mutation site. Journal of Biological Chemistry 263: 13579-13587

Terukina S, Yamazumi K, Okamoto K, Yamashita H, Ito Y, Matsuda M 1989 Fibrinogen Kyoto III: a congenital dysfibrinogen with a γ aspartic acid-330 to tyrosine substitution. Blood 74: 2681-2687

Vali Z, Scherage H A 1988 Localization of the binding site on fibrin for the secondary binding site of thrombin. Biochemistry 27: 1956-1963

Wenzel E, Seyfert U T, Weier B et al 1990 Clinical rheological and haemostaseological aspects of dysfibrinogenemia. In: Matsuda M, Iwanaga S, Takada A, Henschen A (eds) Fibrinogen 4. Current basic and clinical aspects, Elsevier, Amsterdam, p 153-158

Yamazumi K, Terukina S, Onohara S, Matsuda M 1988 Normal plasmic cleavage of the γ-chain variant of "fibrinogen Saga" with an Arg-275 to His substitution. Thrombosis and Haemostasis 60: 476-480

Yamazumi K, Shimura K, Terukina S, Takahashi N, Matsuda M 1989 A γ methionine-310 to threonine substitution and consequent N-glycosylation at γ asparagine-308 identified in a congenital dysfibrinogenemia associated with posttraumatic bleeding, fibrinogen Asahi. Journal of Clinical Investigation 83: 1590-1597

Yamazumi K, Shimura K, Mackawa H, Muramatsu S, Terukina S, Matsuda M 1990 Delayed intermolecular γ-chain cross-linking by factor XIIIa in fibrinogen Asahi characterized by a γ-Met-310 to Thr substitution with an N-glycosylated γ-Asn-308. Blood Coagulation and Fibrinolysis 1: 557-559

Yonekawa O, Vermond A, Nieuwenhuizen W 1990 Localization of a new site in fibrin, involved in the acceleration of the tissue-type plasminogen activator (t-PA). In: Matsuda M, Iwanaga S, Takada A, Henschen A (eds) Fibrinogen 4. Current basic and clinical aspects. Elsevier, Amsterdam, p 111-116

Yoshida N, Ota K, Moroi M, Matsuda M 1988a An apparently higher molecular weight γ-chain variant in a new congenital abnormal fibrinogen Tochigi characterized by the replacement of γ arginine-275 by cysteine. Blood 71: 480-487

Yoshida N, Terukina S, Okuma M, Moroi M, Aoki N, Matsuda M 1988b Characterization of an apparently lower molecular weight γ-chain variant in fibrinogen Kyoto I. The replacement of γ-asparagine 308 by lysine which causes accelerated cleavage of fragment D1 by plasmin and the generation of a new plasmin cleavage site. Journal of Biological Chemistry 263: 13848-13856

Yoshida N, Matsuda M 1990 Fibrinogen Osaka V with a γ Arg-375 to Gly substitution is defective in the high-affinity but normal in the low-affinity calcium binding sites. Xth Fibrinogen Workshop, Rouen (abstract)

Yoshida N, Wada H, Morita K et al 1991 A new congenital abnormal fibrinogen Ise characterized by the replacement of Bβ glycine-15 by cysteine. Blood 77: 1958-1963

CHAPTER 3

A CONGENITALLY ABNORMAL FIBRINOGEN (VLISSINGEN) WITH A SIX-BASE DELETION IN THE γ-CHAIN GENE, CAUSING DEFECTIVE CALCIUM BINDING AND IMPAIRED FIBRIN POLYMERIZATION

Jaap Koopman[1,2], Frits Haverkate[1], Ernest Briët[2] and Susan T. Lord[3]

1) TNO Gaubius Institute for Cardiovascular Research, Leiden, The Netherlands.
2) University Hospital Leiden, The Netherlands.
3) Department of Pathology, University of North Carolina, Chapel Hill, USA.

SUMMARY

A congenitally abnormal fibrinogen (Vlissingen) was isolated from the blood of a young woman suffering from massive pulmonary embolism. Fibrinogen Vlissingen showed an abnormal clotting time with both thrombin and Reptilase^{R}. The release of the fibrinopeptides A and B by thrombin was normal, but fibrin polymerization was impaired both in the presence and absence of Ca^{2+} ions. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis performed according to Laemmli the γ-chain of fibrinogen Vlissingen showed two bands, one normal and one having an apparent molecular mass of about 1500 daltons smaller. The previously described protective effect of Ca^{2+} ions on plasmin degradation of the carboxyl terminus of the γ-chain of normal fibrinogen was only partially detectable in fibrinogen Vlissingen. In addition, the binding of Ca^{2+} ions was decreased. Fibrinogen Vlissingen bound 2.4 Ca^{2+} ions per fibrinogen molecule at pH 7.5, whereas normal fibrinogen bound 3.1 Ca^{2+} ions. At pH 5.8 fibrinogen Vlissingen bound 1.1 Ca^{2+} ions, whereas normal fibrinogen bound 2.0 Ca^{2+} ions per molecule fibrinogen in the D-domains, again indicating a structural change in the carboxyl terminus of fibrinogen. The structural defect was determined by sequence analysis of DNA amplified by use of the polymerase chain reaction. Exons VIII, IX and X of the γ-chain gene were amplified and the DNA sequences of the amplified fragments were determined. A six-base deletion was found in 50% of the fragments corresponding to exon VIII, indicating that the patient was heterozygous for the mutation. This deletion codes for amino acids Asn 319 and Asp 320 in the normal fibrinogen γ-chain. The data indicate that Asn 319 and Asp 320 are crucial for maintaining the integrity of the carboxyl terminal polymerization sites, the protective effect of Ca^{2+} ions on plasmin degradation of the carboxyl terminus of the γ-chain, and the calcium binding domain at the carboxyl terminus of fibrinogen.

INTRODUCTION

Fibrinogen, a soluble plasma protein, is a dimer of three polypeptide chains, called Aα, Bβ and γ. The amino termini of all six chains form a central E domain, while the carboxyl termini of each fibrinogen molecule form two outer D domains. During coagulation fibrinogen is cleaved by thrombin to generate fibrin monomers which then polymerize to form an insoluble fibrin matrix. Fibrin monomer polymerization arises from the interactions of polymerization sites on different molecules. Sites located in the amino terminal part of the molecule are exposed by thrombin cleavage of fibrinopeptides from the Aα and Bβ chains; these sites are designated "A" and "B", respectively.

Complementary polymerization sites, designated "a" and "b", are located in the D domains of fibrinogen which include the carboxyl terminus of the γ-chain (1-3). Human fibrinogen binds three Ca^{2+} ions per molecule with high affinity at pH 7.5. Each D-domain contains one high affinity Ca^{2+} binding site (4-6). Plasmin degradation of the carboxyl terminus of the γ-chain results in loss of Ca^{2+} binding (7). On the other hand, binding of Ca^{2+} ions at this site protects the carboxyl terminus of the γ-chain from plasmin degradation (8). Plasmin degradation of normal fibrinogen in the presence of Ca^{2+} ions results in fragment D_1, with the carboxyl terminus of the γ-chain intact (M_r 38,000), in the absence of Ca^{2+} ions fragment D_3 is formed, with the carboxyl terminus of the γ-chain degraded (M_r 26,000) (8).

More than 200 cases of inherited dysfibrinogenemia have been reported (9), and most of these show prolonged clotting times with both plasma and purified fibrinogen due to a defect in the thrombin catalyzed release of fibrinopeptides or a defect in polymerization of fibrin monomers or both. At least 12 congenitally abnormal fibrinogens have abnormalities in the carboxyl terminus of the γ-chain (10-22) and correspondingly defective fibrin polymerization. In fibrinogen Haifa (γ 275 Arg → His) the protective effect of Ca^{2+} ions on plasmin degradation of the γ-chain was impaired (23), but this was not evident in fibrinogen Saga I (γ 275 Arg → His) (24). Up to now the structural defects in abnormal fibrinogens have been elucidated by amino acid sequence analysis of the purified protein (25). Since the complete cDNA sequences (26-28) and part of the genomic DNA sequences (28,29) of the Aα, Bβ and γ-chain genes are known, the polymerase chain reaction (30) can be employed to determine structural defects in abnormal fibrinogens (31,32). This report describes the functional and structural defects of a congenitally abnormal fibrinogen called fibrinogen Vlissingen. We found a six-base deletion in the γ-chain gene as determined by DNA sequence analysis of polymerase chain reaction-amplified DNA.

This mutation results in a deletion of two amino acids in the protein, affecting fibrin polymerization, Ca^{2+} binding, and the protective effect of Ca^{2+} on plasmin degradation.

EXPERIMENTAL PROCEDURES

Materials

A reversed phase C-18 column, 250 x 4.6 mm, was obtained from Chrompack (Middelburg, The Netherlands). The HPLC device was manufactured by LKB (Biotechnology Inc.). Bovine thrombin was purchased from Leo (Ballerup, Denmark); Reptilase^R from Boehringer (Mannheim); $^{45}CaCl_2$ (3.92 Ci/mmol; 59 μg/ml) from Amersham; water for inorganic trace analysis, with a Ca^{2+} concentration below 3×10^{-7} M, from Fluka. Plasmin was prepared as described (33). Taq DNA polymerase was

obtained from New England Biolabs. Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer. Puc 13 *Sma* I cut/Bap, T7 sequence kit, dATP, dCTP, dGTP and dTTP were purchased from Pharmacia. DNA grade agarose and Chelex-100 were from Bio-rad, aprotinin from Bayer and X-ray film from Kodak.

Fibrinogen purification

Fibrinogen was purified from plasma of the propositus and two normal controls as described by Van Ruijven-Vermeer *et al.* (34). Venous blood was collected in 0.01% (w/v) EDTA and 40 KIU/ml Aprotinin. Plasma was prepared by centrifugation of the blood for 20 min. at 3000 x g and 4°C. The fibrinogen obtained was concentrated by precipitation in 50% saturated $(NH_4)_2SO_4$ solution, centrifuged, dissolved in 0.15 M NaCl and dialysed against this solution for 24 hours at 4°C. The purified fibrinogen solution was stored at -20°C.

Determination of various parameters in plasma

Venous blood was collected in one-tenth volume of 0.11 M sodium citrate. Platelet-poor plasma was prepared by centrifugation at 2300 x g for 30 min. at 4°C. Thrombin and Reptilase^R clotting times were obtained as described (35). The concentration of plasma fibrinogen was measured by radial immunodiffusion (36) and by the functional assay according to Clauss (37). Fibrin(ogen) degradation products were measured in plasma using a monoclonal antibody-based immunoassay (38). Antithrombin III was measured by a chromogenic assay (39), and protein C (40) and protein S (41) were measured by an immunoassay.

Coagulation of purified fibrinogen

Thrombin and Reptilase^R clotting times of purified fibrinogen were determined as described (35). Release of FpA and FpB was determined essentially according to Kehl *et al.* (42) and modified as described previously (35). Fibrin polymerization was determined by measuring the increase in absorbance at 350 nm as a function of time using a spectro-photometer (Pye Unicam SP 1700). Fibrinogen solutions used had been dialysed against 0.1 M Tris-HCl pH 7.5 containing 1 mM $CaCl_2$ or 1mM EDTA. The fibrinogen concentration was determined by measuring the absorbance at 280 nm ($A^{1\%,1cm}$ = 15.0) and adjusted to 0.25 mg/ml. To initiate polymerization, 10 μl of thrombin (10 NIH/ml) or undiluted Reptilase^R solution was added to 0.7-ml aliquots of these fibrinogen solutions.

SDS-PAGE

SDS-PAGE was performed according to Laemmli (43) or Weber and Osborn (44).

Plasmin degradation of fibrinogen

Plasmin degradation of fibrinogen in the presence of Ca^{2+} or EDTA was performed as described (8). Purified fibrinogen was dialysed against 0.04 M sodium 5,5-dietylbarbiturate, pH 7.8, containing 0.1 M NaCl, and the fibrinogen concentration was adjusted to 0.5 mg/ml. To 0.5 ml of fibrinogen solution was added 10 μl of 0.1 M $CaCl_2$ or 0.1 M EDTA. After mixing and adding 10 μl of a plasmin solution containing 0.13 mg/ml, the samples were incubated for 120 min at 37 °C. The reaction was stopped by adding an equal volume of incubation buffer containing 0.1 M Tris-HCl, pH 6.8, 2 % (w/v) SDS, 7 M urea and 5% v/v 2-mercapthoethanol, the samples were boiled for 10 min and 25 μl of each sample was loaded on a 10% SDS-PAGE gel.

Calcium binding of fibrinogen

Ca^{2+} binding of fibrinogen was performed essentially as described by Marguerie *et al.* (45). Fibrinogen solutions were treated with EGTA, and buffer solutions were prepared in water with a Ca^{2+} concentration below 3×10^{-7} M and treated with Chelex-100 to remove traces of Ca^{2+} ions (45). Ca^{2+} binding was measured at 15 different Ca^{2+} concentrations *i.e.* 1×10^{-4} M; 8,6,4,3,2,1.5 and 1×10^{-5} M; and 8,6,4,3,2,1.5, and 1×10^{-6} M in 5 mM Tris-HCl, pH 7.5, or 5 mM sodium acetate, pH 5.8, buffer, both containing 0.5 M NaCl.

A 0.25 ml volume of fibrinogen solution (3.0 mg/ml) was dialysed against 100 ml of the Ca^{2+}-free buffers containing one of the above mentioned Ca^{2+} concentrations and 10 μl $^{45}CaCl_2$ stock solution. After 48 hours of dialysis at 25 °C, 0.1 ml of inner and outer fluid was added to 10 ml of scintillation fluid and counted on a liquid scintillation counter (1900CA Tricarb, Packard). The data were quantified according to Scatchard (46).

Isolation of genomic DNA

Genomic DNA was isolated from the blood cells which remained after plasma had been collected for fibrinogen purification. The cells were stored at -70 °C until use. The isolation of genomic DNA was performed as described by Gustafson *et al.* (47).

DNA amplification, cloning and sequencing

Primer pair 4a (5'GAGGAGGGTCAGCATGTGATGGTTGAATTCCCTTC 3') and 4b (5'AACTTGGAATCTAAGAAAGGAAAACATACC 3') and primer pair 5a (5' ATGTACATCTACGACTTGTTTTAG 3') and 5b (5'AAAAAAGGAATTCTCTTTTG-AAACGGTC 3') were used for amplification of a 348- and a 780-bp fragment of the fibrinogen γ-chain gene including exon VIII (4a/4b) or exon IX, intron I and exon X (5a/5b) respectively. Amplification was performed in a 100 μl reaction volume containing 1 μg of genomic DNA, 0.2 mM each of dATP, dCTP, dGTP and dTTP, and 0.3 μM each of primer a and b in 1 X reaction buffer (10 mM Tris-HCl, pH 8.3 at 25 °C, 50 mM KCl,

3.0 mM $MgCl_2$ and 0.01% (w/v) gelatin). The DNA was denatured at 95°C for 8 min after which 1.0 unit Taq DNA polymerase was added. Each cycle consisted of a 1 min 95°C, 0.5 min 60°C and 3-min 72°C incubation. After 30 cycles, 10 µl of a sample was run on a 1.5% (w/v) agarose gel, from which the band with the expected size was isolated and cloned into Puc 13/*SmaI*/Bap. Individual clones were sequenced using the T7 sequence kit.

RESULTS

Clinical and laboratory data

A 23 year old woman was hospitalized suffering from massive pulmonary embolism as diagnosed from X-ray and perfusion scan. The laboratory studies revealed that the functional fibrinogen values were lower than the gravimetric and immunological values and that both the thrombin and Reptilase^R clotting time were prolonged (Table 1). The fibrin(ogen) degradation products and protein C concentrations were slightly increased, while antithrombin III activity and protein S antigen were within the normal range (Table 1). The father as well as the daughter of the propositus showed low functional fibrinogen values and prolonged clotting times by thrombin and Reptilase^R, but no clinical symptoms.

Table 1. Parameters of normal plasma and the patient's plasma (Vlissingen).

	Normal	Vlissingen
Thrombin time (s)	18.6	57.5
Reptilase^R time (s)	20.1	41.3
Fibrinogen concentration (mg/ml)		
- Clauss	2 to 4	0.4
- Gravimetric	2 to 4	1.8
- Immunologic	2 to 4	3.1
FDP (µg/ml)	< 0.5	0.8
Antithrombin III (% of normal)	75-120	83
Protein C (% of normal)	75-135	153
Protein S (% of normal)	67-125	91

SDS-PAGE analysis of purified fibrinogen

Purified fibrinogen Vlissingen analyzed by SDS-PAGE in the Laemmli system (43) showed two types of γ-chains: the normal γ-chain with an apparent M_r of 48,000 and the γ-chain variant γ-Vlissingen with an apparent M_r of 46,500 (Fig. 1). Higher concentrations of 2-mercapthoethanol and longer reduction times had no effect on the subsequent electrophoretic migration rate of γ-Vlissingen, indicating that the cleavage of intrachain disulfide bonds was complete. In contrast, SDS-PAGE performed according to Weber and Osborn (44) showed no difference in electrophoretic migration between normal γ-chain and the γ-variant. Similar observations have been reported for a number of other abnormal fibrinogens, all having a single amino acid substitution in the carboxyl terminus of the γ-chain (11,13,14,18,20,21,22).

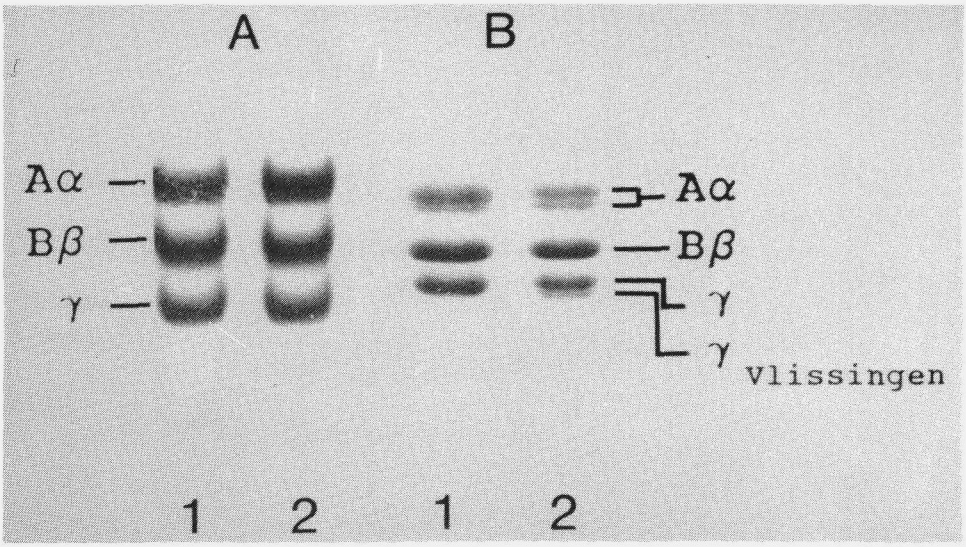


Figure 1. SDS-PAGE of purified fibrinogen after reduction with 2-mercapthoethanol.
(A) 8% SDS-PAGE gel according to Weber and Osborn (B). 10% SDS-PAGE gel according to Laemmli. Lane 1, normal fibrinogen, lane 2, fibrinogen Vlissingen.

Fibrinopeptide release and fibrin polymerization

The HPLC profiles of the FpA and FpB released by thrombin or Reptilase^R from fibrinogen Vlissingen were normal (data not shown). As shown in Fig. 2, the rate of thrombin catalyzed fibrinopeptide release from fibrinogen Vlissingen was equivalent to that of normal fibrinogen. In contrast, polymerization of fibrinogen Vlissingen induced by thrombin or Reptilase^R was impaired. When thrombin was used to induce polymerization, the impairment was more pronounced in the presence of EDTA (Fig. 3B) than in the presence of Ca^{2+} (Fig. 3A). When Reptilase^R was used in the presence

of EDTA (Fig. 3D) no polymerization was detected; the addition of calcium ions only partially restored polymerization (Fig. 3C).

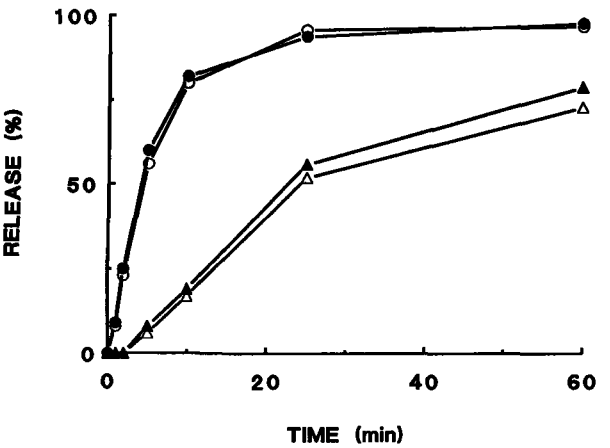


Figure 2. Percent FpA and FpB release from purified fibrinogen by thrombin as determined by HPLC. Normal FpA (●), Normal FpB (▲), Vlissingen FpA (○) and Vlissingen FpB (△).

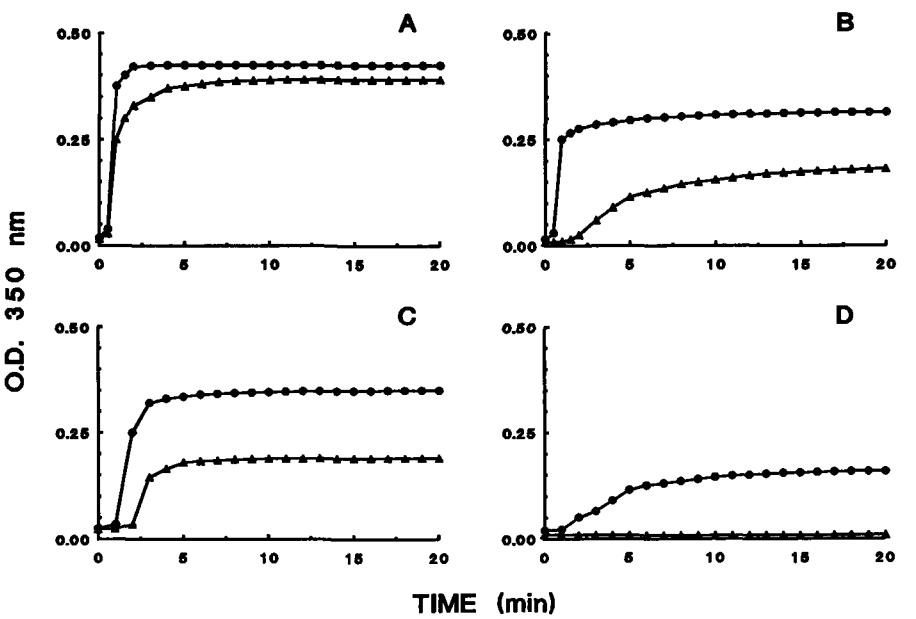


Figure 3. Coagulation profiles of purified fibrinogen.
Rate and degree of coagulation of normal (●) and Vlissingen (▲) fibrinogen induced by thrombin in the presence of (A) Ca^{2+}(1 mM) or (B) EDTA (1 mM) and by Reptilase^{R} in the presence of (C) Ca^{2+}(1 mM) or (D) EDTA (1 mM).

Plasmic digestion of fibrinogen in the presence of calcium or EDTA

The above results prompted us to study the γ-chain more closely. Since the carboxyl terminus of the γ-chain of normal fibrinogen is protected against plasmin degradation by Ca^{2+} ions (8), we analyzed reduced plasmin digests of fibrinogen Vlissingen formed in the presence of Ca^{2+} or EDTA. As shown by SDS-PAGE in Fig. 4, the plasmin digest of fibrinogen Vlissingen formed in the presence of Ca^{2+} ions, differed from that of normal fibrinogen. With fibrinogen Vlissingen a band with the electrophoretic mobility of the γ-chain of normal fragment D_3 was formed in the presence of Ca^{2+} ions; a band equivalent to the γ-chain of normal fragment D_1 was also present in this plasmin digest. The plasmin degradation products formed in the presence of EDTA from normal fibrinogen and fibrinogen Vlissingen had the same electrophoretic mobility. This result indicates that fibrinogen Vlissingen is heterozygous for the defect, which is consistent with the two γ-chains demonstrated in Fig. 1B. As the protective effect of Ca^{2+} ions against plasmin degradation of the carboxyl terminus of the γ-chain, is related to the Ca^{2+} binding to this part of the molecule, we determined the Ca^{2+} binding to fibrinogen Vlissingen.

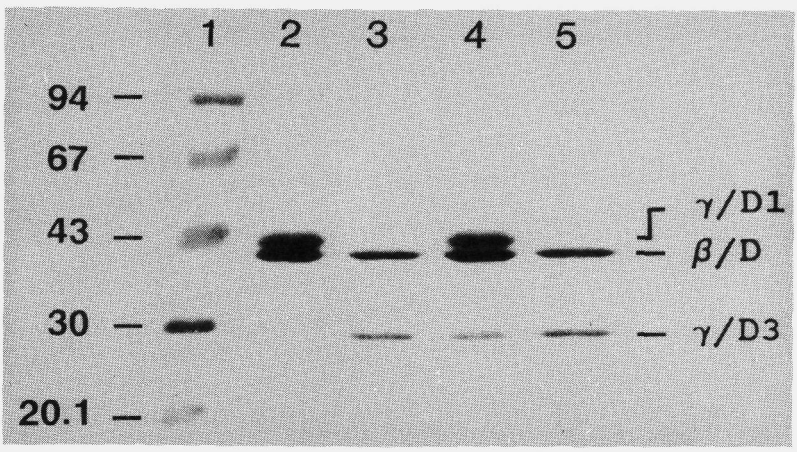


Figure 4. Plasmic degradation of the fibrinogen γ-chain analyzed by SDS-PAGE according to Laemmli after reduction with 2-mercapthoethanol.
Lane 1, low molecular weight standard (Pharmacia); lanes 2 and 3, plasmic digest of normal fibrinogen in the presence of Ca^{2+} and EDTA respectively; lanes 4 and 5, plasmic digest of fibrinogen Vlissingen in the presence of Ca^{2+} and EDTA respectively.

Calcium binding to fibrinogen

Using equilibrium dialysis, we quantitively compared Ca^{2+} binding of fibrinogen isolated from the propositus with that of normal fibrinogen. We measured binding at pH 7.5,

where three moles of Ca^{2+} bind per mole of fibrinogen (5,6) and at pH 5.8, where fibrinogen (bovine) binds only two moles of Ca^{2+} per mole of fibrinogen (45). Scatchard analysis of the calcium binding data (Fig. 5A) show at pH 7.5 approximately 3 Ca^{2+} binding sites with a K_d of 2.6 x 10^{-5} M in normal fibrinogen, which is in agreement with previously reported data (5,6). In contrast, fibrinogen isolated from the propositus contained 2.4 binding sites with a K_d of 2.8 x 10^{-5} M (Fig. 5A). At pH 5.8, normal fibrinogen showed 2.0 Ca^{2+} binding sites with a K_d of 2.4 x10^{-5} M and fibrinogen isolated from the propositus showed only 1.1 sites with a K_d of 1.8 x10^{-5} M (Fig. 5B). The observed decrease in the number of binding sites from 3 at pH 7.5 to 2 at pH 5.8 is the same as that reported for bovine fibrinogen (45). In contrast to the results with bovine fibrinogen, our results obtained with human fibrinogen show that the K_d at pH 7.5 does not differ significantly from that at pH 5.8.

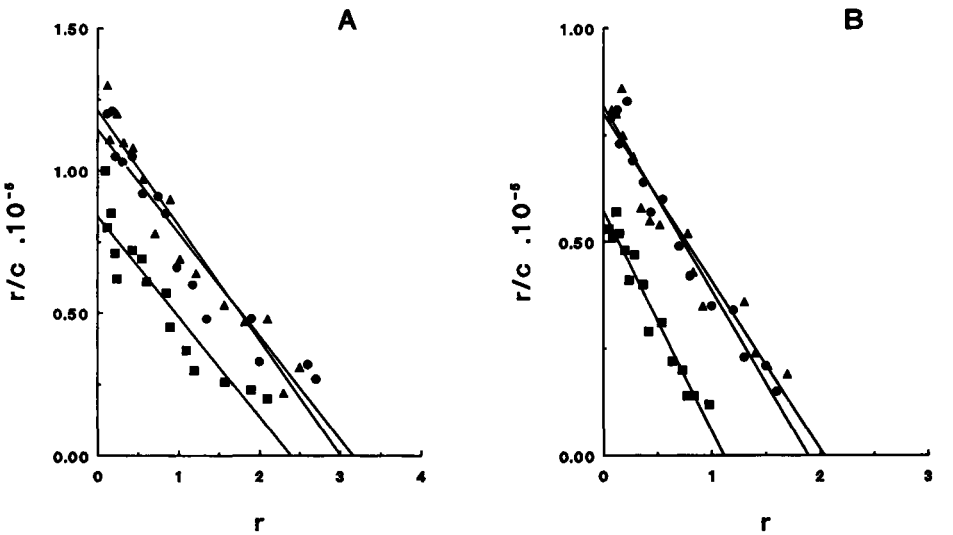


Figure 5. Scatchard plot of Ca^{2+} binding to purified fibrinogen.
Measured at pH 7.5 (A) and 5.8 (B) for two normal samples of fibrinogen (●▲) and fibrinogen Vlissingen (■). "r" is the number of Ca^{2+} ions bound per molecule of fibrinogen and "c" is the equilibrium concentration (M). The Ca^{2+} concentration was varied between 10^{-4} M and 10^{-6} M.

Amplification and DNA sequence analysis of fibrinogen γ-chain gene exon VIII, IX and X

Based on evidence implicating the γ-chain, we determined the DNA sequence of polymerase chain reaction-amplified genomic DNA coding for the carboxyl terminus of the γ-chain. After 30 cycles of amplification, the polymerase chain reaction samples

contained DNA fragments of the size predicted from the genomic DNA sequence (29). These fragments were cloned and individual isolates were sequenced. All the clones containing the γ-chain exon IX and X fragment showed the normal γ-chain gene sequence. We sequenced 12 clones containing the exon VIII fragment. Five clones had the normal sequence, but seven clones had a six-base deletion (Fig. 6) corresponding to amino acids Asn 319 and Asp 320. Since about half of the clones containing the exon VIII fragment showed this deletion, we conclude that the patient is heterozygous for this mutation.

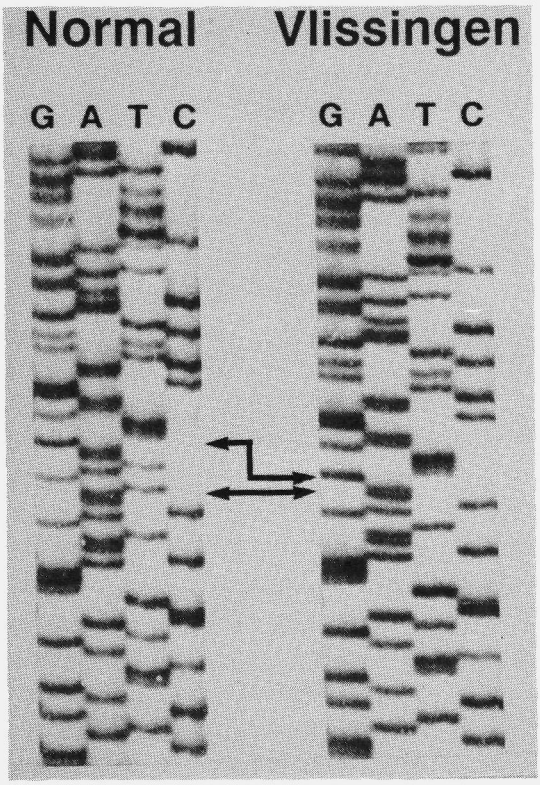


Figure 6. DNA sequence analysis of individual clones containing the amplified γ-chain gene exon VIII fragment, coding for normal fibrinogen and fibrinogen Vlissingen (arrows indicate the deletion).

DISCUSSION

Using the polymerase chain reaction, we have identified a six-base deletion in the gene coding for the γ-chain of the abnormal fibrinogen designated fibrinogen Vlissingen. SDS-PAGE analysis of plasmic digests of fibrinogen Vlissingen in the presence of Ca^{2+} ions or EDTA show that the protective effect of Ca^{2+} ions is only partially present in fibrinogen Vlissingen. This protective effect is associated with the calcium binding site located in the carboxyl terminus of the γ-chain. We, therefore, designed oligonucleotide

primers which spanned the γ-chain carboxyl terminal exons, amplified these genomic segments, and cloned and sequenced the amplified products. Sequence analysis demonstrated that the patient was heterozygous for a deletion where the codons for Asn-319 and Asp-320 of the γ-chain were missing. The size of this deletion probably arises from its location within a sequence repeat where the repeat unit is six bases. Deletion of a repeat unit, by either unequal crossing-over during meiosis or strand slippage during DNA replication, is a commonly noted mutation.

On SDS-PAGE using the method of Laemmli, γ-Vlissingen showed an apparent M_r approximately 1500 smaller then the normal γ-chain. The two amino acid deletion, however, justified a decrease of only 265 Da. Such unexpected changes in mobility on Laemmli gels have been observed previously with mutant chains, even when only a single amino acid substitution was found. These changes have been ascribed to changes in hydrophobicity or local conformation, which then alters the mobility of the protein-SDS complexes (48,49).

The interaction of Ca^{2+} ions with fibrinogen Vlissingen was measured by equilibrium dialysis. Scatchard analysis of the data showed a decrease in the number of high-affinity binding sites for Ca^{2+} ions in fibrinogen Vlissingen as compared with normal fibrinogen. As has been described for bovine fibrinogen (45), we found that the number of high affinity binding sites in human fibrinogen depends on the pH. At both pH values 5.8 and 7.5, the number of sites was decreased in fibrinogen Vlissingen. The difference in the number of Ca^{2+} binding sites between normal fibrinogen and fibrinogen Vlissingen is the clearest at pH 5.8, because under these conditions two identical high affinity binding sites remain in normal fibrinogen, being those in the carboxyl terminus of the two D domains (45). We therefore conclude that the deleted amino acids are essential for the calcium binding associated with the D domain of fibrinogen. Previously, this binding site was localized in the region γ 303-336 (50). On the basis of amino acid sequence analogy with known calcium binding sites in a number of proteins and the results obtained with different spectrophotometric methods, it has been suggested that the amino acids involved in calcium binding are γ Asp-316, Asp-318, Asp-320, Phe-322, Gly-324 and Glu-328 (51). Since amino acids Asn-319 and Asp-320 are missing in fibrinogen Vlissingen, our results are consistent with this hypothesis.

As the thrombin-catalyzed release of FpA and FpB was normal and the defect is localized in the carboxyl terminus of the γ-chain, we conclude that the prolonged clotting time of fibrinogen Vlissingen is due to defective fibrin polymerization. Since the presence of Ca^{2+} ions is known to increase the rate of fibrin polymerization (52) by a number of mechanisms (53,54), the loss of the high affinity calcium binding site in the D domain may fully account for the altered polymerization. However, this is unlikely because the results with fibrinogen Vlissingen using Reptilase^R (Fig. 3D) or thrombin (Fig. 3B) show that polymerization in the absence of calcium is also altered.

The polymerization defect in fibrinogen Vlissingen with Reptilase^R in the absence of Ca^{2+} indicates that the "a" polymerization site is defective. This is in conjunction with the data of Laudano and Doolittle (53), who demonstrated that the presence of Ca^{2+} ions do not significantly alter the interaction between the carboxyl terminal "a" polymerization site and a peptide analog of the amino-terminal "A" polymerization site. Whether there is an additional influence of the defective calcium binding on polymerization of fibrinogen Vlissingen is difficult to determine, because our measurement of polymerization requires formation of aggregates through multiple interactions, and because the propositus with fibrinogen Vlissingen is heterozygous. Thus it is not possible to interpret our results more specifically. However, our data clearly demonstrate an association of this small γ-chain deletion with altered fibrin polymerization.

In summary, we have used the polymerase chain reaction to determine the structural defect in an abnormal fibrinogen, and have found a six-base deletion encoding Asn 319 and Asp 320 of the γ-chain. Studies on the patient's plasma and purified fibrinogen indicated that the mutation is associated with reduced calcium binding and impaired fibrin polymerization. This study demonstrates the effectiveness of studies on dysfibrinogenemias where 1) the primary abnormality of this large complex protein can readily be identified by DNA sequence analysis, and 2) the altered protein can be functionally characterized in vitro.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Dana Fowlkes for synthesizing the oligonucleotides. This work was supported by NATO grant CRG 860110 and NIH grant HL 31048.

REFERENCES

1. Olexa, S.A., and Budzynski, A.Z. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1374-1378
2. Varadi, A., and Scheraga, H.A. (1986) *Biochemistry* **25**, 519-528
3. Nieuwenhuizen, W.M., Voskuilen, M., Vermond, A., Haverkate, F., and Hermans, J. (1982) *Biochim. Biophys. Acta* **707**, 190-192
4. Lindsey, G.C., Brown, G., and Purves, L.R. (1978) *Thromb. Res.* **13**, 345-350
5. Nieuwenhuizen, W., Vermond, A., Nooijen, W.J., and Haverkate, F. (1979) *FEBS Lett.* **98**, 257-259
6. Nieuwenhuizen, W., Ruijven-Vermeer, I.A.M., Nooijen, W.J., Vermond, A., and Haverkate, F. (1981) *Thromb. Res.* **22**, 653-657
7. Nieuwenhuizen, W., and Haverkate, F. (1983) in *Molecular Biology of Fibrinogen and Fibrin* (Mosesson, M.W., and Doolittle, R.F., eds) Vol. 408, pp. 92-96, Annals of the New York Academy of Sciences, New York
8. Haverkate, F., and Timan, G. (1977) *Thromb. Res.* **10**, 803-812

9. Ebert, R. (1990) *Index of variant human fibrinogens,* 1990 Edition (Ebert, R.F. ed) Published by Ebert, R.F., Rockville, P.O. Box 5811, Maryland 20855, USA
10. Schmelzer, C.H., Ebert, R.F., and Bell, W.R. (1989) *Thromb. Res.* **56,** 307-317
11. Ebert, R.F., and Bell, W.R. (1988) *Thromb. Res.* **51,** 251-258
12. Terukina, S., Matsuda, M., Yoshida, N., Yamazumi, K., Takeda, Y., and Takano, T. (1987) *Thromb. Haemostas.* **58,** 515
13. Terukina, S., Matsuda, M., Hirata, H., Takeda, Y., Miyata, T., Takao, T., and Shimonishi, Y. (1988) *J. Biol. Chem.* **263,** 13579-13587
14. Yoshida, N., Ota, K., Moroi, M., and Matsuda, M. (1988) *Blood* **71,** 480-487
15. Reber, P., Furlan, M., Henschen, A., Kaudewitz, H., Barbui, T., Hilgard, P., Nenci, G.G., Berrettini, M., and Beck, E.A. (1986) *Thromb. Haemostas.* **56,** 401-406
16. Siebenlist, K.R., Mosesson, M.W., Di Orio, J.P., Tavori, S., Tatarsky, I., and Rimon, A. (1989) *Thromb. Haemostas.* **62,** 875-879
17. Yamazumi, K., Terukina, S., Onohara, S., and Matsuda, M. (1988) *Thromb. Haemostas.* **60,** 476-480
18. Yoshida, N., Terukina, S., Okuma, M., Moroi, M., Aoki, N., and Matsuda, M. (1988) *J. Biol. Chem.* **263,** 13848-13856
19. Yamazumi, K., Shimura, K., Terukina, S., Takahashi, N., and Matsuda, M. (1989) *J. Clin. Invest.* **83,** 1590-1597
20. Miyata, T., Furukawa, K., Iwanaga, S., Takamatsu, J., and Saito, H. (1989) *J. Biochem. (Tokyo)* **105,** 10-14
21. Terukina, S., Yamazumi, K., Okamoto, K., Yamashita, H., Ito, Y., and Matsuda, M. (1989) *Blood* **74,** 2681-2687
22. Reber, P., Furlan, M., Rupp, C., Kehl, M., Henschen, A., Mannucci, P.M., and Beck, E.A. (1986) *Blood* **67,** 1751-1756
23. Soria, J., Soria, C., Samama, M., Tabori, S., Kehl, M., Henschen, A., Nieuwenhuizen, W., Rimon, A., and Tatarski, I. (1987) *Thromb. Haemostas.* **57,** 310-313
24. Yamazumi, K., Terukina, S., Onohara, S., and Matsuda, M. (1988) *Thromb. Haemostas.* **60,** 476-480
25. Henschen, A., Kehl, M., Southan, C., Lottspeich, F., and Georgopoulos, D. (1983) in *Fibrinogen-Structure, Functional Aspects, Metabolism* (Haverkate, F., Henschen, A., Nieuwenhuizen, W., and Straub, P.W., eds) Vol. 2, pp. 125-144, Walter de Gruyter & Co., Berlin
26. Kant, J.A., Lord, S.T., and Crabtree, G.R. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3953-3957
27. Chung, D.W., Que, B.G., Rixon, M.W., Mace, M., and Davie E.W. (1983) *Biochemistry* **22,** 3244-3250
28. Chung, D.W., Chan, W-Y., and Davie E.W. (1983) *Biochemistry* **22,** 3250-3256
29. Rixon, M.W., Chung, D.W., and Davie, E.W. (1985) *Biochemistry* **24,** 2077-2086
30. Saiki, R.K., Bugawan, G.T., Horn, K.B., and Erlich, H.A. (1986) *Nature* **324,** 163-166
31. Koopman, J., Haverkate, F., Lord, S.T., and Lane, D. (1988) in *Fibrinogen 3. Biochemistry, Biological Functions, Gene Regulation and Expression* (Mosesson, M.W., Amrani, D.L., Siebenlist, K.R., and Diorio, J.P., eds) pp. 275-278, Elsevier Science Publishers, B.V., Amsterdam
32. Koopman, J., and Haverkate, F. (1990) in *Fibrinogen 4. Current Basic and Clinical Aspects* (Matsuda, M., Iwanaga, S.I., Takada, A., and Henschen, A., eds.) pp. 179-183, Elsevier Sience Publishers, B.V., Amsterdam
33. Nieuwenhuizen, W., and Gravesen, M. (1981) *Biochim. Biophys. Acta* **668,** 81-88.
34. Van Ruijven-Vermeer, I.A.M., and Nieuwenhuizen, W. (1978) *Biochem. J.* **169,** 653-658
35. Haverkate, F., Koopman, J., Kluft, C., D'Angelo, A., Cattaneo, M., and Mannucci, P.M. (1986) *Thromb. Haemostas.* **55,** 131-135
36. Mancini, G., Carbonara, A.O., and Heremans, J.F. (1965) *Immunochem.* **2,** 235-254
37. Clauss, A. (1957) *Acta Haematol.* **17,** 237-246
38. Koopman, J., Haverkate, F., Koppert, P., Nieuwenhuizen, W., Brommer, E.J.P., and Van der Werf, W.G.C. (1988) *J. Lab. Clin. Med.* **109,** 75-84

39. Abildgaard, U., Lie, N., and Odegaard, O.R. (1977) *Thromb. Res.* **11**, 549-553
40. Bertina, R.M., Broekmans, A.W., van der Linden, I.K., and Mertens, K. (1982) *Thromb. Haemostas.* **48**, 1-5
41. Bertina, R.M., van Wijngaarden, A., Reinalda-Poot, J., Poort, S.R., and Bom, V.J. (1985) *Thromb. Haemostas.* **53**, 268-272
42. Kehl, M., Lottspeich, F., and Henschen, A. (1981) *Hoppe Seyler's Z. physiol. Chem.* **362**, 1661-1664
43. Laemmli, U.K (1970) *Nature* **227**, 680-685
44. Weber, K., and Osborn,M. (1969) *J. Biol. Chem.* **244**, 4406-4412
45. Marguerie, G., Chagniel, G., and Suscillon, M. (1977) *Biochim. Biophys. Acta* **490**, 94-103
46. Scatchard, G. (1949) *Ann. NY Acad. Sci.* **51**, 660-672
47. Gustafson, S., Proper, J.A., Bowie, E.J.W., and Sommer, S.S. (1987) *Anal. Biochem.* **165**, 294-299
48. de Jong, W.W., Zweers, A., and Cohen, L.H. (1978) *Biochem. Biophys. Res. Commun.* **82**, 532-53952
49. Noel, D., Nikaido, K., and Ames, F-L. (1979) *Biochemistry* **18**, 4159-4165
50. Dang, C.V., Bell, W.R., Ebert, R.F., and Starksen, N.F. (1985) *Archiv. Biochem. Biophys.* **238**, 452-457
51. Dang, C.V., Ebert, R.F., and Bell, W.R. (1985) *J. Biol. Chem.* **260**, 9713-9719
52. Ratnoff, O.D., and Potts, A.M. (1954) *J. Clin. Invest.* **33**, 206-210
53. Laudano, A.P., and Doolittle. R.F. (1981) *Science* **212**, 457-459
54. Häberli, A., Straub, P.W., Dietler, G., and Känzig, W. (1987) *Biopolymers* **26**, 27-43

CHAPTER 4

ABNORMAL FIBRINOGENS IJMUIDEN (BβArg$_{14}$ → Cys) AND NIJMEGEN (BβArg$_{44}$ → Cys) FORM DISULFIDE-LINKED FIBRINOGEN-ALBUMIN COMPLEXES

Jaap Koopman[1,2], Frits Haverkate[1], Jos Grimbergen[2], Luzia Engesser[1], Irena Nováková[3], Anton F.J.A. Kerst[4] and Susan T. Lord[5]

1 Gaubius Institute TNO, Leiden, The Netherlands.
2 Department of Hematology, University Hospital Leiden, The Netherlands.
3 Saint Radboud Ziekenhuis, Nijmegen, The Netherlands.
4 Zeeweg Ziekenhuis, Velsen, The Netherlands.
5 Department of Pathology and Curriculum in Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, U.S.A.

SUMMARY

The molecular defects in two congenital abnormal fibrinogens, IJmuiden and Nijmegen, were determined by sequence analysis of genomic DNA amplified by the polymerase chain reaction. Both propositi are heterozgyous, IJmuiden having a $B\beta Arg_{14} \rightarrow$ Cys substitution and Nijmegen a $B\beta Arg_{44} \rightarrow$ Cys substitution. Clotting induced by thrombin or Reptilase^R was impaired in both fibrinogens, indicating defective fibrin polymerization. Immunoblot analysis of both purified fibrinogens demonstrated that some of the abnormal molecules were linked by disulfide bonds to albumin. In addition, abnormal high molecular weight fibrinogen complexes with M_rs between 600,000 and 700,000 were present. Fibrinogen-albumin and high molecular weight complexes were also detected in the patients' plasmas. Quantitative analysis demonstrated that of the total plasma fibrinogen in the IJmuiden patients, 20% was linked to albumin and 5% was present as high molecular weight complexes. In plasma Nijmegen, 13% was linked to albumin and 7% was present as high molecular weight complexes. These results demonstrate that the additional abnormal cysteine in fibrinogens IJmuiden and Nijmegen resulted in the formation of disulfide-linked complexes with other proteins, predominantly albumin. We also found that a significant fraction of the abnormal fibrinogen molecules contained free sulfhydral groups.

INTRODUCTION

The fibrinogen molecule is a dimer of three polypeptides, Aα, Bβ, and γ, linked together by 29 disulfide bonds (1). During coagulation fibrinogen is converted to an insoluble fibrin matrix by thrombin catalyzed removal of fibrinopeptides from the Aα and the Bβ chains. Congenital dysfibrinogenemia is a disorder in which a fibrinogen structural abnormality results in altered functional characteristics. A number of abnormal fibrinogens have been described (2), some associated with bleeding disorders and some associated with thrombosis. The structural defects of approximately 75 cases are known, with 21 cases of arginine substituted by cysteine, 14 cases of $A\alpha Arg_{16} \rightarrow$ Cys, 2 cases of $B\beta Arg_{14} \rightarrow$ Cys and 5 cases of $\gamma Arg_{275} \rightarrow$ Cys. For a few cases the consequences of an additional cysteine have been studied. For homozygous fibrinogen Metz (3) and heterozygous fibrinogens Kawaguchi and Osaka I (4) it has been reported that $A\alpha Cys_{16}$ participates in an intramolecular disulfide bond between two abnormal Aα-chains. Consequently, in heterozygous individuals, abnormal molecules are homodimers; that is, both Aα chains in one fibrinogen molecule are abnormal. In contrast, analysis of another heterozygous $A\alpha Cys_{16}$ mutant, Stoney Brook I (5), indicated that heterodimers are

present. Homodimers of abnormal chains have been postulated for the two heterozygous cases of $B\beta Arg_{14} \rightarrow$ Cys, Christchurch II (6) and Seattle I (7). The additional Cys in heterozygous fibrinogen Osaka II (8), $\gamma Arg_{275} \rightarrow$ Cys, was linked to a free cysteine molecule. Free sulfhydryl groups have not been detected in these abnormal fibrinogens.

This report describes the structural defects in two fibrinogens isolated from patients suffering from thrombophilia. These are fibrinogen IJmuiden, $B\beta Arg_{14} \rightarrow$ Cys and fibrinogen Nijmegen, $B\beta Arg_{44} \rightarrow$ Cys. We found that these abnormal fibrinogens circulate as disulfide linked fibrinogen-albumin complexes, as disulfide linked high molecular weight fibrinogen complexes and as molecules with free sulfhydryl groups.

MATERIALS AND METHODS

Materials

Unspecified materials were obtained from sources previously described (9). Crotalus Atrox venom, DNA grade (A-6013) and Ultra Low Gelling (A-5030) agarose, were from Sigma. CH-activated Sepharose 4B, Gelatine-Sepharose 4B, precast polyacrylamide gradient gels (2-16%), SDS-PAGE low molecular weight protein calibration kit, T7 DNA sequence kit and an FPLC system equipped with a Superose 12 column were obtained from Pharmacia (Uppsala). Rabbit anti-human albumin antiserum was purchased from Behringwerke AG (Marburg, Germany), peroxidase conjugated (HRP) goat anti-human albumin and goat anti-rabbit immunoglobulins were from Nordic Immunological Laboratory (Tilburg, NL), and rabbit anti-human fibrinogen $B\beta$ 1-42 from IMCO (Stockholm). A pool of rabbit anti-human fibrinogen IgGs was prepared as described (10). The monoclonal anti-fibrinogen antibody (11) conjugated with HRP (Y18/HRP) was a generous gift of Dr. W. Nieuwenhuizen (Gaubius Laboratory, Leiden, NL). Nitrocellulose was from Schleicher and Schuell (Dassel, Germany). Polystyrene microtiter plates (Immulon) were from Greiner (Alphen a/d Rijn, NL). Ellman's reagent (DTNB) and 4-chloro-1-naphthol were from Aldrich (Beerse, Belgium). PBS, pH 7.4, contained 0.15 M NaCl, 0.01 M Na_2HPO_4 and 1.6 mM KH_2PO_4.

Coagulation Studies

Blood was collected and plasma was prepared as described (12). Thrombin and Reptilase^R clotting times were performed as described (13). The plasma concentration of fibrinogen was determined functionally according to Clauss (14) and immunologically according to Mancini (15). Release of FpA and FpB from purified fibrinogen was determined as described (13). Coagulation profiles of purified fibrinogen dialyzed against 0.1 M Tris/HCl, pH 7.5, were determined by measuring the increase in absorbance at 350 nm (9).

Protein Purification

Fibrinogen was purified from plasma (16), contaminating fibronectin removed by adsorption to a gelatin-sepharose column, and the fibrinogen concentrated by precipitation in 50% saturated $(NH_4)_2SO_4$. The precipitate was dissolved in 0.15 M NaCl, dialyzed against this solution for 24 hrs at 4°C and stored at -20°C. Fibronectin was eluted from the gelatin-sepharose column with 0.05 M Tris/HCl, pH 7.5, containing 1.0 M arginine (17), dialyzed against distilled water for 24 hrs at 4°C, and lyophilized. Rabbit anti-human albumin IgGs were purified from serum by precipitation with 18% Na_2SO_4 (18) and the precipitate dissolved in and dialyzed against distilled water for 24 hrs at 4°C, and lyophilized.

SDS-PAGE

SDS-PAGE was performed on 5-25% gradient gels according to Laemmli (19) or on 2-16% precast gels using an electrophoresis buffer of 0.04 M Tris, 0.02 M sodium acetate, 0.002 M EDTA, 0.02% SDS, pH 7.4, and a sample buffer of 0.01 M Tris/HCl, 0.001 M EDTA, 1% SDS, pH 8.0. Molecular weights were estimated using a low molecular weight calibration kit and a mixture of monoclonal IgM, fibronectin and fibrinogen with molecular weights of 900 kD, 450 kD and 340 kD, respectively.

Immunoblot Analysis

SDS-PAGE gels were electroblotted onto nitrocellulose (20) for 16 hrs at 400 mA and 10°C. Intact proteins and Crotalus Atrox venom digests were transferred to 0.45 μm and 0.1 μm nitrocellulose sheets, respectively. The sheets were incubated with 0.01 M Tris/HCl, pH 7.4, with 0.15 M NaCl, 0.5% gelatin and 0.05% Tween 80 for 2 hrs. Antibodies were diluted in 0.01 M Tris/HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween 80 and crossreacting bands visualized with 4-chloro-1-naphthol (21).

Cotalus Atrox Venom Digestions

Fibrinogen was dialyzed against 0.05 M Tris/HCl, pH 7.5, 0.1 M NaCl, 5 mM EDTA, and the concentration adjusted to 2 mg/ml. Lyophilized venom was dissolved in this buffer to 0.1 mg/ml and 5 μl added to 0.5 ml of fibrinogen. The sample was incubated for 1 hr at 37°C and the reaction was stopped by adding an equal volume of 0.1 M Tris/HCl, pH 6.8, 2% SDS and 8 M urea, followed by boiling for 5 min. When reducing disulfide bonds, 5% (v/v) 2-mercaptoethanol was added before boiling.

Purification of fibrinogen-albumin complexes

Purified rabbit anti-human albumin IgGs were immobilized to activated CH Sepharose 4B following the manufacturer's guidelines. The amount of IgG coupled, calculated from the difference in IgG concentration in the gel supernatant before and after coupling, was

approximately 3 mg/ml wet gel. The immunoadsorbent was packed in a column (bed volume 2 ml) and equilibrated with 6 mM disodium tetraborate, 0.2 M boric acid and 5 mM EDTA, pH 7.4. Purified fibrinogen was diluted to 0.5 mg/ml with borate buffer, and 5 ml was applied to the column. The column was washed with 25 ml of borate buffer, followed by 10 ml of borate buffer containing 1 M NaCl to elute non-specifically bound protein. Albumin complexes were eluted with borate buffer containing 8 M urea and fractions containing protein were pooled, dialyzed against distilled water for 16 hours at 4°C, lyophilized and dissolved in PBS to a concentration of 0.1 mg/ml.

Enzyme immunoassays

Microtiter plate wells were incubated with 0.12 ml of rabbit anti-fibrinogen IgGs (20 μg/ml) for 16 hours at 4°C and washed three times with PBS and 0.05% Tween 20 (PBS/Tween). Samples (0.1 ml) diluted in PBS/Tween were added, incubated for 1 hr at room temperature, and the wells washed as above. Bound fibrinogen was measured by incubation for 1 hour at room temperature with 0.1 ml of rabbit anti-fibrinogen/HRP, prepared as described (10). Bound fibrinogen-albumin complexes were measured by incubating with 0.1 ml of goat anti-albumin/HRP for 1 hr at room temperature. The wells were washed and bound HRP quantitated by incubation with 0.2 ml of substrate solution containing H_2O_2 and 3,3',5,5'-tetramethylbenzidine (22). After 30 min 50 μl of 2 M H_2SO_4 was added and the absorbance at 450 nm measured on a Titertek Multiscan.

Gel filtration of plasma

Plasma (0.25 ml) was diluted with PBS (1:5) and applied to a FPLC Superose 12 column equilibrated with PBS. Fractions were assayed for fibrinogen and/or fibrinogen-albumin complexes using the enzyme immunoassay described above.

Titration of free sulfhydryl groups

Free sulfhydryl groups in fibrinogen were determined using DTNB (23) in the presence of urea (24). Purified fibrinogen was dissolved to 10 mg/ml in 0.05 M Tris/HCl, pH 8.2, 1 mM EDTA, 8 M urea. Five μl of 10 mM DTNB was added to 0.5 ml of fibrinogen and the sample incubated for 10 min at room temperature. The absorbance at 412 nm was measured and reactive sulfhydryls were determined from the molar extinction coefficient = 13,600 $M^{-1}cm^{-1}$ (23).

DNA amplification and sequence analysis

Genomic DNA was isolated from blood cells (25). Primers β1a (5'GGTGTTGGAATA-GTTACATTCC3') and β1b (5'ATCAGTGCACCCACCAAGTCTGGG3') amplified the DNA coding for amino acids Bβ 9 to 72. Amplification by PCR (26) was performed in 100 μl containing 1 μg genomic DNA, 0.2 mM each of dATP, dCTP, dGTP and dTTP,

0.3 μg each of primer β1a and β1b in 10 mM Tris/HCl, pH 8.3 at 25°C, 50 mM KCl, 3.0 mM $MgCl_2$ and 0.001% gelatin. The samples were heated at 94°C for 4 min, 2 units Taq DNA polymerase (Perkin-Elmer Cetus) were added, and the samples incubated for 30 cycles of 1 min 94°C, 0.5 min 58°C and 1.5 min 72°C. Amplified DNA was precipitated with ethanol, dried, dissolved in 20 μl of distilled water, and run on a 1.0% (w/v) ultra low gelling agarose gel. The band with the size predicted by the genomic sequence (27) was excised in approximately 20 μl, and heated to 55°C. One μl of the melted agarose was mixed with 1 μl of primer, β1a (60 ng), and 2 μl 5X annealing buffer (T7 sequence kit), and the volume was brought up to 14 μl with distilled water. The mixture was heated to 95°C for 3 min and immediately put on ice. Labeling (5 min) and termination (10 min) reactions were performed using the T7 DNA sequencing kit as specified by the manufacturer.

RESULTS

Coagulation studies

The thrombin clotting times for plasma IJmuiden (25.0s) and Nijmegen (20.2s), and the Reptilase^R clotting times (26.0s and 22.7s, respectively) were slightly prolonged compared to normal plasma (18.5s for thrombin and 20.0s for Reptilase^R). The functionally determined fibrinogen concentration in plasma IJmuiden (0.9 mg/ml) and Nijmegen (1.2 mg/ml) was less than half that determined immunologically (2.7 mg/ml and 3.5 mg/ml, respectively). Clotting of purified fibrinogen induced by either enzyme was impaired for both abnormal fibrinogens. Thrombin catalyzed release of FpB from fibrinogen IJmuiden was half that of normal fibrinogen. All other FpA and FpB release from either abnormal fibrinogen with either protease were normal.

DNA Sequence Analysis

We amplified the DNA from exon II of the Bβ-chain which codes for amino acids 9 to 72. DNA sequence analysis (Fig. 1) demonstrated that the amplified Bβ fragment from fibrinogen IJmuiden had a single base substitution in the codon for Arg_{14} (CGT), changing this to the codon for Cys (TGT). The corresponding Bβ gene fragment from fibrinogen Nijmegen had a similar mutation, changing the codon for Arg_{44} (CGT) to Cys (TGT). Direct sequence determination of the amplified fragments showed both the normal and the abnormal sequence indicating that these patients are heterozygous.

Analysis of fibrinogen cleavage with Crotalus Atrox venom

Crotalus Atrox venom cleaves the Bβ chain of fibrinogen between amino acids 42 and 43 (28). When purified fibrinogen IJmuiden was digested with venom and analyzed by

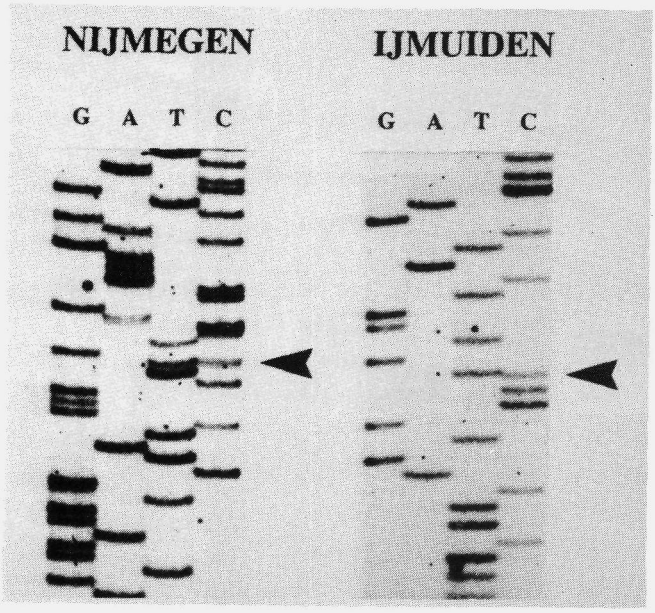


Figure 1. DNA sequence analysis of the amplified Bβ gene fragments. Direct sequence analysis of amplified fragments demonstrated heterozygous mutations at the positions indicated by the arrows. In both cases C was substituted by T.

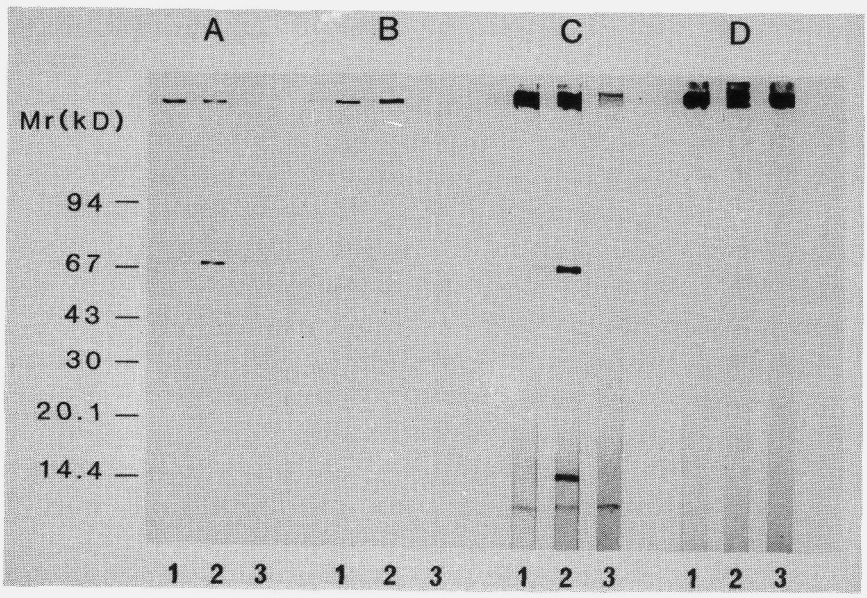


Figure 2. Immunoblot analysis of Crotalus Atrox digests of fibrinogen (panels A and C) and intact fibrinogen (panels B and D). Samples of fibrinogen Nijmegen, lanes 1, fibrinogen IJmuiden, lanes 2, and normal fibrinogen, lanes 3, were electrophoresed on a 5-25% polyacrylamide gradient SDS gel. Proteins were blotted onto nitrocellulose and developed with goat anti-albumin/HRP, panels A and B, or with rabbit anti-Bβ 1-42 followed by goat anti-rabbit IgG/HRP, panels C and D.

immunoblotting with a rabbit antibody to Bβ 1-42, four crossreacting species were found as shown in Fig. 2C, lane 2. Two of these, one which stays at the top of the gel and a second which has an M_r ~ 6000, were present in normal fibrinogen and probably are residual intact fibrinogen and the Bβ 1-42 cleavage product, respectively. The remaining two species with M_rs ~ 14,000 and 68,000 (Fig. 2C, lane 2) were not present in normal fibrinogen digests (Fig. 2C, lane 3). The band representing residual fibrinogen was more intense in fibrinogen IJmuiden than in normal fibrinogen (Fig. 2C, lane 3). SDS-PAGE analysis of reduced samples demonstrated that digests of fibrinogen IJmuiden contained slightly greater amounts of intact Bβ chain than digests of normal fibrinogen (data not shown), indicating that the snake venom activity was slightly inhibited by the IJmuiden mutation. No abnormal bands were seen in the reduced IJmuiden samples.

These results suggest that some of the Bβ 1-42 peptide in fibrinogen IJmuiden was linked to other proteins or peptides by disulfide bonds. The species with M_r ~ 14,000 is probably Bβ 1-42 dimer, a product previously suggested for two other $B\beta Arg_{14} \rightarrow Cys$ substitutions in abnormal fibrinogens Christchurch II (6) and Seattle I (7). To determine whether the additional band with M_r ~ 68,000 was due to complex formation with albumin, as described previously for an antithrombin III variant (29), we prepared blots with unreduced samples and developed these with anti-albumin/HRP (Fig. 2A and 2B). Fibrinogen IJmuiden digests (Fig. 2A, lane 2) contained two crossreacting species. One that remained at the top of the gel was also present with untreated fibrinogen IJmuiden, indicating that albumin is complexed with fibrinogen IJmuiden. The second had M_r ~ 68,000. Since this species also reacted with anti-Bβ 1-42, it is probably a disulfide linked complex between albumin and Bβ 1-42.

Immunoblot analysis of fibrinogen Nijmegen ($B\beta Arg_{44} \rightarrow Cys$) with anti-Bβ 1-42 demonstrated that after incubation with Crotalus Atrox venom a substantial amount of fibrinogen Nijmegen remained intact (Fig. 2C, lane 1), indicating that the venom did not efficiently cleave the mutated chain. A reduced amount of the species with M_r ~ 6,000 was present indicating that the normal Bβ chain was cleaved. SDS-PAGE analysis of reduced samples confirmed that approximately half of the Bβ chains in fibrinogen Nijmegen remained intact after incubation with Crotalus Atrox venom (data not shown). Immunoblot analysis showed only one species at the top of the gel which reacted with the anti-albumin antibodies (Fig. 2A and 2B, lane 1). This indicates that fibrinogen Nijmegen is also linked to albumin by disulfide bonds. Normal fibrinogen did not react with the anti-albumin antibodies (Fig. 2A and 2B, lane 3).

Analysis of intact fibrinogen

Purified fibrinogen samples were analyzed on 2-16% gradient SDS-PAGE gels stained with Coomassie blue. Normal fibrinogen (Fig. 3A, lane 3) contained two species previously shown (30,31) to be intact fibrinogen (HMW Fbg, M_r = 340,000) and

fibrinogen with a partially degraded Aα-chain (LMW Fbg, M_r = 300,000). These two species were present in both abnormal fibrinogens. In addition, each abnormal fibrinogen contained three minor species, one with M_r ~ 400,000 and two with M_rs between 600,000 and 700,000. Immunoblots prepared from similar gels and developed with a monoclonal anti-fibrinogen antibody (Fig. 3B) demonstrated that these minor species contained fibrinogen. Two additional novel crossreacting species of M_r = 360,000 and M_r between 600-700,000 were present. Similar blots developed with anti-albumin antibodies (Fig. 3C) showed that the novel species with M_rs = 360,000 and 400,000 contained albumin. These results indicate that fibrinogens Nijmegen and IJmuiden contain two fibrinogen-albumin complexes, which are probably albumin linked to LMW Fbg and HMW Fbg, respectively. The novel larger species may be dimers formed from HMW Fbg and LMW Fbg, or they may be complexes of fibrinogen with other proteins.

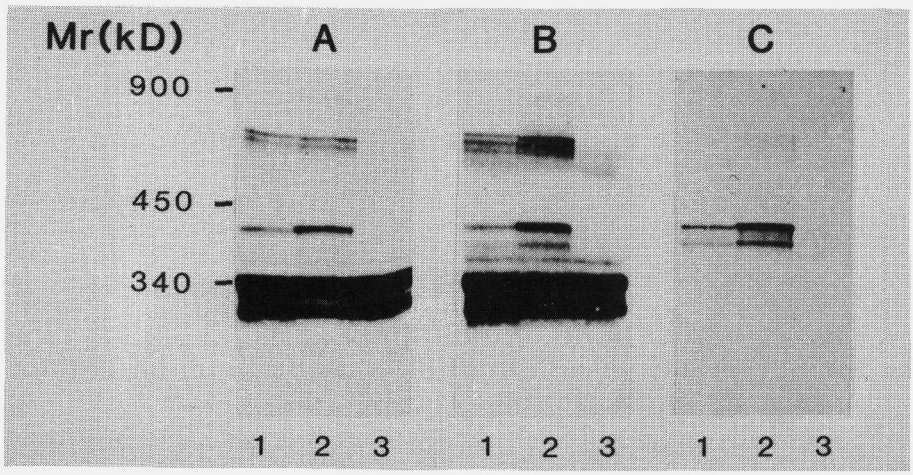


Figure 3. SDS-PAGE and immunoblot analysis of purified fibrinogen. Samples of fibrinogen Nijmegen, lanes 1, fibrinogen IJmuiden, lanes 2, and normal fibrinogen, lanes 3, were electrophoresed on 2-16% polyacrylamide gradient SDS gels and either stained with Coomassie blue (Panel A) or transferred to nitrocellulose and developed with a monoclonal antibody to fibrinogen (Y18/HRP, panel B) or with goat anti-albumin/HRP (panel C).

Determination of free sulfhydryl groups

The number of sulfhydryl groups present in these fibrinogens was determined by titration of the denatured purified proteins with DTNB (23,24). Less than 0.05 mol SH/mole fibrinogen was found with normal protein, while fibrinogen IJmuiden contained 0.18 ± 0.05 mol SH/ml Fibrinogen and fibrinogen Nijmegen contained 0.13 ± 0.05 mol SH/mol fibrinogen. Thus, several Cys residues were present as free sulfhydryl groups.

Measurement of fibrinogen-albumin complexes

To detect the presence of fibrinogen-albumin complexes in plasma, we developed an enzyme immunoassay specific for these complexes. To obtain reference standards, we isolated complexes from purified abnormal fibrinogens by affinity chromatography on a rabbit anti-albumin IgG Sepharose column as described in methods. Purified complexes were added to normal plasma to create reference samples with different fibrinogen/ fibrinogen-albumin ratios. Dose response curves of the patients' plasmas were compared to reference curves (Fig. 4). Complexes with plasma IJmuiden were estimated as 20% of total fibrinogen (Fig. 4A) and complexes with plasma Nijmegen as 13% of total fibrinogen (Fig. 4B).

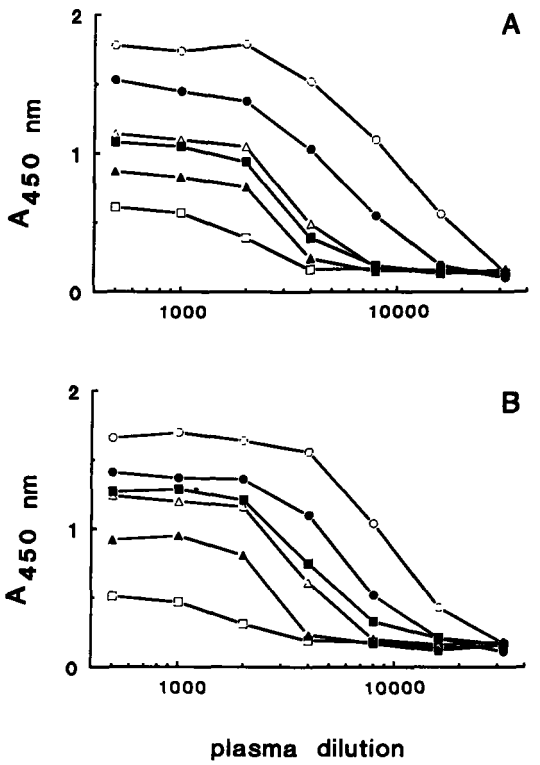


Figure 4. Enzyme immunoassays for fibrinogen-albumin complexes in plasma. Dose response curves were determined for plasmas Nijmegen (■, panel A) and IJmuiden (■, panel B). Standard curves were prepared by mixing normal plasma with 50% (○), 25% (●), 15% (△), 10% (▲), and 5% (□) of purified fibrinogen Nijmegen-albumin complexes (panel A) or fibrinogen IJmuiden-albumin complexes (panel B).

Measurement of larger fibrinogen complexes

To measure the concentration of high molecular weight fibrinogen complexes (M_r = 600-700,000), samples were separated by gel filtration and fractions assayed for fibrinogen (Fig. 5A) and fibrinogen-albumin complexes (Fig. 5B), as described in methods. Fibrinogen-albumin complexes were present in fractions 9-12 for both abnormal plasmas.

In addition, both plasmas contained a small fibrinogen crossreactive peak at fraction 6 indicating the presence of larger fibrinogen complexes. Analysis of the peak areas demonstrated that these larger complexes were approximately 5% and 7% of total fibrinogen in the IJmuiden and Nijmegen plasmas, respectively.

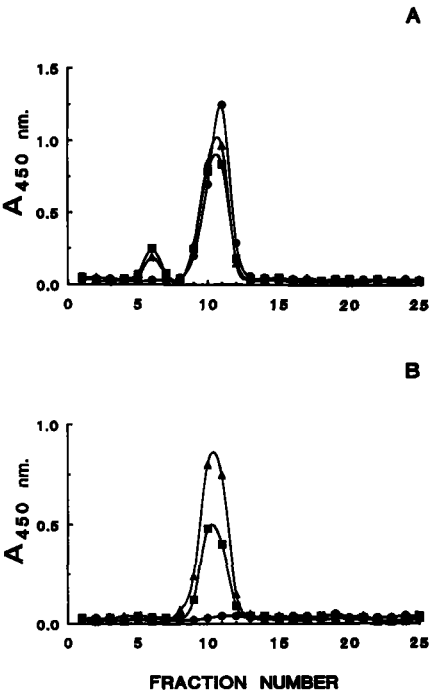


Figure 5. FPLC analysis of plasma samples. Normal (●), Nijmegen (■) and IJmuiden (▲) samples were run over a Superose 12 column and the collected fractions were analyzed by immunoassay for fibrinogen (panel A) or fibrinogen-albumin complexes (panel B).

DISCUSSION

We have identified the structural defect in two congenital abnormal fibrinogens, designated IJmuiden and Nijmegen. Both were heterozygous for a single base substitution (C → T) changing codons for Arg to Cys. The defect in fibrinogen IJmuiden $B\beta Arg_{14}$ → Cys, has also been found in fibrinogens Christchurch II (6) and Seattle I (7). The defect in Nijmegen, $B\beta_{44}Arg$ → Cys, has not been previously reported. We examined the fate of these abnormal Cys residues and found, for the first time, that abnormal fibrinogens circulate as disulfide linked complexes with albumin. The disulfide bonds presumably form between the additional cysteine introduced by the mutation and the free sulfhydryl group present in albumin (32). Both abnormal fibrinogens contained two complexes, presumably albumin linked to HMW fibrinogen and to LMW fibrinogen. It is unknown whether fibrinogen becomes linked to albumin during or after synthesis of these proteins for albumin and fibrinogen are both synthesized in hepatocytes. Disulfide

linked albumin complexes have also been detected with a variant of antithrombin III (Northwick Park, $Arg_{393} \rightarrow$ Cys (29)), which is also synthesized in hepatocytes.

We also identified three high molecular weight fibrinogen complexes in both abnormal fibrinogens. These could be disulfide linked fibrinogen dimers with bonds between the two forms of fibrinogen giving three species, HMW-HMW, HMW-LMW, and LMW-LMW dimers. Crotalus Atrox venom digestion of purified fibrinogen IJmuiden demonstrated the presence of disulfide linked Bβ 1-42 dimers ($M_r \sim 14{,}000$). This product could arise from cleavage of fibrinogen dimers, but the $M_r \sim 14{,}000$ band is more intense than expected from the fibrinogen dimer species. This suggests that intramolecular disulfide bonds between the Bβ chains of fibrinogen IJmuiden are also present, in agreement with the reports on fibrinogen Christchurch II (6).

We did not determine whether fibrinogens IJmuiden and Nijmegen are linked to small molecules such as cysteine, as was reported for fibrinogen Osaka II (8), but we did find that a substantial fraction of the mutant molecules contained free sulfhydryl groups. If we assume that half of the Bβ chains are mutated in these heterozygous samples, then 36% of the IJmuiden mutant chains and 26% of the Nijmegen mutant chains exist as free sulfhydryls. (This also assumes that the novel Cys is that being measured as a free sulfhydryl.) Similarly, albumin complexes account for 40% of the IJmuiden mutant chains and 26% of the Nijmegen mutant chains, and high molecular weight complexes account for 10% of the IJmuiden and 14% of the Nijmegen mutant chains. These novel forms of the altered fibrinogens complicate interpretation of functional studies. For example, the previously described defective t-PA binding to fibrin Nijmegen (12) could arise either directly from the loss of an essential residue, $B\beta Arg_{44}$, or indirectly from the presence of disulfide linked complexes or free sulfhydryl groups.

In summary, we have used the polymerase chain reaction to determine the structural defects in abnormal fibrinogens IJmuiden, $B\beta Arg_{14} \rightarrow$ Cys, and Nijmegen, $B\beta Arg_{44} \rightarrow$ Cys. Analysis of the purified abnormal fibrinogens and the corresponding plasmas demonstrated that the additional mutant Cys residues formed disulfide linked complexes with albumin. This finding severely complicates structure-function analysis whereby amino acid substitutions are associated with altered functions of the fibrinogen molecule.

We thank Dr. D. Fowlkes for oligonucleotide synthesis and Dr. W. Nieuwenhuizen for Mab Y18. This work was supported by USPHS grant # HL 31048 (STL) and NATO grant # CRG 860110 (STL and FH).

REFERENCES

1. Doolittle, R.F. (1984) *Ann. Rev. Biochem.* 53, 195-229.
2. Ebert, R.F. (1991) *Index of variant human fibrinogens* (CRC Press, Boca Raton, FL).

3. Mosesson, M.W., DiOrio, J.P., Muller, M.F., Shainoff, J.R., Siebenlist, K.R., Amrani, D.L., Homandberg, G.A., Soria, J, Soria, C. & Samama, M. (1987) *Blood* **69**, 1073-1081.
4. Miyata, T., Terukina, S., Matsuda, M., Kasamatsu, A., Takeda, Y., Murakami, T. & Iwanaga, S. (1987) *J. Biochem.* (Tokyo) **102**, 93-101.
5. Galanakis, D.K., Henschen, A., Peerschke, E.I.B. & Kehl, M. (1989) *J. Clin. Invest.* **84**, 295-304.
6. Kaudewitz, H., Henschen, A., Soria, C., Soria, J., Bertrand, O. & Heaton, D. (1986) in *Fibrinogen and its Derivatives,* eds. Müller-Berghaus, G., Scheefers-Borchel, V., Sellmayer, E. & Henschen, A. (Elsevier, Amsterdam), pp. 31-36.
7. Pirkle, H., Kaudewitz, H., Henschen, A., Theodor, I. & Simmons, G. (1987) in *Fibrinogen 2. Biochemistry, Physiology and Clinical Relevance,* eds. Lowe, G.D.O., Douglas, J.T., Forbes, C.D. & Henschen, A. (Elsevier, Amsterdam), pp. 49-52.
8. Terukina, S., Matsuda, M., Hirata, H., Takeda, Y., Miyata, T., Takao, T. & Shimonishi, Y. (1988) *J. Biol. Chem.* **263**, 13579-13587.
9. Koopman, J., Haverkate, F., Briët, E. & Lord, S.T. (1991) *J. Biol. Chem.* **266**, 13456-13461.
10. Koopman, J., Haverkate, F., Koppert, P., Nieuwenhuizen, W., Brommer, E.J.P. & Van der Werf, W.G.C. (1987) *J. Lab. Clin. Med.* **109**, 75-84.
11. Koppert, P.W., Huijsmans, C.M.G. & Nieuwenhuizen, W. (1985) *Blood* **66**, 503-507.
12. Engesser, L., Koopman, J., De Munk, G., Haverkate, F., Nováková, I., Verheijen, J.H., Briët, E. & Brommer, E.J.P. (1988) *Thromb. Haemostas.* **60**, 113-120.
13. Haverkate, F., Koopman, J., Kluft, C., D'Angelo, A., Cattaneo, M. & Mannucci, P.M. (1986) *Thromb. Haemostas.* **55**, 131-135.
14. Clauss, A. (1957) *Acta Haematol.* **17**, 237-246.
15. Mancini, G., Carbonara, A.O. & Heremans, J.F. (1965) *Immunochemistry* **2**, 235-254.
16. Van Ruijven-Vermeer, I.A.M. & Nieuwenhuizen, W. (1978) *Biochem. J.* **169**, 653-658.
17. Vuento, M. & Vaheri, A. (1978) *Biochem. J.* **175**, 333-336.
18. Keckwick, R.A. (1940) *Biochem. J.* **34**, 1248-1257.
19. Laemmli, U.K. (1970) *Nature* **227**, 680-685.
20. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.
21. Hong, C.S., Stadler, B.M., Walti, M. & De Weck, A.L. (1986) *J. Immunol. Methods* **95**, 195-202.
22. Bos, E.S., Van der Doelen, A.A., Van Rooy, N. & Schuurs, A.H.W.M. (1981) *J. Immunoassay* **2**, 187-204.
23. Ellman, G.L. (1959) *Arch. Biochem. Biophys.* **82**, 70-77.
24. Takahashi, N. & Hirose, M. (1990) *Anal. Biochem.* **118**, 359-365.
25. Gustafson, S., Proper, J.A., Bowie, E.J.W. & Sommer, S.S. (1987) *Anal. Biochem.* **165**, 294-299.
26. Saiki, R.K., Bugawan, T.L., Horn, G.T. & Erlich, H.A. (1986) *Nature* **324**, 163-166.
27. Chung, D.W., Que, B.G., Rixon, M.W., Mace, M. & Davie, E.W. (1983) *Biochemistry* **22**, 3244-3250.
28. Pandya, B.V., Cierniewski, C.S. & Budzynski, A.Z. (1985) *J. Biol. Chem.* **260**, 2994-3000.
29. Erdjument, H., Lane, D.A., Ireland, H., Panico, M., Di Marzo, V., Blench, I. & Morris, H.R. (1987) *J. Biol. Chem.* **262**, 13381-13384.
30. Mosesson, M.W., Galanakis, D.K. & Finlayson, J.S. (1974) *J. Biol. Chem.* **249**, 4656-4664.
31. Lipinska, I., Lipinski, B. & Gurewich, V. (1974) *J. Lab. Clin. Med.* **84**, 509-516.
32. Dugaiczyk, A., Low, S.W. & Dennison, O.E. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 71-75.

CHAPTER 5

FIBRINOGEN MARBURG: A HOMOZYGOUS CASE OF DYSFIBRINOGENEMIA, LACKING AMINO ACIDS Aα 461-610 (LYS 461 AAA →STOP TAA)

Jaap Koopman[1,2], Frits Haverkate[1], Jos Grimbergen[1,2]
Rudolf Egbring[3] and Susan T. Lord[4].

[1] Gaubius Laboratory IVVO-TNO, Leiden, The Netherlands.
[2] Dept. of Haematology, University Hospital Leiden, The Netherlands.
[3] Medical University Clinic Marburg/Lahn, Germany.
[4] Dept. of Pathology, University of North Carolina, Chapel Hill, USA.

SUMMARY

In the Aα-chain gene coding for an abnormal fibrinogen (Fibrinogen Marburg) we identified a single-base substitution (A → T), which changes the codon Aα 461 AAA (Lys) to TAA (Stop). The propositus was found to be homozygous for the mutation, whereas the father and five siblings were heterozygous, and three other siblings contained only the normal sequence. The stop codon at position 461 results in the deletion of the carboxyl-terminal segment Aα 461-610. Purified fibrinogen Marburg contained an Aα-chain with a relative molecular weight of approximately 47,000. The FpA release by thrombin was not affected by this deletion, whereas the fibrin polymerization was strongly decreased. The binding of endothelial cells to immobilized fibrinogen Marburg was almost completely abolished as compared to normal fibrinogen. Fibrinogen Marburg contained a substantial amount of albumin linked to the fibrinogen molecule by disulfide bonds, and these fibrinogen-albumin complexes were also present in plasma. The plasma fibrinogen concentration of the propositus was measured by three different methods: a functional method (less than 0.25 mg/ml), an immunological method using polyclonal antibodies (0.6 mg/ml) and an immunological method based on two monoclonal antibodies specific for the amino-terminus and carboxyl-terminus of the Aα-chain (<0.05 mg/ml). Using the two immunological methods, it appeared that only 10-15% of the plasma fibrinogen of the heterozygous siblings was abnormal.

INTRODUCTION

Fibrin formation begins with the thrombin catalyzed release of fibrinopeptide A (FpA) from the amino-terminus of the Aα-chains of fibrinogen. The cleavage of FpA and fibrinopeptide B (FpB) results in the exposure of polymerization sites in the amino-terminal part of fibrinogen, designated "A" and "B" site, respectively (1). Complementary polymerization sites to the "A" and "B" sites, designated "a" and "b", are located in the carboxyl-terminal region of the fibrinogen molecule (1). Assembly of the mature three-dimensional fibrin matrix starts with the formation of double stranded fibrils (2-6) followed by branching and lateral association of these fibrils (7-9). The initial fibrin formation arises predominantly from the interaction between the "A" and "a" sites (4), while lateral association is more dependent on the interaction between the "B" and "b" sites (4,9). The carboxyl-terminus of the Aα-chain is thought to play an important role in the branching and lateral association step of fibrin formation (10). In addition to being a precursor of fibrin, fibrinogen also binds to several cell types, of which platelets and endothelial cells are the best studied. This binding is mediated through the interaction

of a fibrinogen-specific receptor on the membrane of these cells (11) with distinct sites on the fibrinogen molecule. It has recently been suggested that the Arg-Gly-Asp containing sequence in the carboxyl-terminus of the Aα-chain (Aα 572-574) is the major site that interacts with the integrin $\alpha_v\beta_3$ on endothelial cells (12).

Of the 76 structurally identified congenitally abnormal fibrinogens, 73 showed a single amino acid substitution and two a deletion in the fibrinogen molecule (13). From the cases with known structural defects, 68 are heterozygous for the molecular defect, while 8 are homozygous. Molecular defects introducing additional cysteines by amino acid substitutions are described in 25 cases. In fibrinogens Metz (14) and Kawaguchi (15) (Aα 16 Arg → Cys), Seattle I (16) and Christchurch II (17) (Bβ 14 Arg→Cys) intramolecular disulfide bond formation between two abnormal chains was suggested. In fibrinogen Osaka II (γ 275 Arg → Cys) the additional Cys was linked to a free cysteine molecule (18). Recently, fibrinogen-albumin complexes were detected in fibrinogens IJmuiden (Bβ 14 Arg → Cys) and Nijmegen (Bβ 44 Arg → Cys) (19). Congenital dysfibrinogenemias associated with a low plasma fibrinogen concentration are described in 24 cases with unknown structural defects (13).

The present report describes the structural defect in the previously reported fibrinogen Marburg (20) inferred from DNA analysis (Aα 461 AAA (Lys) → TAA (Stop)). Evidence is presented that this mutation affects fibrin polymerization and prevents the binding of fibrinogen Marburg to endothelial cells. Furthermore, disulfide linked fibrinogen-albumin complexes are detected in the plasma of the propositus.

MATERIALS AND METHODS

Parameters determined in plasma

Blood was collected by venepuncture and anti-coagulated with 1/10 volume of 0.11 M trisodium citrate. Plasma was prepared by centrifugation of the blood at 2300 xg for 30 min at 4 °C. Thrombin and Reptilase^R clotting times were performed as described (21). Fibrin(ogen) degradation products were measured in plasma using a monoclonal antibody based immunoassay (22). The functional plasma fibrinogen concentration was determined according to Clauss (23). The immunological fibrinogen concentration was determined by radial immunodiffusion according to Mancini (24). The plasma fibrinogen concentration was also measured by two enzyme immunoassays (EIA) using a monoclonal antibody specific for the amino-terminus of the human fibrinogen Aα-chain (Mab Y18, 25) as capture antibody. The bound fibrinogen was detected by rabbit anti fibrinogen IgGs conjugated with HRP (r-a-Fbg/HRP, 22) or with a monoclonal antibody specific for the carboxyl-terminus of the fibrinogen Aα-chain conjugated with HRP (Mab G8/HRP, 26). The assays were performed by incubating microtiter plates (655001,

Greiner, Alphen a/d Rijn , The Netherlands) with 0.12 ml per well of Mab Y18 dissolved in PBS to a concentration of 3 μg/ml for 16 hours at 4°C. The coated plates were washed three times with PBS containing 0.05% Tween 20 (PBS/Tween), after which 0.1 ml of plasma diluted in PBS/Tween was added to the wells and incubated for 30 min at room temperature. After washing three times with PBS/Tween, 0.1 ml of r-a-Fbg/HRP or Mab G8/HRP was added to the wells. The plates were incubated for 60 min at room temperature, washed four times with PBS/Tween after which 0.2 ml of substrate solution containing TMB and H_2O_2 (27) was added. The reaction was stopped by adding 50 μl of 2 M H_2SO_4, and the absorbance at 450 nm was measured on a multiscan (Flow laboratories, Irvine, England). Pooled plasma containing 2.5 mg/ml fibrinogen, determined gravimetrically (27), was used as a standard in all fibrinogen assays.

Purification of fibrinogen

Fibrinogen was purified from plasma by precipitation with β-alanine essentially as described by Straughn and Wagner (28). β-alanine was added to plasma to a final concentration of 1 M and incubated on ice for 30 min. The precipitate was removed by centrifugation for 30 min at 3000 xg, 4°C. To the supernatant, β-alanine was added to a final concentration of 3.0 M and incubated for 60 min on ice. The precipitated fibrinogen was collected by centrifugation for 30 min at 10,000 xg, 4°C and dissolved in 0.15 M NaCl, 5 mM trisodium citrate solution. The fibrinogen was reprecipitated by adding β-alanine to a final concentration of 3.0 M, and incubating for 30 min on ice. The fibrinogen was collected by centrifugation for 30 min at 10,000 xg, 4°C and dissolved in 0.05 M Tris/HCl pH 7.5 to a concentration of approximately 2 mg/ml. After dialyses against 0.05 M Tris/HCl pH 7.5 for 16 hours at 4°C, contaminating fibronectin was removed by adsorption to gelatin-sepharose (Pharmacia) (29). The yield of this purification was approximately 60% for normal fibrinogen and only 20% for fibrinogen Marburg.

Coagulation of purified fibrinogen

Fibrinogen solutions were first dialyzed against 0.1 M Tris/HCl pH 7.5, containing 1 mM $CaCl_2$ or 1 mM EDTA, and the fibrinogen concentration, determined by measuring the absorbance at 280 nm ($A^{1\%,\,1\,cm}$ = 15.0), was adjusted to 0.2 mg/ml. To initiate coagulation, 10 μl of thrombin (10 NIH units/ml, Leo, Denmark) solution was added to 0.7 ml aliquots of these fibrinogen solutions. Coagulation of purified fibrinogen was measured as absorbance at 350 nm as a function of time using a spectrophotometer (Pye Unicam SP 1700).

FpA release from purified fibrinogen by thrombin was determined by radio-immunoassay (Mallinckrodt Diagnostics, Maryland Heights, M.O.). Fibrinogen was

diluted to 0.1 mg/ml with 0.05 M Tris/HCl pH 7.5, and to 100 μl of this fibrinogen solution, 25 μl of thrombin solution (0.05 NIH units/ml, Leo, Denmark) was added. The reaction was stopped at different time intervals by adding 25 μl of Hirudin solution (10 U/ml, Sigma, St.Louis, USA). The fibrin and residual fibrinogen was removed by precipitation with 200 μl of bentonite solution. The supernatant was diluted 1:100, 1:1000, and 1:10000 with 0.05 M Tris/HCl buffer pH 7.5 and the FpA concentration was measured according to the manufacturers instructions.

SDS-PAGE of purified fibrinogen and plasma

SDS-PAGE of purified fibrinogen and plasma after reduction with 2-mercaptoethanol was performed on 10% gels according to Laemmli (30). Electrophoresis of plasma without reduction was performed on 2-16% precast gradient gels (Pharmacia). The electrophoresis buffer contained 0.04 M Tris, 0.02 M sodium acetate, 0.002 M EDTA, 0.2% w/v SDS, pH 7.4, while the sample buffer contained 0.01 M Tris/HCl, 0.001 M EDTA, 1% w/v SDS, pH 8.0.

Immunoblot analysis

After separation on the SDS-PAGE gels, proteins were electroblotted onto nitrocellulose (31) for 16 hours at 400 mA and 10°C. The blots were blocked by incubation with 0.01 M Tris/HCl pH 7.4, 0.15 M NaCl, 0.5% (w/v) gelatin and 0.05% (v/v) Tween 80 for 2 hours at room temperature. All antibodies were diluted with 0.01 M Tris/HCl pH 7.4, 0.15 M NaCl, 0.05% (v/v) Tween 80. Mab Y18/HRP, goat anti-human albumin conjugated to HRP (g-a-albumin/HRP, Nordic, Tilburg, The Netherlands) and r-a-Fbg/HRP were diluted 1:2000, 1:2500 and 1:300, respectively. The blots were incubated for 2 hours with the antibody solutions at room temperature, and subsequently washed 5 times, 10 min with dilution buffer. Cross-reacting bands were visualized by incubation with 4-chloro-1-naphthol (32).

Polymerase chain reaction and DNA sequencing

Genomic DNA was isolated from blood cells as described previously (33). Oligonucleotides α5a (5'TGGGGCACATTTGAAGAGGTGTCA 3') and α5b (5'GGAACTTACAGTCGACCACAAAAACAGACC 3') were used to amplify the part of the Aα-chain gene, coding for amino acid 391 to 625 and 121 base pairs of 3'untranslated sequence (34). Amplification by PCR (35) was performed in a 100 μl reaction volume containing 1 μg genomic DNA, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM each of primer α5a and α5b in 1X reaction buffer (10 mM Tris/HCl pH 8.3 at 25°C, 50 mM KCl, 3.0 mM $MgCl_2$ and 0.001% (w/v) gelatin). The DNA was denatured at 94°C for 4 min and 2.0 units Taq DNA polymerase was added. Cycles consisted of a 1 min 94°C, 0.5 min 58°C and 1.5 min 72°C incubation. After 30 cycles,

the amplified DNA was precipitated with ethanol, dried, dissolved in 20 μl distilled water and run on a 1% (w/v) ultra low gelling agarose (A-5030, Sigma). The band with the appropriate size, as predicted by the cDNA sequence of the Aα-chain gene (34), was cut out of the gel in a volume of approximately 20 μl and heated to 55 °C. 1 μl of the melted agarose was mixed with 1 μl of primer α5a (60 ng), and 2 μl of 5X annealing buffer (T7 sequence kit, Pharmacia), the volume was brought up to 14 μl with distilled water. The mixture was heated to 95 °C for 3 min and immediately after this put on ice. Labelling (5 min) and termination (10 min) reactions were performed using the T7 DNA sequence kit (Pharmacia) according to the manufacturers instructions.

Detection of mutation in family members by restriction analysis

PCR samples of the family members containing the Aα-fragment were incubated with the restriction endonuclease Dde I (New England Biolabs). After incubation, the restriction digests were analyzed on a 2% (w/v) agarose gel (A-6013, Sigma) and stained with ethidium bromide. DNA molecular size markers were prepared by Rsa I digestion of M13mp18 DNA.

Titration of free sulfhydryl groups in fibrinogen

Free sulfhydryl groups in fibrinogen were determined using DTNB (36). Purified fibrinogen was dissolved to 5 mg/ml in 0.05 M Tris/HCl pH 8.2 containing 1 mM EDTA and 8 M urea (37). To 0.5 ml of the fibrinogen solution, 5 μl of a 10 mM DTNB solution was added, the sample was incubated for 10 min at room temperature. The absorbance at 412 nm was measured, and reactive sulfhydryls were determined on the basis of a molar extinction coefficient of 13,600 $M^{-1}.cm^{-1}$ (36).

Binding of endothelial cells to fibrinogen

Human endothelial cells were isolated by collagenase digestion of fresh human umbilical veins and propagated as described (38). Cells forming confluent monolayers were detached with a solution containing trypsin and EDTA, sedimented by centrifugation at 250 xg for 10 min and resuspended in medium 199 containing 1% BSA. The cells were washed twice with medium 199 containing 1% BSA, and finally resuspended in this medium at a concentration of 5×10^4 cells/ml. Microtiter plates (655001, Greiner) were coated with fibrinogen or BSA by incubation for 16 hours at 4 °C with 50 μl of protein solution (10 μg/ml) in PBS. Before use the plates were washed three times with PBS. 50 μl of cell suspension was added to the wells of the coated plates, and incubated for 2 hours at 37 °C. The wells were washed three times with PBS to remove unattached cells. After washing, the number of cells was determined by microscopic examination of random fields, from which the percentage of attached cells was calculated.

RESULTS

Clinical and laboratory data

A 20-year old woman suffered from a uterine hemorrhage after delivery of her first child by caesarian section. The patient received 2 liter fresh blood during the operation. On the 7th postoperative day a pulmonary embolism occurred, for which the patient was treated with phenprocoumon. Embolism occurred repetitiously and the patient developed a deep pelvic thrombosis. A second pelvic thrombosis developed at the 36th postoperative day, followed by a third after the patient's dismissal from the hospital. The mother of the patient had died from apoplexia after a long period of hypertension. All other family members did not show any clinical symptoms. The laboratory studies on the plasma of the propositus (Table 1) revealed severely prolonged thrombin and Reptilase^R clotting times as compared to normal plasma. The functional fibrinogen value was lower than the immunological value and the immunological value itself was much lower than that of normal plasma.

Table 1. Coagulation parameters measured in normal plasma and plasma of the proprositus (Marburg).

	Normal	Propositus
Thrombin clotting time (s)	19	> 180
Reptilase^R clotting time (s)	21	> 180
Functional fibrinogen concentration (mg/ml)	2.5	< 0.25
Immunological fibrinogen concentration (mg/ml)	2.5	0.6
Fibrin(ogen) degradation products (μg/ml)	< 0.25	< 0.25

SDS-PAGE and Immunoblot analysis of purified fibrinogen and plasma

Purified fibrinogen Marburg analyzed after reduction by SDS-PAGE on a 10% gel according to Laemmli (Fig. 1A, lane 2) showed a very faint Coomassie stained band at the Aα-chain position of normal fibrinogen (lane 1). Immunoblot analysis using r-a-Fbg/HRP demonstrated that this faint band did not cross-react with the antibody against fibrinogen (Fig. 1B, lane 2), indicating that the protein was not related to fibrinogen. Furthermore, the γ-chain band in fibrinogen Marburg (Fig. 1B, lane 2) is more intense than the corresponding band in normal fibrinogen (lane 1). Two additional faint bands with M_r's smaller than normal γ-chain are present in fibrinogen Marburg. Incubation of the blots with Mab Y18/HRP, specific for the amino-terminus of the Aα-chain (24),

demonstrated that the Aα-chain of fibrinogen Marburg (Fig. 1C, lane 2) had the same electrophoretic mobility as the γ-chain of normal fibrinogen (lane 1). The additional bands in fibrinogen Marburg also reacted with Mab Y18/HRP (Fig. 1C, lane 2), indicating that these bands were degraded Aα-chains. Normal fibrinogen also shows degradation of the Aα-chain (Fig. 1C, lane 1).

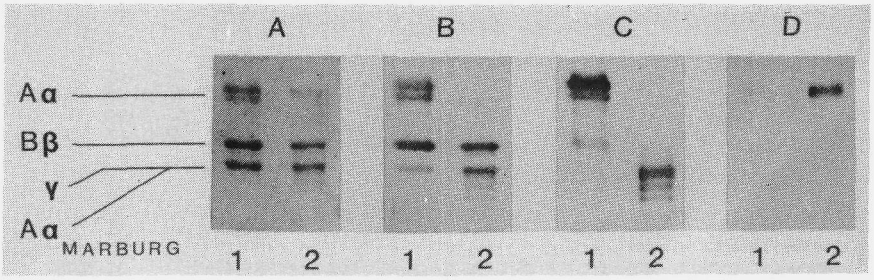


Figure 1. Coomassie stained 10% SDS-PAGE gel (panel A) and immunoblots after incubation with r-a-Fbg/HRP (panel B), Mab Y18/HRP (panel C) and g-a-albumin/HRP (panel D) of purified reduced normal fibrinogen (1) and fibrinogen Marburg II.1 (2).

Figure 1D reveals that the faint Coomassie stained band present in fibrinogen Marburg at the position of normal Aα-chain (Fig. 1A, lane 2) cross-reacts with the g-a-albumin/HRP conjugate. This indicates that fibrinogen Marburg (Fig. 1D, lane 2) contains albumin whereas normal fibrinogen (lane 1) does not. Since normal fibrinogen was purified in exactly the same way as fibrinogen Marburg, it is unlikely that the presence of albumin is due to a purification artifact. To determine whether fibrinogen Marburg-albumin complexes are present in plasma, normal plasma and plasma of the propositus were run on 2-16% precast gradient SDS-PAGE gels (Pharmacia) followed by immunoblot analysis. The blots incubated with Mab Y18/HRP demonstrated that normal plasma (Fig. 2A, lane 1) contained two intense bands representing high molecular weight fibrinogen (HMW Fbg) and low molecular weight fibrinogen (LMW Fbg) (39,40). Plasma of the propositus contained at least seven bands which cross-reacted with the monoclonal antibody (Fig. 2A, lane 2). Five of these were discrete bands with M_r's of approximately 270, 300, 330, 370, and 440 x 10^3, and two were diffuse bands with M_r's of approximately 400 and 530 x 10^3. Incubation of the blots with g-a-albumin/HRP revealed that the three bands with M_r's of 330, 370 and 440 x 10^3 also contained albumin (Fig. 2B, lane 2). Normal plasma did not contain any band which cross-reacted with the g-a-albumin/HRP. The immuno blot analysis of purified fibrinogen Marburg and plasma indicates that fibrinogen Marburg is missing a part of the Aα-chain, which results in the formation of fibrinogen-albumin complexes. This deletion is apparently not located in the amino-terminal part of the Aα-chain of fibrinogen Marburg, because fibrinogen Marburg reacts with Mab Y18/HRP.

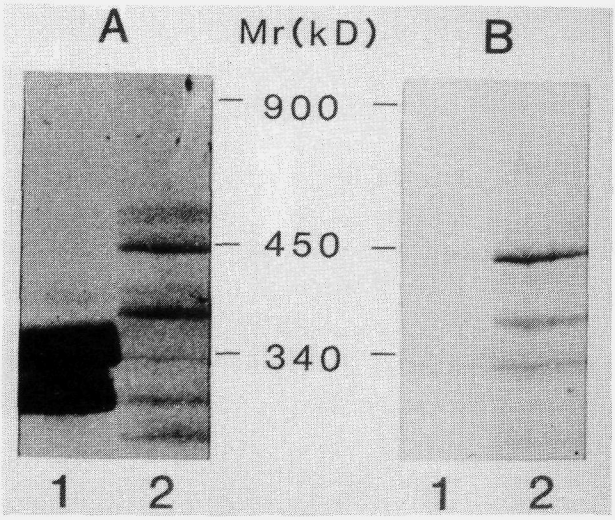


Figure 2. Immunoblot of normal plasma (1) and plasma of the propositus (2) after separation of the plasma proteins on a 2-16% gradient SDS-PAGE gel, using Mab Y18/HRP (panel A) and g-a-albumin/HRP (panel B).

Coagulation of purified fibrinogen

Clotting of purified fibrinogen Marburg induced by thrombin in the presence of Ca^{2+} or EDTA (Fig. 3) was impaired as compared to normal fibrinogen. The rate of FpA release of fibrinogen Marburg by thrombin was similar to that of normal fibrinogen as measured by radio-immunoassay. These results suggest that the reduced clotting of fibrinogen Marburg is due to an impairment in fibrin polymerization.

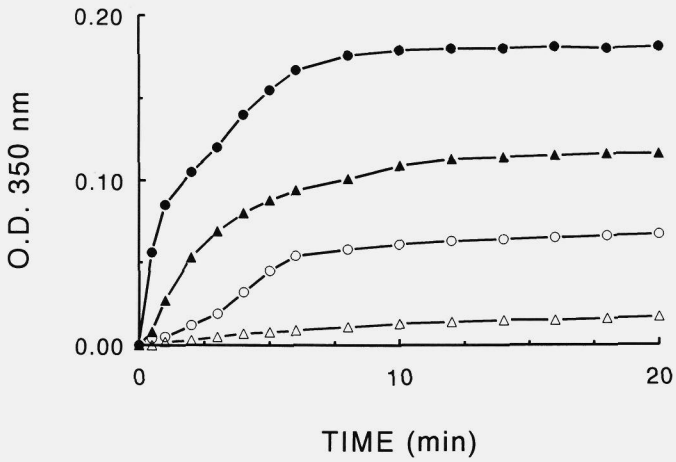


Figure 3. Thrombin induced coagulation profiles of normal fibrinogen (● ▲) and fibrinogen Marburg II.1 (○ △) in the presence of Ca^{2+} (● ○) or EDTA (▲ ●).

Amplification and DNA sequence analysis of fibrinogen Aα-chain gene exon V

Based on the evidence from the immunoblot analysis using Mab Y18/HRP, we amplified the genomic DNA which codes for the carboxyl-terminal amino acids (391 to 625) of the Aα-chain from fibrinogen Marburg. The amplified Aα-chain gene fragments of both normal and Marburg had the size predicted from the genomic DNA sequence. DNA sequence analysis of the amplified fragments demonstrated that the fragment derived from genomic DNA coding for fibrinogen Marburg had a single base mutation (Fig. 4) in the codon for lysine at position 461 in the Aα-chain. This mutation changed the codon AAA (Lys) to TAA, a stop codon. The normal sequence was not present in fragments amplified from Marburg DNA, indicating that the propositus was homozygous for this mutation. This is in agreement with the absence of normal Aα-chain in fibrinogen Marburg (Fig. 1B and C). Furthermore, the presence of the stop codon at position 461 in the Aα-chain gene of fibrinogen Marburg is in good agreement with the size of the Aα-chain of fibrinogen Marburg on SDS-PAGE gels (Fig. 1C).

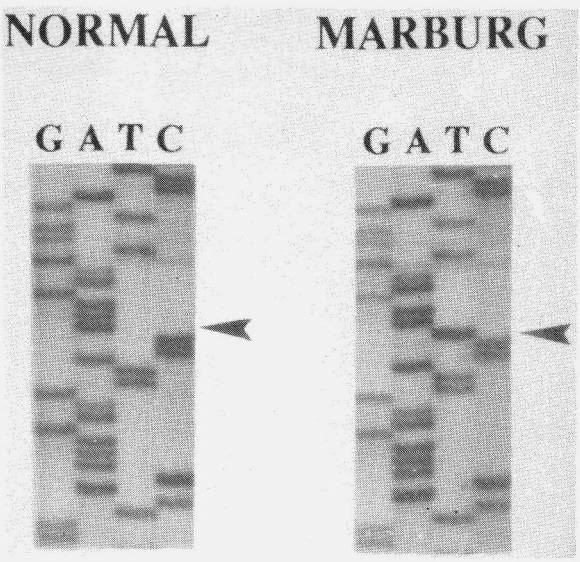


Figure 4. DNA sequence of the amplified Aα gene fragment derived from normal genomic DNA and genomic DNA of the propositus (Marburg II.1) (arrows indicate the mutation).

Determination of free sulfhydryl groups in purified fibrinogen

The mutation in fibrinogen Marburg deletes amino acid 461 to 610 in the Aα-chain including a cysteine residue at position 472 which normally forms an intramolecular disulfide bond with the cysteine at position 442 in the Aα-chain. To determine the redox state of the cysteine at position 442 in fibrinogen Marburg, the number of free sulfhydryl groups was determined by titration of denatured purified fibrinogen with DTNB (Ellman's reagent). Normal fibrinogen and fibrinogen Marburg did not contain any detectable free sulfhydryl groups (< 0.1 mol SH/mol fibrinogen).

Binding of endothelial cells to purified fibrinogen

The Arg-Gly-Asp sequence in the carboxyl-terminus of the Aα-chain (residues 572-574) is recognized by intergrin-type receptors on endothelial cells (12). Because this sequence is deleted in fibrinogen Marburg, the binding of endothelial cells to purified fibrinogen was determined. To microtiter plate wells coated with normal fibrinogen, approximately 57% of the endothelial cells remained attached after washing with PBS (Fig. 5). Wells coated with fibrinogen Marburg or BSA retained less than 10% of the endothelial cells after washing with PBS. These results demonstrate that endothelial cells bind much more weakly to fibrinogen Marburg than to normal fibrinogen, in agreement with the results of Cheresh et al. (12).

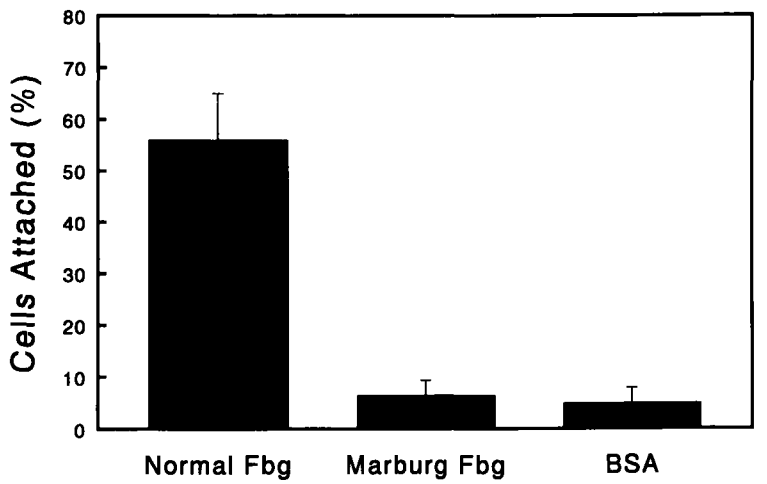


Figure 5. Percentage of endothelial cells bound to immobilized normal fibrinogen, fibrinogen Marburg II.1 and Bovine serum albumin. Each bar represents the mean ± SD of five replicates.

Detection of mutation in genomic DNA of family members

The mutation found in the Aα gene fragment of fibrinogen Marburg creates a recognition-sequence for the restriction endonuclease Dde I in the mutated Aα gene fragment. The additional Dde I site results in the formation of a 195 bp band for the Aα PCR amplified fragment of Marburg as compared to the 236 bp band for normal Aα PCR amplified fragment. Restriction analysis with Dde I of the amplified fragments (Fig. 6) confirmed that the propositus (II.1) was homozygous for the defect. The father (I.1), three sisters (II.2, II.3 and II.6) and the son (III.2) of the propositus showed both the 195 bp and the 236 bp bands, indicating that they were heterozygous for the mutation. The restriction digests of the other three family members (II.4, II.5 and III.1) containing only

the 236 bp band, were similar to normal. The mother of the propositus (I.2) was not studied, but from the hereditary pattern (Fig. 8) it is most likely that she was heterozygous for the mutation. The homozygous propositus is the only individual with clinical symptoms, indicating that fibrinogen Marburg is a clinically recessive dysfibrinogenemia.

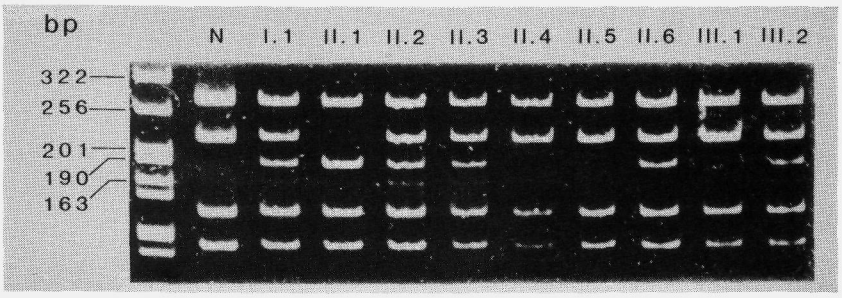


Figure 6. Ethidium Bromide stained gel of restriction digests by Dde I of amplified Aα-chain gene fragment from a normal individual (N), the propositus (II.1), and his family members (I.1, II.2, II.3, II.4, II.5, II.6, III.1 and III.2). DNA molecular size marker: M13mp18 RSA I digest.

Detection of abnormal fibrinogen in plasma of family members

To determine whether the mutation found in the genomic DNA of the family members corresponded with abnormal fibrinogen in plasma, immunoblot analysis of reduced plasma samples was performed using Mab Y18/HRP (Fig. 7). Reduced normal plasma contained one intense band corresponding to intact Aα-chain of normal fibrinogen and several faint bands with lower M_r's. The propositus (II.1) contained one intense band corresponding to the shorter Aα-chain or fibrinogen Marburg and one faint band with a much lower M_r. The heterozygous family members (I.1, II.2, II.3, II.6, and III.2) all showed two intense bands corresponding to normal and Marburg Aα-chain. The three normal family members (II.4, II.5, and III.1) only contained the intense band corresponding to normal Aα-chain. The difference in intensity between the normal Aα-chain band and the Marburg Aα-chain band on the immuno blot (Fig. 7) suggested that the amount of abnormal fibrinogen present in the heterozygous individuals was substantially smaller than the amount of normal fibrinogen concentration.

To quantitate the amount of normal and abnormal fibrinogen in plasma of the heterozygous individuals, plasma fibrinogen concentrations were determined by enzyme immunoassays, using Mab Y18 as a capture antibody and Mab G8/HRP or r-a-Fbg/HRP for detection (Table 2). When plasma of normal family members was analyzed, the fibrinogen concentrations measured by the two EIA's was virtually the same. The results obtained with plasma of the propositus (II.1) demonstrate that crossreactivity with Mab G8/HRP was undetectable with fibrinogen Marburg (< 0.05 mg/ml), whereas the EIA

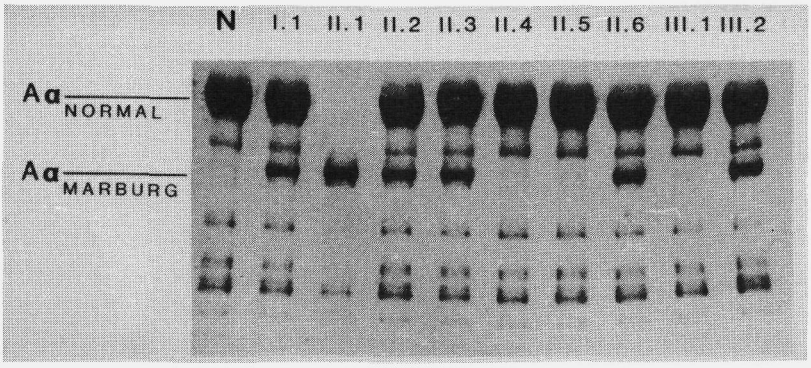


Figure 7. Immunoblot of reduced normal plasma (N), plasma of the propositus (II.1) and her family members (I.1, II.2, II.3, II.4, II.5, II.6, III.1 and III.2) after electrophoresis on a 10% SDS-PAGE gel and incubation with Mab Y18/HRP, a monoclonal antibody specific for fibrinogen.

Table 2. Plasma thrombin clotting times (T.T), fibrinogen with intact carboxyl-terminal Aα-chain concentrations measured by Mab Y18-Mab G8/HRP EIA (Y18/G8) and total fibrinogen concentrations measured by Mab Y18-r-a-Fbg/HRP EIA (Y18/r-a-Fbg) in normal plasma, plasma of the propositus (II.1) and her family members (I.1, II.2, II.3, II.4, II.5, II.6, III.1 and III.2).

		Fibrinogen concentration (mg/ml)	
	T.T. (s)	Y18/G8	Y18/r-a-Fbg
Normal	19	2.5	2.5
I.1	25	1.7	1.9
II.1	> 180	< 0.05	0.5
II.2	23	1.6	1.7
II.3	27	1.8	2.1
II.4	20	2.6	2.5
II.5	18	2.8	2.9
II.6	26	1.6	1.8
III.1	20	2.5	2.5
III.2	24	1.9	2.1

combining Mab Y18 and r-a-Fbg/HRP gave a similar value to that determined by radial immunodiffusion (Table 1). These results indicate that the monoclonal antibody G8 does not react with fibrinogen Marburg, which is in good agreement with the results of

Hoegee-de Nobel et al. (26) who localized the epitope for Mab G8 in the carboxyl-terminus of the Aα-chain. Analysis of the heterozygous family members demonstrated that the plasma fibrinogen levels measured by the Mab Y18-Mab G8/HRP EIA were only 10-20% lower than those determined by the Mab Y18-r-a-Fbg/HRP EIA. This small difference indicates that in heterozygous individuals the majority (80-90%) of the plasma fibrinogen molecules is normal, which is in agreement with the results of the immunoblot analysis (Fig. 7). Furthermore, the total fibrinogen concentration in plasma of heterozygous family members (1.7 - 2.1 mg/ml) was slightly lower than that in plasma of normal individuals (2.5 - 2.9 mg/ml). The plasma thrombin clotting times in the heterozygous family members (23 - 27s) were slightly prolonged as compared to normal individuals (18 - 20s), indicating that the abnormal fibrinogen molecules inhibited the clotting of normal fibrinogen.

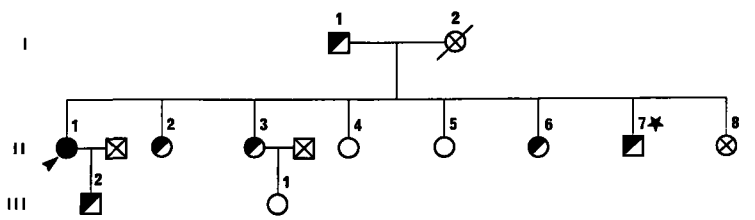


Figure 8. Pedigree of the family, the arrow indicates the propositus, homozygous (■●) and heterozygous (◪◭) individuals are indicated. Other symbols: (□) man, (○) woman, (⊘) deceased, (⊠⊗) not studied, (*) studied previously.

DISCUSSION

SDS-PAGE and immunoblot analysis of the abnormal fibrinogen Marburg demonstrated that this fibrinogen (II.1) contained a shorter Aα-chain polypeptide which we thought to arise from a deletion in the carboxyl-terminus. Following this evidence, we amplified the genomic DNA of the propositus which codes for the carboxyl-terminus of the Aα-chain from amino acid 391 through the stop codon. Sequence analysis of the amplified fragment demonstrated that the propositus was homozygous for a single base substitution (A → T) in the codon for Aα Lys 461 (AAA) resulting in a Stop codon (TAA) at this position. The mutation in the Aα-chain gene of fibrinogen Marburg predicts an abnormal fibrinogen with a M_r of approximately 300,000, containing a shorter Aα-chain with a M_r of approximately 47,000, consistent with our protein gel analysis.

Coagulation studies on purified fibrinogen Marburg (II.1) indicate that the deleted Aα-segment contains residues critical for fibrin polymerization. We found that thrombin catalyzed clotting was impaired while FpA release was normal, as is expected for

defective fibrin polymerization. This is consistent with the report of Hasegawa and Sasaki (10) who demonstrated that reaggregation of fibrin monomers prepared from purified low molecular weight fibrinogen (fraction F2) was abnormal in morphology, turbidity, elasticity, and clotting velocity. Low molecular weight fibrinogen lacks a carboxyl-terminal fragment from one of the Aα-chains.

Similarly, our results demonstrate that this deleted Aα-segment contains residues critical for fibrinogen binding to endothelial cells. Cherish et al. (12) demonstrated that the Arg-Gly-Asp containing site, Aα-residues 572-574, is essential for endothelial cell binding. Since fibrinogen Marburg lacks this Arg-Gly-Asp containing domain, the loss of endothelial cell adhesion to this defective fibrinogen confirms the previous findings.

The amino acid stretch (Aα 461 to 610) lacking in fibrinogen Marburg contains a cysteine residue at position 472 which normally forms an intra-molecular disulfide bridge with Aα Cys 442 (41). Purified fibrinogen and plasma of the Marburg propositus contained fibrinogen-albumin complexes, most likely due to disulfide bond formation between the free SH group in albumin (42) and the unpaired Cys at position 442 in the Aα-chain(s) of fibrinogen Marburg. Plasma of the Marburg propositus (II.1) contained three fibrinogen-albumin complexes with different M_r's. The fibrinogen-albumin complex with a M_r of 440,000 is consistent with a complex formation between fibrinogen Marburg and two albumin molecules. The complex with a M_r of 370,000 probably contains only one albumin molecule per fibrinogen Marburg molecule. The fibrinogen-albumin complex with a M_r of 330,000 is consistent with a complex of the low molecular weight form of fibrinogen Marburg (M_r 270,000) and albumin. Similar complexes have been reported for the abnormal fibrinogens Nijmegen (Bβ 44 Arg → Cys) and IJmuiden (Bβ 14 Arg → Cys) (19) and for the antithrombin III variant Northwick Park (393 Arg → Cys) (43, 44). The absence of free sulfhydryl goups suggests that the unpaired cysteines in fibrinogen Marburg, which are not linked to albumin, are linked to other small molecules as was reported for fibrinogen Osaka II (18) or form intra molecular disulfide bonds between the two sulfhydryl groups as demonstrated for fibrinogens Metz (14) and Kawaguchi (15).

The presence of the mutation in the genomic DNA of the family members was determined by restriction analysis of the amplified DNA fragment coding for the carboxyl-terminus of the Aα-chain. The plasmas of the five heterozygous individuals (I.1, II.2, II.3, II.6 and III.2), contained abnormal fibrinogen. However, in the heterozygous family members the concentration of abnormal fibrinogen was less than 10-20% of the total fibrinogen concentration. The plasma fibrinogen concentration of the homozygous propositus was also very low (0.6 mg/ml). These low values of fibrinogen Marburg in plasma indicate that the synthesis, assembly, secretion and/or plasma lifetime of fibrinogen Marburg is decreased. A decrease in plasma lifetime has been reported for fibrinogen Philadelphia (45), for which the structural defect is not yet known.

The clinical symptoms of the propositus consisted of severe hemorrhage after delivery followed by repeated thrombotic events. The severe hemorrhage may be explained by the low fibrinogen concentration and the impaired clotting of fibrinogen Marburg. To explain the repeated thrombotic events, we postulate that fibrinolysis of fibrin Marburg is impaired. The role of the carboxyl-terminus of the Aα-chain in the stimulatory effect of fibrin on t-PA induced plasminogen activation has been previously described (46-48). The absence of clinical symptoms in the heterozygous family members is probably due to the relatively low concentration of abnormal fibrinogen in the plasma of these individuals. From these results we can conclude that fibrinogen Marburg (Aα 461 Lys → Stop) is a clinically recessive hypo-dysfibrinogenemia.

ACKNOWLEDGEMENTS

We like to thank Dr. Dana Fowlkes for synthesizing the oligonucleotides and Ria van den Hoogen for technical assistance.

REFERENCES

1. Kudryk BJ, Collen D, Woods KR, Blombäck B: Evidence for localization of polymerization sites in fibrinogen. J Biol Chem 249:3322, 1974
2. Shainoff JR, Dardik BN: Fibrinopeptide B and aggregation of fibrinogen. Science 204:200, 1979
3. Shainoff JR, Dardik BN: Fibrinopeptide B in fibrin assembly and metabolism: physiologic significance in delayed release of the peptide. Ann N Y Acad Sci 408:254, 1983
4. Olexa SA, Budzynski AZ: Evidence for four different polymerization sites involved in human fibrin formation. Proc Natl Acad Sci USA 77:1374, 1980
5. Olexa SA, Budzynski AZ, Doolittle RF, Cottrell BA, Greene TC: Structure of fragment E species from human cross-linked fibrin. Biochemistry 21:6139, 1981
6. Budzynski AZ, Olexa SA, Pandya BV: Fibrin polymerization sites in fibrinogen and fibrin fragments. Ann N Y Acad Sci 408:301, 1983
7. Carr ME Jr, Shen LL, Hermans J: Mass-length ratio of fibrin fibers from gel permeation and light scattering. Biopolymers 16:1, 1977
8. Hantgan RR, Hermans J: Assembly of fibrin: a light scattering study. J Biol Chem 254:11272, 1979
9. Mosesson MW: Fibrin polymerization and its regulatory role in hemostasis. J Lab Clin Med 116:8, 1990
10. Hasegawa N, Sasaki S: Location of the binding site "b" for lateral polymerization of fibrin. Thromb Res 57:183, 1990
11. Hynes RO: Integrins: A family of cell surface receptors. Cell 48:549, 1987
12. Cheresh DA, Berliner SA, Vicente V, Rugger ZM: Recognition of distinct adhesive sites on fibrinogen by related integrins on platelets and endothelial cells. Cell 58:945, 1989
13. Ebert RF: Index of variant human fibrinogens, 1991 edition. Ebert RF (ed) Boca Raton, Florida, CRC Press, 1991

14. Mosesson MW, DiOrio JP, Muller MF, Shainoff JR, Siebenlist KR, Amrani DL, Homandberg GA, Soria J, Soria C, Samama M: Studies on the ultra structure of fibrin lacking fibrinopeptide B (β-fibrin). Blood 69:1073, 1987
15. Miyata T, Terukina S, Matsuda M, Kasamatsu M, Takeda Y, Murakami T, Iwanaga S: An amino acid substitution of Aα arginine-16 to cysteine which forms an extra interchain disulfide bridge between the two Aα-chains. J Biochem (Tokyo) 102:93, 1987
16. Pirkle H, Kaudewitz H, Henschen A, Theodor I, Simmons G: Substitution of Bβ-14 arginine by Cyst(e)ine in fibrinogen Seattle I, in Lowe GDO, Douglas JT, Forbes CD, Henschen A (eds): Fibrinogen 2. Biochemistry, Physiology and Clinical Relevance. Amsterdam, Elsevier, 1987, p 49
17. Kaudewitz H, Henschen A, Soria C, Soria J, Bertrand O, Heaton D: The molecular defect of the genetically abnormal fibrinogen Christchurch II, in Müller-Berghaus G, Scheefers-Borchel V, Sellmayer E, Henschen A (eds): Fibrinogen and its Derivatives. Amsterdam, Elsevier, 1986, p 31
18. Terukina S, Matsuda M, Hirata H, Takeda Y, Miyata T, Takao T, Shimonishi Y: Substitution of γ Arg-275 by Cys in an abnormal fibrinogen, Fibrinogen Osaka II: Evidence for a unique solitary cystine structure at the mutation site. J Biol Chem 263:13579, 1988
19. Koopman J, Haverkate F, Grimbergen J, Engesser L, Nováková I, Kerst A, Lord ST: Formation of fibrinogen-albumin and fibrinogen-fibrinogen complexes by disulphide bridges in fibrinogen IJmuiden (Bβ Arg 14 → Cys) and Nijmegen (Bβ Arg 44 → Cys). Thromb Haemostas 65:822, 1991
20. Fuchs G, Egbring R, Havemann K: Fibrinogen Marburg, a new genetic variant of fibrinogen. Blut 34:107, 1977
21. Haverkate F, Koopman J, Kluft C, D'Angelo A, Cattaneo M, Mannucci PM: Fibrinogen Milano II: a congenital dysfibrinogenaemia associated with juvenile arterial and venous thrombosis. Thromb Haemostas 55:131, 1986
22. Koopman J, Haverkate F, Koppert P, Nieuwenhuizen W, Brommer EJP, Van der Werf WGC: New enzyme immunoassay of fibrin-fibrinogen degradation products in plasma using a monoclonal antibody. J Lab Clin Med 109:75, 1987
23. Clauss A: Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. Acta Haematol 17:237, 1957
24. Mancini G, Carbonara AO, Heremans JF: Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochem 2:235, 1965
25. Koppert PW, Huijsmans CMG, Nieuwenhuizen W: A monoclonal antibody, specific for human fibrinogen, fibrinopeptide A-containing fragments and not reacting with free fibrinopeptide A. Blood 66:503, 1985
26. Hoegee-de Nobel E, Voskuilen M, Briët E, Brommer EJP, Nieuwenhuizen W: A monoclonal antibody-based quantitative enzyme immunoassay for the determination of plasma fibrinogen concentrations. Thromb Haemostas 60:415, 1988
27. Astrup T, Brakman P, Nissen U: The estimation of fibrinogen, a revision. Scand J Clin Lab Invest 17:237, 1957
28. Straughn III W, Wagner RH: A simple method for preparing fibrinogen. Thromb Diath Haemorrh 16:198, 1966
29. Vuento M, Vaheri A : Dissociation of fibrònectin from gelatin-agarose by amino compounds. Biochem J 175:333, 1978
30. Laemmli UK: Cleavage of structural proteins during the assembly of the HAED of bacteriophage T4. Nature 227:680, 1970
31. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350, 1979
32. Hong CS, Stadler BM, Wälti M, De Weck AL: Dot immunobinding assay with monoclonal anti-IgE antibodies for the detection and quantitation of human IgE. J Immunol Methods 95:195, 1986

33. Gustafson S, Proper JA, Bowie EJW, Sommer SS: Parameters affecting the yield of DNA from human blood. Anal Biochem 165:294, 1987
34. Kant JA, Lord ST, Crabtree GR: Partial mRNA sequences from human Aα, Bβ, and γ fibrinogen chains: Evolutionary and functional implications. Proc Natl Acad Sci USA 80:3953, 1983
35. Saiki RK, Geleand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA: Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 39:487, 1988
36. Ellman GL: Tissue sulfhydryl groups. Arch Biochem Biophys 82:70, 1959
37. Takahashi N, Hirose M: Determination of sulfhydryl groups and disulfide bonds in a protein by polyacrylamide gel electrophoresis. Anal Biochem 188:359, 1990
38. Jaffe EA, Nachmann RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins. J Clin Invest 52:2745, 1973
39. Mosesson MW, Galanakis DK, Finlayson JS: Comparison of human plasma fibrinogen subfractions and early plasmic fibrinogen derivatives. J Biol Chem 249:4656, 1974
40. Lipinska I, Lipinski B, Gurewich V: Fibrinogen heterogeneity in human plasma. Electrophoretic demonstration and characterization of two major fibrinogen components. J Lab Clin Med 84:509, 1974
41. Henschen A, Lottspeich F, Kehl M, Southan C: Covalent structure of fibrinogen. Ann NY Acad Sci 408:28, 1983
42. Dugaiczyk A, Law SW, Dennison OE: Nucleotide sequence and the encoded amino acids of human serum albumin mRNA. Proc Natl Acad Sci USA 79:71, 1982
43. Erdjument H, Lane DA, Ireland H, Panico M, Di Marzo V, Blench I, Morris HR: Formation of a covalent disulfide -linked antithrombin-albumin complex by an antithrombin variant, antithrombin "Northwick Park". J Biol Chem 262:13381, 1987
44. Erdjument H, Lane DA, Panico M, Dimarzo V, Morris HR: Single amino acid substitution in tthe reactive site of antithrombin leading to thrombosis. J Biol Chem 263:5589, 1988
45. Martinez J, Holburn RR, Shapiro SS, Erslev AJ: Fibrinogen Philadelphia. A hereditary hypodysfibrinogenemia characterized by fibrinogen hypercatabolism. J Clin Invest 53:600, 1974
46. Suenson E, Petersen LC: Fibrin and plasminogen structures essential to stimulation of plasmin formation by tissue-type plasminogen activator. Biochim Biophys Acta 870:510, 1986
47. Koopman J, Engesser L, Nieveen M, Haverkate F, Brommer EJP: Fibrin polymerization associated with tissue-type plasminogen activator (t-PA) induced Glu-plasminogen activation, in Muller-Berghaus G, Scheefers-Borchel U, Selmayer E, Henschen A (eds):Fibrinogen and its derivatives. Biochemistry, Physiology and Pathophysiology. Amsterdam, Elsevier, 1986, p 315
48. Soria J, Soria C, Bertrand O, Mirshahi M, Dunn FW, Svenson E, Desvignes P, Bonnet P, Samama M, Caen JP: Role of fibrin polymerization in fibrinolysis: comparison between polymerisable and unpolymerisable fibrins in the potentiating effect of plasminogen activation by tissue-type plasminogen activator, in Lane D, Henschen A, Jasani MK (eds): Fibrinogen-Fibrin formation and fibrinolysis Vol. 4. Berlin, Walter de Gruyter & Co., 1986, p 153

CHAPTER 6

FIBRINOGEN NAPLES: A CONGENITAL DYSFIBRINOGENAEMIA ASSOCIATED WITH JUVENILE ARTERIAL AND VENOUS THROMBOSIS

F. Haverkate[1], J. Koopman[1], C. Kluft[1], A. D'Angelo[2], M. Cattaneo[2] and P.M. Mannucci[2]

1) TNO Gaubius Institute for Cardiovascular Research, Leiden, The Netherlands.
2) A. Bianchi Bonomi Hemophilia and Thrombosis Centre and Institute of Internal Medicine, University of Milan, Italy.

SUMMARY

A congenitally abnormal fibrinogen was isolated from blood of a young man with deep-vein thrombosis. Two other affected members of his family had three episodes of severe arterial thrombosis. The fibrinogen showed a delayed clotting by thrombin, but a normal clotting by Arvin^{R}, Reptilase^{R}, and prothrombin-staphylocoagulase complex. Analysis of the fibrinopeptides A and B by High Performance Liquid Chromatography did not reveal an abnormal peptide structure. The rate of release of A and B peptides by thrombin was strongly delayed, whereas the rate of release of fibrinopeptide A by Arvin^{R} appeared to be normal. The fibrin polymerization rate was normal. Interactions between the abnormal fibrinogen, platelets and the fibrinolytic system were also normal. Evidence is presented that the defective interaction between fibrinogen Naples and thrombin is associated with a defective binding of thrombin to the fibrin moiety of the abnormal fibrinogen.

INTRODUCTION

More than 100 cases of inherited dysfibrinogenaemia have been reported (1). The hereditary transmission is autosomally dominant. Only four homozygous cases have been described (1). Abnormal fibrinogens are usually characterized by a delayed thrombin clotting time of plasma or purified fibrinogen. The structural defect in the fibrinogen molecule has been localized in approximately 20 cases (1). The clinical phenomena can be roughly divided into three groups: no symptom, bleeding episodes, and thrombotic episodes. Up to now, 20 cases of dysfibrinogenaemia associated with arterial or venous thrombosis have been reported (1). To elucidate the cause underlying thrombosis, careful studies of the clinical symptoms, the laboratory findings, and the fibrinogen structure are required. The present report describes our findings obtained with fibrinogen Naples, which is associated with severe episodes of juvenile arterial and venous thrombosis.

MATERIALS AND METHODS

Venous blood was collected from the propositus in tubes containing trisodium citrate (final concentration 0.011 mol/l). Platelet-poor plasma was prepared by centrifugation at 2300 xg for 30 min at 4 °C.

Fibrinogen was determined in plasma functionally according to Clauss (2), gravimetrically according to Astrup et al. (3), and immunologically according to Mancini

(4), using a 1% (w/v) agarose (BDH, Poole, United Kingdom) solution in 0.03 mol/l sodium diethylbarbiturate buffer, pH 8.6 containing 5 mmol/l EDTA.

The diluted blood clot lysis time method was performed with 10% blood (5). *The euglobulin clot lysis time method* was performed by clotting 0.2 ml of euglobulin fraction (EF) with 0.1 ml bovine thrombin solution (10 NIH/ml) containing 0.075 mol/l NaCl and 0.025 mol/l $CaCl_2$. The activity of a 30 μl drop of the EF prepared at pH 5.9 with a plasma dilution of 1/10 (6) was determined on plasminogen-rich bovine fibrin plates (7) and results expressed in diameters of lysis zones in the plates, after 18 h of incubation at 37°C. The C1-inactivator resistant activator activity determined (8) represents specifically the extrinsic activator activity in blood. The total plasminogen activator plus proactivator level in plasma was assayed in the EF by using dextran sulphate (9).

Clotting times. Thrombin, Arvin^R and Reptilase^R clotting times were determined in plasma (200 μl) after preincubation at 37°C for 3 min and subsequently addition of 50 μl of the enzyme solution. Bovine thrombin (Leo, Copenhagen, Denmark) 7 NIH/ml, or undiluted Arvin^R (Knoll, Ludwigshafen, W. Germany) or undiluted Reptilase^R (Boehringer, Mannheim, W. Germany) were used. The thrombin-coagulase time was performed with reagent from Boehringer according to the manufacturer's directions (10). The prothrombin time (PT) was determined (10) with the Manchester Comparative Reagent (overseas version, Laboratori Baldacci, Pisa). Clotting times of solutions of purified fibrinogen (2 mg/ml) in 200 μl buffered saline (PBS), pH 7.4, containing 0.15 mol/l NaCl, 1.6 mmol/l KH_2PO_4 and 10 mmol/l Na_2HPO_4 were also determined as in plasma. The enzyme solutions (50 μl) contained bovine thrombin. 10 NIH/ml, or purified human thrombin (11) 20 NIH/ml, or undiluted Arvin^R, or a 1:1 (v/v) mixture of a 1:10 diluted solution of purified staphylocoagulase (by the courtesy of Dr. J. Lindhout, Department of Biochemistry, State University, Maastricht, The Netherlands) and a solution of human prothrombin, 4.3 IU/ml (12).

Purification of proteins

Fibrinogen from the propositus' plasma and from a pool of three normal plasmas was purified essentially according to Van Ruyven-Vermeer and Nieuwenhuizen (13). Plasma was obtained from venous blood containing 0.01% (w/v) disodium EDTA and 40 KIU/ml Trasylol (Bayer, Leverkusen, W. Germany) by centrifugation at 3000 xg for 20 min at 4°C. The purified fibrinogen was concentrated by precipitation in 50% saturated ammonium sulphate solution. After centrifugation the precipitate was dissolved in PBS and dialysed at 4°C against this buffer. Fibrin monomer solution was prepared from fibrinogen with thrombin as described previously (14).

The polymerization time was assessed after addition of 0.15 ml of the fibrin monomer solution (0.6%, w/v) in 0.02 mol/l acetic acid to 0.45 ml of 0.05 mol/l diethylbarbiturate buffer pH 9.0 containing 0.1 mol/l NaCl. The final pH of the solution was 7.4.

The preparation of the samples of *fibrinopeptides A and B*, and the subsequent analysis by High Performance Liquid Chromatography (HPLC) were performed essentially according to Kehl et al. (15) with a device manufactured by LKB (Bromma, Sweden). A C-18 reversed phase column, 250 x 4.6 mm (Chrompack, Middelburg, The Netherlands), was used and a gradient solution system was applied using the following solvents: A) 0.025 mol/l sodium phosphate buffer pH 6.0; B) 0.05 mol/l sodium phosphate buffer pH 6.0 mixed with an equal volume of acetonitrile (Uvasol, Merck, Darmstadt, W. Germany). The detection wavelength was 206 nm. Maximal release of fibrinopeptides A and B was determined after incubation at 37°C overnight of 200 µl fibrinogen solution (4 mg/ml) in PBS with 10 µl of enzyme solution. The enzyme solutions used contained bovine thrombin (20 NIH/ml) or Arvin^R (1:5 diluted) or a mixture of staphylocoagulase (1:10 diluted) and human prothrombin (4.3 IU/ml). The rate of release of the fibrinopeptides A and B was determined after incubation at 37°C for different time intervals of 200 µl fibrinogen solution (4 mg/ml) in PBS with 10 µl of enzyme solution. The enzyme solutions used contained bovine thrombin (2 NIH/ml) or Arvin^R (1:10 diluted) or a 1:1 mixture of staphylocoagulase (1:20 diluted) and human prothrombin (2.2 IU/ml). The reaction was stopped by placing the samples in boiling water for 90 sec. The precipitated fibrinogen and fibrin were removed by centrifugation at 10,000 xg for 10 min, after which 50 µl of the supernatant was injected into the HPLC column.

To determine *thrombin binding to fibrin*, different volumes (0.1-0.3 ml) of bovine thrombin (5 NIH/ml) were added to 0.1 ml of fibrinogen solution (2 mg/ml). The total volume was made up with PBS to 0.4 ml, and incubated for 30 min at 37°C. To achieve complete coagulation of fibrinogen, 0.1 ml of Arvin^R was subsequently added, followed by incubation for 30 min at 37°C. The fibrin formed was removed by centrifugation for 10 min at 10,000 xg and the residual thrombin activity measured in the supernatant with the synthetic substrate Chromozyme TH (Boehringer, Mannheim, W. Germany), according to the instructions of the manufacturer. The amount of thrombin bound to the fibrin was calculated by subtracting the amount of thrombin recovered in the supernatant (equilibrium concentration) from the total amount of thrombin added. The specific activity of the thrombin used was determined by active site titration with p-nitrophenyl-p'-guanidobenzhoate-HCl (PNGB, Polysciences, Warrington, U.S.A.) (16). One NIH unit of thrombin corresponded to 14 pmol PNGB.

Platelet studies

The interaction between fibrinogen Naples and platelets was studied in several systems. Propositus' platelet rich plasma (PRP) obtained by centrifugation at 150 xg for 10 min of citrated venous blood was placed in an aggregometer (Elvi Chronolog, Milano) at 37°C and stimulated with varying concentrations of aggregating agents (ADP,

arachidonic acid and collagen). The aggregation curves were evaluated for 3 min after the addition of the agent, and compared with those of three healthy subjects. In addition, varying concentrations of purified fibrinogen Naples or normal fibrinogen were tested for their ability to restore platelet aggregation in PRP obtained from an afibrinogenemic patient. ADP-induced aggregation was also tested in the presence of acetylsalicylic acid (ASA, 5 x 10^{-4} mol/l) in order to evaluate aggregation independently of the platelet release reaction. Finally, fibrinogen Naples or normal fibrinogen were tested for their ability to support the aggregation of platelets from the propositus or normal donors washed according to the method of Mustard et al. (17). In some experiments the platelets were also exposed to chymotrypsin (8 U/ml, Sigma Chemical Co, St. Louis, Mo) for 30 min during the first washing. Platelets exposed to chymotrypsin interact directly with fibrinogen and aggregate without the addition of stimulating agents (18).

Binding of fibrinolytic components to fibrin Naples

Serum from non-anticoagulated propositus' blood was separated from the clot by centrifugation after incubation in a glass tube for 4 h at 37°C. Serum was also prepared from citrated plasma clotted with calcium and thrombin (0.05 M and 20 NIH/ml). α_2-antiplasmin activity in propositus' plasma and after clotting of the plasma was measured by the immediate plasmin inhibition test described before (19). t-PA activity from plasma was measured in the euglobulin fraction of plasma as described (8) and expressed in IU/ml (20). Binding of plasminogen and plasma urokinase to fibrin Naples was assessed in the plasma fraction prepared by precipitation and activation with 30 mg/l dextran sulphate (MW 500,000, Pharmacia) of the supernatant of euglobulin fraction (1:10 dilution, pH 5.9) (8). Clots were formed by addition of calcium/thrombin (0.05 M and 20 NIH/ml) to 0.5 ml of the fraction, separated by a spatula and centrifugation and washed three times in buffered saline and compressed on filter paper. Clots were then placed on one of the following bovine plasminogen-rich fibrin plates (7): plates which incorporated 5 U/ml streptokinase (Kabi), plates with no addition and plates which incorporated an excess of anti-human urokinase rabbit IgG (30 μg/ml) (21). The lysed zones after 18 h of incubation at 37°C were recorded.

CASE REPORT

The propositus (II.3, Fig. 1) (male, born in 1951) was admitted in 1983 to the Institute of Internal Medicine of the University of Milano with symptoms of deep-vein thrombosis extending into the femoroiliac axis that developed three weeks after abdominal surgery for acute pancreatitis. He belongs to a family in whom congenital dysfibrinogenemia associated with severe thrombotic symptoms had been previously

documented by Quattrone et al. (22). His brother (II.2, Fig. 1) had a stroke at the age of 21, with no abnormality of the extra- and intracranial vessels revealed by angiography. A few days after that episode, during the hospital stay, he developed acute arterial insufficiency of both limbs, with severe rest pain and no palpable peripheral pulses. Angiography revealed complete thrombotic occlusion of the abdominal aorta below the renal arteries. The propositus' sister (II.1, Fig. 1) had a stroke at the age of 25 due to thrombotic occlusion of the internal carotid artery documented by angiography. Another brother of the propositus (II.4) and his parents (I.1 and I.2) (who are first cousins) are asymptomatic and not available for investigation. Coagulation studies carried out in the propositus' brother and sister by Quattrone et al. (22) revealed a prolonged thrombin clotting time but normal Reptilase^R and thrombin-coagulase time; the functional fibrinogen assay gave lower values than the immunoassay. The asymptomatic parents and brother, as well as our propositus II 3, were not studied by Quattrone et al. (22), who did not carry out any further characterization of the abnormal fibrinogen.

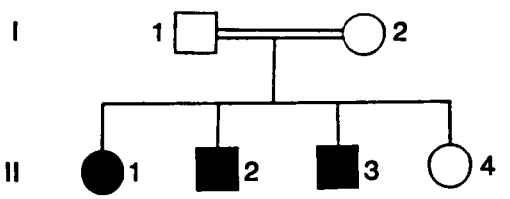


Figure 1. Pedigree of the family. The propositus, with symptoms of venous thromboembolism, is II.3. His brother (II.2) and sister (II.1) had symptoms of arterial thrombosis, whereas the parents I.1 and I.2 (first cousins) and the brother II.4 are asymptomatic.

RESULTS

Laboratory assays with the patients's plasma

Table 1 shows the coagulation parameters of the propositus' plasma as compared with normal plasma. The prothrombin time was prolonged, the thrombin time markedly prolonged and the functional fibrinogen assay (Clauss) gave lower values than did the gravimetric and immunological assays. The clotting by Arvin^R and Reptilase^R, which are known to split off only the fibrinopeptide A, were normal. The clotting by the staphylocoagulase-prothrombin complex (thrombin-coagulase time) was also normal.

Table 2 shows that the fibrinolysis assays performed were found to lie within the normal range or were slightly elevated due to a higher level of extrinsic tissue-type plasminogen activator.

Table 1. Coagulation parameters of the propositus's plasma as compared with normal plasma.

	Normal	Naples
Prothrombin time (sec)	13	21
Thrombin time (sec)	21	300
Fibrinogen (mg/ml)		
Clauss	1.58-3.54	1.9
Gravimetrical		3.2
Immunological		3.4
Arvin^{R} time (sec)	23	23
Reptilase^{R} time (sec)	20	20
thrombin-coagulase time (sec)	19	22

Table 2. Fibrinolysis parameters of the patient's plasma as compared with normal plasma.

	Normal	Naples
Dilute blood clot lysis time (min)	>119	248
Euglobulin clot lysis time (min)	281	167
Fibrin plate assay:		
Euglobulin fraction (EF) (mm)	9-15	18
EF with C1-inactivator (mm)	6-8	9
EF with dextran sulphate (BAU/ml)	85-115	110

Laboratory assays with the patient's purified fibrinogen

The clotting of purified fibrinogen Naples by both bovine and human thrombin was strongly delayed (Table 3). The delay in clotting with human thrombin was more pronounced than with the bovine product. The clotting times obtained with Reptilase^{R}, Arvin^{R}, and the staphylocoagulase-prothrombin complex (Table 3) were normal or even shorter, as compared with normal purified fibrinogen, in agreement with the corresponding clotting times in plasma. Calcium ions (10 mmol/l) had no significant influence on the clotting of fibrinogen Naples by thrombin (not shown), indicating that the enzymatic, non-calcium dependent step is the rate-limiting step associated with the abnormality. This was confirmed by measuring the polymerization time of purified fibrin monomers prepared from fibrinogen Naples. The polymerization time did not differ significantly from that of normal fibrin monomers: 68 and 77 sec, respectively.

Table 3. Clotting time (sec) of purified fibrinogen Naples as compared with normal purified fibrinogen

Clotting enzyme	Normal	Naples
Bovine thrombin	23	90
Human thrombin	18	190
Reptilase^{R}	65	38
Arvin^{R}	23	16
Prothrombin-staphylocoagulase complex	28	30

Release of fibrinopeptides A and B

The release of fibrinopeptides A and B from purified fibrinogen Naples, as measured by HPLC, after prolonged incubation with thrombin, is shown in Fig. 2. The profile obtained is similar to that of normal fibrinogen. It indicates that the peptides A and B apparently have a normal structure, and that the amount of the peptides released after prolonged incubation with thrombin, as reflected by the peak heights, are normal.

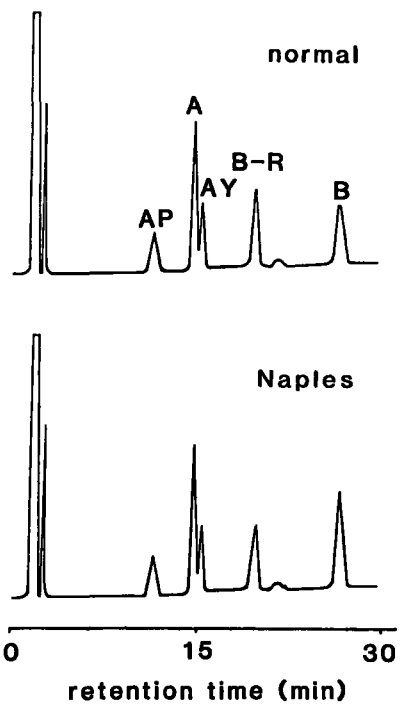


Figure 2. HPLC profiles of fibrinopeptides released after prolonged thrombin action. One molecule of fibrinogen released two A and two B peptides. AP: fibrinopeptide A, phosphorylated; A: fibrinopeptide A; AY; Des-arg-fibrinopeptide A; B-R: Des-arg-fibrinopeptide B; B: fibrinopeptide B.

However, the rate of release of both the fibrinopeptides A and B by thrombin, as determined by HPLC at different incubation times, is considerably retarded (Fig. 3), in agreement with the delayed clotting with thrombin. The rate of release of fibrinopeptide A by Arvin^R from fibrinogen Naples appeared to be similar to that from normal fibrinogen (Fig. 4), in agreement with the normal clotting with Arvin^R (Table 1). The total amount of A and B peptides released by the prothrombin-staphylocoagulase complex as well as the rate of release were normal, in agreement with the normal thrombin-coagulase time (data not shown).

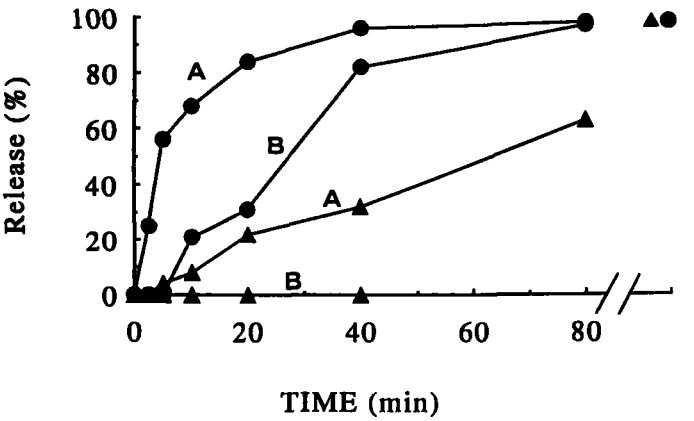


Figure 3. Release of fibrinopeptides A and B by thrombin as a function of time, as determined by HPLC. Normal fibrinogen (●), fibrinogen Naples (▲). The points located beyond 80 min have been measured after 16 h.

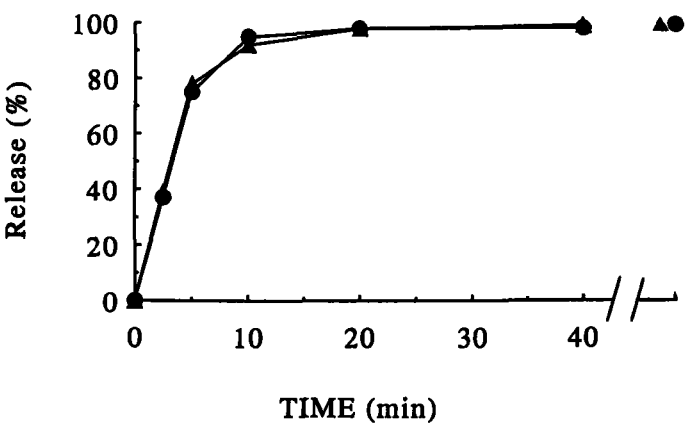


Figure 4. Release of fibrinopeptide A by Arvin^R with time, as determined by HPLC. Normal fibrinogen (●), fibrinogen Naples (▲). The points located beyond 40 min have been measured after 16 h.

Binding of thrombin to fibrin

It is known that normal fibrin binds thrombin (23). As fibrinogen Naples has a defective interaction with thrombin, we investigated whether this was associated with a defective binding of thrombin to the fibrin moiety. The binding was determined at a molar ratio fibrinogen:thrombin in the range 30:1 to 170:1. Under these conditions, the amount of thrombin bound to fibrin Naples, expressed on a molar basis, was about 3 times less than the amount bound to normal fibrin (Fig. 5). Defective thrombin binding was also observed when Arvin^{R} was omitted from the incubation mixture and complete clotting of fibrinogen Naples was obtained only with high thrombin concentrations and prolonged incubation times.

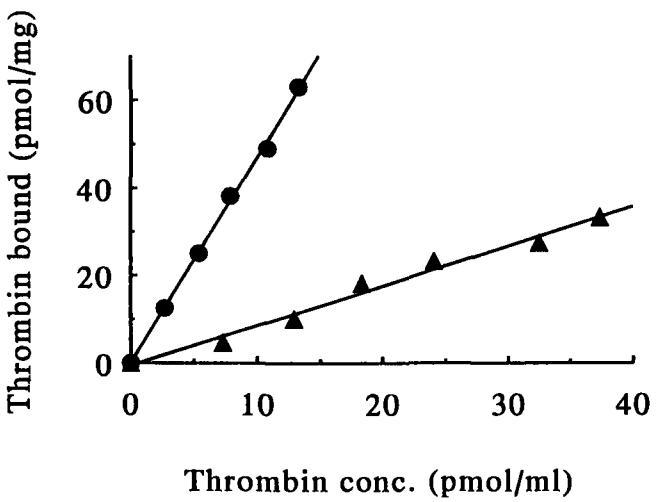


Figure 5. Binding of bovine thrombin to fibrin. Horizontal axis: thrombin equilibrium concentration after binding; vertical axis: thrombin bound to clotted fibrin. Arvin^{R} was added in all experiments to ensure total clotting. Normal fibrinogen (●), fibrinogen Naples (▲).

Platelet studies

Propositus' PRP aggregated as normal PRP to varying concentrations of ADP, collagen or arachidonic acid. The aggregation of PRP from an afibrinogenemic patient exposed to several concentrations of arachidonic acid, collagen or ADP (the latter both in the presence and absence of ASA) was restored to the same degree by purified fibrinogen Naples and normal fibrinogen. Similar degrees of aggregation were obtained when the two fibrinogen preparations were added to washed platelet suspension exposed to aggregating agents or to chymotrypsin-treated washed platelet suspensions (data not shown).

Binding of fibrinolytic components to fibrin Naples

α_2-antiplasmin in serum from whole blood and clotted citrated plasma of the propositus was lower compared to the plasma level (99% of pooled plasma) by respectively 35 and 39%, due to factor XIII-mediated binding of the inhibitor to the fibrin. Such reduction is well within the normal range (35 ± 6%; n = 12). Binding of endogenous tissue-type plasminogen activator (t-PA) was assessed using plasma enriched in t-PA after administration of DDAVP (desamino D-arginine-vasopressin, 0.4 μg/Kg) to the propositus. The t-PA activity dropped from 15.5 IU/l to 0.25 IU/l upon removal of the clot formed with calcium and thrombin, indicating 95% binding of t-PA to the fibrin clot. Binding of plasminogen and plasma urokinase to fibrin Naples was assessed by placing washed clots from a plasma fraction (see methods) upon special fibrin plates with incorporated streptokinase or with and without excess of antiurokinase IgG, respectively. After incubation for 18 h at 37°C the lysis zone was identical for the propositus and a pooled normal plasma (32 mm), indicating normal binding of plasminogen to fibrin Naples. The spontaneous fibrinolytic activity of propositus' clots on plasminogen-rich fibrin plates was sensitive to inhibition by urokinase antibodies to a similar way as clots from pooled normal plasma (propositus' plasma with no added IgG: 21.7 mm; propositus' plasma with added IgG: 17.8 min; pooled normal plasma with no added IgG: 20.4 mm; pooled normal plasma with added IgG: 17.3 mm).

DISCUSSION

We have investigated an abnormal fibrinogen present in three siblings and associated with early and unusually severe episodes of arterial thrombosis in two of them. The question whether the abnormality, called Naples after the name of the city where the propositus lives, is due to homozygosity or heterozygosity for an abnormal allele remains unanswered. As the parents of the propositus are cousins and asymptomatic, homozygosity in their siblings is possible. On the other hand, the propositus and his siblings had approximately one-half of their fibrinogen functionally active, whereas homozygotes usually have abnormal fibrinogen molecules with no measurable functional fibrinogen (1).

The clotting of fibrinogen Naples by thrombin was considerably retarded, both in plasma and in purified fibrinogen preparations. Surprisingly, clotting by Arvin^R and Reptilase^R, known to split off only the fibrinopeptide A, and by the prothrombin-staphylocoagulase complex, known to split off both the fibrinopeptides A and B, were normal. Prolonged thrombin time with normal Reptilase^R time have been previously described in a family with dysfibrinogenemia and bleeding symptoms (fibrinogen Houston) exhibiting defective fibrin polymerization (24). Further investigations by HPLC

indicated a delayed release of the fibrinopeptides A and B from fibrinogen Naples by thrombin, with no abnormality of the peptide structure being demonstrable. Prolonged incubation with thrombin resulted in the release of two A and two B peptides, as for normal fibrinogen. Hence, the defect of fibrinogen Naples manifests itself in the rate of enzymatic release of the peptides. The subsequent fibrin polymerization step proceeds normally as can be deduced from the normal polymerization rate of purified fibrin Naples.

The delayed release of the fibrinopeptides from fibrinogen Naples by thrombin, the defective thrombin binding, and the normal clotting by Arvin^{R}, Reptilase^{R} and the prothrombin-staphylocoagulase complex indicate the existence of a specific thrombin interaction site present in normal fibrinogen, which is defective in fibrinogen Naples. This site is unlikely to be located in the fibrinopeptides A and B as these peptides in fibrinogen Naples appear to be normal. Our thrombin binding experiments suggest that this thrombin interaction site is located in the fibrin moiety of the normal fibrinogen molecule, and is defective in the fibrin Naples.

It is intriguing that fibrinogen Naples shows a defective interaction with thrombin, and not with the prothrombin-staphylocoagulase complex. Staphylocoagulase activates prothrombin by unmasking the active site possibly through a conformational change, not by limited proteolysis (12). The results obtained suggest that the active prothrombin complex differs significantly from thrombin in its interaction with fibrinogen.

A possible explanation for the unusually severe thrombotic tendency associated with fibrinogen Naples, reflected by juvenile arterial thrombosis in two siblings of the propositus, could be an enhanced platelet-fibrinogen interaction. Thorsen et al. (25) have shown that an abnormal fibrinogen associated with a thrombotic tendency (Oslo I) acted as a more efficient cofactor of ADP-induced platelet aggregation than normal fibrinogen. Fibrinogen Naples interacted normally with platelets, as shown by its normal ability to support the aggregation of platelet-rich plasma from an afibrinogenemic patient and of washed platelets exposed to ADP or pre-incubated with chymotrypsin.

Another possible explanation for thrombotic episodes could be a decreased fibrinolytic activity caused by a defect in the patient's fibrin structure resulting in decreased binding to fibrin of components of the fibrinolytic system. No decrease in fibrinolytic activity was found using different clot lysis methods in which the patient's own fibrinogen was present. A decreased binding of fibrinolytic components (α_2-antiplasmin, tissue-type plasminogen activators, plasminogen and urokinase) to fibrin Milano in vitro could also be excluded.

The abnormal fibrinogen Naples has some features in common with fibrinogen New York I, both being characterized by defective thrombin binding and an associated thrombotic tendency (26). The two abnormal fibrinogens, however, are not identical, because Reptilase^{R} and Ancrod^{R} clotting times were prolonged in fibrinogen New York

but not in fibrinogen Naples. Fibrinogen New Orleans II, another abnormality associated with thrombosis, shows delayed clotting by thrombin and normal clotting by Ancrod^{R} (27); the thrombin-fibrin interaction has not been investigated. Liu et al. (26) have postulated that the thrombotic tendency associated with fibrinogen New York I might be related to defective thrombin binding by the abnormal fibrin, which could lead to high plasma levels of free thrombin and subsequent processes such as platelet activation and fibrin formation in the circulation. In our propositus, however, we found no evidence supporting this hypothesis. On three different occasions at a time when the propositus received no anticoagulant treatment, plasma antithrombin III, fibrinopeptide A (an index of circulating thrombin) and platelet serotonin, ADP and β-thromboglobulin (indexes of activated platelets, ref. 28 and 29), were within the normal range (data not shown). However, our failure to detect the indirect signs of enhanced thrombin formation does not rule out the thrombogenic mechanism proposed by Liu et al. (26), because such signs might be only transiently present, particularly during triggering conditions such as, for instance, surgery, immobilization and trauma.

ACKNOWLEDGEMENTS

This work was carried out within the framework of the Concerted Action "Thrombosis and Disabilities" of the Commission of the European Communities. PMM was supported in part by a CNR Grant, Progetto Finalizzato Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie. Thanks to A.F.H. Jie for excellent technical assistance.

REFERENCES

1. **Henschen A, Kehl M, Southan C, Lottspeich F, Geogopoulos D.** Genetically abnormal fibrinogens - some current characterization strategies. In: Fibrinogen, structure, functional aspects, metabolism. Haverkate F, Henschen A, Nieuwenhuizen W, Straub PW (eds). Walter de Gruyter, Berlin 1983; pp 125-144.
2. **Clauss A.** Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. Acta Haematol 1957; 17: 237-246.
3. **Astrup T, Brakman P, Nissen U.** The estimation of fibrinogen, a revision. Scand J Clin Lab Invest 1965; 17: 57-65.
4. **Mancini G, Carbonara AO, Heremans JF.** Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochem 1965; 2: 235-254.
5. **Chohan IS, Vermylen J, Singh I, Balakrishnan K, Verstraete M.** Sodium acetate buffer. A diluent of choice in the clot lysis time technique. Thromb Diath Haemorrh 1975; 33: 226-229.
6. **Kluft C, Brakman P, Veldhuyzen-Stolk EC.** Screening of fibrinolytic activity in plasma euglobin fractions on the fibrin plate. In: Progress in chemical fibrinolysis and thrombolysis. Davidson JF, Samama MM, Desnoyers PC (eds). Raven Press, New York 1976; 2: 57-65.
7. **Haverkate F, Brakman P.** Fibrin plate assay. In Progress in chemical fibrinolysis and thrombolysis. Davidson JF, Samama MM, Desnoyers PC (eds). Raven Press, New York 1975; 1: 151-159.

8. **Kluft C.** C1-resistant fibrinolytic activity in plasma euglobulin fractions: its relation to vascular activator in blood and its role in euglobulin fibrinolysis. Thromb Res 1978; 13: 135-151.
9. **Kluft C.** Studies on the fibrinolytic system in human plasma: quantitative determination of plasminogen activators and proactivators. Thromb Haemostas 1979; 41: 365-383.
10. **Mannucci PM, Federici AB, Sirchia G.** Haemostasis testing during massive blood replacement. A study of 172 cases. Vox Sang 1982; 42: 113-123.
11. **Bertina RM, Broekmans AW, Krommenhoek van Es T, Van Wijngaarden A.** The use of functional and immunologic assay for plasma protein C in the study of the heterogeneity of congenital protein C deficiency. Thromb Haemostas 1984; 51: 1-5.
12. **Hendrix H, Lindhout T, Mertens K, Engels W, Hemker HC.** Activation of human prothrombin by stoichiometric levels of staphylocoagulase. J Biol Chem 1983; 258: 3637-3644.
13. **Van Ruyven-Vermeer IAM, Nieuwenhuizen W.** Purification of rat fibrinogen and its constituent chains. Biochem J 1978; 169: 653-658.
14. **Haverkate F, Timan G.** Preparation of highly purified bovine fibrin plates. In: Progress in Chemical fibrinolysis and thrombosis. Davidson JF, Samama MM, Desnoyers PC (eds). Raven Press, New York 1976; 2: 67-71.
15. **Kehl M, Lottspeich F, Henschen A.** Analysis of human fibrinopeptide by high performance liquid chromatography. Hoppe Seyler's Z Physiol Chem 1981; 362: 1661-1664.
16. **Chase T, Shaw E.** Titration of trypsin, plasmin and thrombin with p-nithrophenyl-p'-guanidinobenzoate HCl. Methods Enzymol 1970; 19: 20-27.
17. **Mustard JF, Perry DW, Ardlie NG, Packham MA.** Preparation of suspensions of washed platelets from humans. Br J Haematol 1972; 22: 193-204.
18. **Greenberg JP, Packham MA, Guccione MA, Harfenist EJ, Orr JL, Kinlough-Rathbone RL, Perry DW, Mustard JF.** The effect of pretreatment of human or rabbit platelets with chymotrypsin on their responses to human fibrinogen and aggregating agents. Blood 1979; 54: 753-765.
19. **Kluft C, Vellenga E, Brommer EJP, Wijngaards G.** A familial hemorrhagic diathesis in a Dutch family: an inherited deficiency of α_2-antiplasmin. Blood 1982; 59: 1169-1180.
20. **Gaffney PJ, Curtis AD.** A collaborative study of a proposed international standard for tissue plasminogen activator (t-PA). Thromb Haemostas 1985; 53: 134-136.
21. **Wijngaards G, Kluft C, Groeneveld E.** Demonstration of urokinase-related fibrinolytic activity in human plasma. Br J Haematol 1982; 51: 165-169.
22. **Quattrone A, Colucci M, Donati MB, Mussoni L, Roncaglioni MC, Semeraro N, Carlomagno S, Bonavita V.** Cerebral thrombosis in two young siblings with dysfibrinogenemia. Neurosci Lett 1979; 3, suppl 1: 54-62.
23. **Liu CY, Nossel HL, Kaplan KL.** The binding of thrombin by fibrin. J Biol Chem 1979; 254: 10421-10425.
24. **Weinger RS, Rudy C, Moake JL, Olson JD, Cimo PL.** Fibrinogen Houston: A dysfibrinogen exhibiting defective fibrin monomer aggregation and -chain cross-linkages. Am J Hematol 1980; 9: 237-248.
25. **Thorsen LI, Stormorken H, Brosstad F, Gogstad G, Solum NO.** A dysfibrinogen, Oslo I, acting as a more efficient cofactor in ADP-stimulated aggregation than normal fibrinogen. Thromb Haemostas 1983; 50: 202.
26. **Liu CY, Nossel HL, Kaplan KL.** Defective thrombin binding by abnormal fibrin associated with recurrent thrombosis. Thromb Haemostas 1979; 42: 79 (abstr).
27. **Andes WA.** Fibrinogen New Orleans II: a new dysfibrinogenemia with venous thrombosis. Blood 1982; 60 (suppl 1): 206a.
28. **Capitanio A, Mannucci PM, Ponticelli C, Pareti FI.** Detection of circulating released platelets after renal transplantation. Transplantation 1982; 33: 298-301.
29. **Pareti FI, Gugliotta L, Mannucci L, Guarini A, Mannucci PM.** Biochemical and metabolic aspects of platelet dysfunction in chronic myeloproliferative disorders. Thromb Haemostas 1982; 47: 84-89.

CHAPTER 7

THE MOLECULAR BASIS OF FIBRINOGEN NAPLES ASSOCIATED WITH DEFECTIVE THROMBIN BINDING AND THROMBOPHILIA: HOMOZYGOUS SUBSTITUTION OF Bβ 68 Ala → Thr

J. Koopman[1,2], F. Haverkate[2], S.T. Lord[3],
J. Grimbergen[1] and P.M. Mannucci[4]

1) University Hospital Leiden, The Netherlands.
2) Gaubius Laboratory IVVO-TNO Leiden, The Netherlands.
3) Department of Pathology, University of North Carolina Chapel Hill, USA.
4) A. Bianchi Bonomi Hemophilia and Thrombosis Centre and Institute of Internal Medicine, University of Milan, Italy.

ABSTRACT

In an abnormal fibrinogen (fibrinogen Naples) associated with congenital thrombophilia we have identified a single base substitution (G → A) in the Bβ-chain gene that results in an amino acid substitution of Alanine by Threonine at position 68 in the Bβ-chain of fibrinogen. The propositus and two siblings were found to be homozygous for the mutation, whereas the parents and another sibling were found to be heterozygous. Individuals homozygous for the defect had a severe history of both arterial and venous thrombosis, while heterozygous individuals had no clinical symptoms. The three homozygotes had a prolonged thrombin clotting time in plasma whereas the heterozygotes had a normal thrombin clotting time. FpA and FpB release from purified fibrinogen by human α-thrombin was delayed in both the homozygous propositus and a heterozygous family member. Release of FpA from normal and abnormal NDSK corresponded to that found with the intact fibrinogens, indicating a decreased interaction of thrombin with the NDSK part of fibrinogen Naples. Binding studies showed that fibrin from homozygous abnormal fibrinogen bound less than 10% of active site inhibited α-thrombin as compared with normal fibrin, while fibrin formed from heterozygous abnormal fibrinogen bound approximately 50% of α-thrombin. These results suggest that the mutation of Bβ Ala 68 → Thr affects the binding of α-thrombin to fibrin and that defective binding results in a decreased release of FpA and FpB in both homo - and heterozygous abnormal fibrinogens.

INTRODUCTION

The fibrinogen molecule is involved in the final phase of blood coagulation and consists of pairs of Aα, Bβ and γ chains linked by 29 disulphide bonds (1). The conversion of fibrinogen to fibrin is initiated by thrombin catalysed cleavage of fibrinopeptides A (FpA) and B (FpB) from the amino-terminus of the Aα and Bβ chains, respectively. The interaction of thrombin with fibrin(ogen) involves multiple sites on both thrombin and fibrin(ogen). An important domain of fibrinogen which interacts with the catalytic site of thrombin is located in the Aα chain between amino acids 1 and 23 (2,3,4,5,6). The interaction of thrombin with fibrin through a site independent of the catalytic site (anion-binding exosite) (7,8,9,10,11,12) is located in a CNBr derived fragment of the amino-terminal part of the fibrin(ogen) molecule (NDSK) (13,14,15). Binding of α-thrombin to fibrin by the anion-binding exosite results in the removal of thrombin from solution (7,9). It has been proposed that this interaction plays an important role in the regulation of thrombus formation in vivo by limiting the amount of free active thrombin in the

circulation (7). Congenitally abnormal fibrinogens are valuable tools for structure-function studies of human fibrinogen. They also provide a basis for correlating the molecular defect with the clinical symptoms of affected individuals. Many abnormal fibrinogens have been described (16), of which a large number show defective release of fibrinopeptides due to a mutation at or near the thrombin cleaved bond (17,18,19,20). The structural defect of an abnormal fibrinogen (fibrinogen New York I) with impaired thrombin binding to fibrin (21) has been determined. This fibrinogen lacks amino acids 9 to 72 in the Bβ-chain (22). The patient, who was shown to be heterozygous, suffered from thrombotic episodes (23). Fibrinogen Naples (Milano II) (24,25) is another abnormal fibrinogen associated with congenital thrombophilia. Preliminary work on fibrinogen Naples demonstrated a defective interaction between bovine thrombin and fibrin of the propositus (24). In this paper we report the structural defect of fibrinogen Naples, inferred from genetic analysis of patient DNA using the polymerase chain reaction (26). The presence of the mutation in the family members, the binding of human α-thrombin to fibrin and the relationship between the defect and clinical symptoms was also determined.

METHODS

Patients

The propositus (II.3) developed post-operative deep-vein thrombosis at the age of 33. His sister (II.1) had a stroke at the age of 25 due to thrombotic occlusion of the internal carotid artery and his brother (II.2) had a stroke and thrombosis of the abdominal aorta at the age of 21. Another brother (II.4) of the propositus is asymptomatic, as are his parents (I.1 and I.2) (who are first cousins).

Coagulation studies on plasma

Blood was collected by venepuncture and anti-coagulated with 1/10 volume of 0.11 M trisodium citrate. Plasma was prepared by centrifugation at 2300 g for 30 min at 4 °C. Thrombin clotting times were performed at 37 °C with 200 μl plasma and 50 μl of human α-thrombin (1000 NIH/mg, Protogen, Läufelfingen, Switzerland), dissolved in 0.15 M NaCl containing 0.025% (w/v) gelatine to a concentration of 25 NIH/ml. Reptilase^R (Boehringer Mannheim, Mannheim, Germany) clotting time was performed as described (24). Fibrinogen concentration was determined functionally according to Clauss (27) and immunologically according to Mancini (28).

Purification of fibrinogen and NDSK

Fibrinogen was purified from plasma as described (29) and dialyzed against PBS for 24

hours at 4°C. NDSK was purified after CNBr digestion of purified fibrinogen (30) by a FPLC system equipped with a Superose 12 column (Pharmacia, Uppsala, Sweden). The column was equilibrated with 10% acetic acid containing 0.1 M NaCl and run at a flow rate of 1.0 ml/min. The CNBr digest, 6 mg protein in 0.3 ml, was injected and 0.5 ml fractions were collected. Fractions 12 to 15 (Fig. 1) were pooled and analyzed on SDS-PAGE (31). The purified fibrinogen NDSK showed a M_r of about 65000 and a purity of about 90% (Fig. 1). Pooled fractions were dialyzed against distilled water, lyophilized and dissolved in PBS to a concentration of 0.7 mg/ml. The fibrinogen and NDSK concentrations were measured spectrophotometrically at 280 nm (fibrinogen, $A^{1\%,1cm}$ = 15.0, NDSK, $A^{1\%,1cm}$ = 12.0); the yield of this purification was approximately 65%.

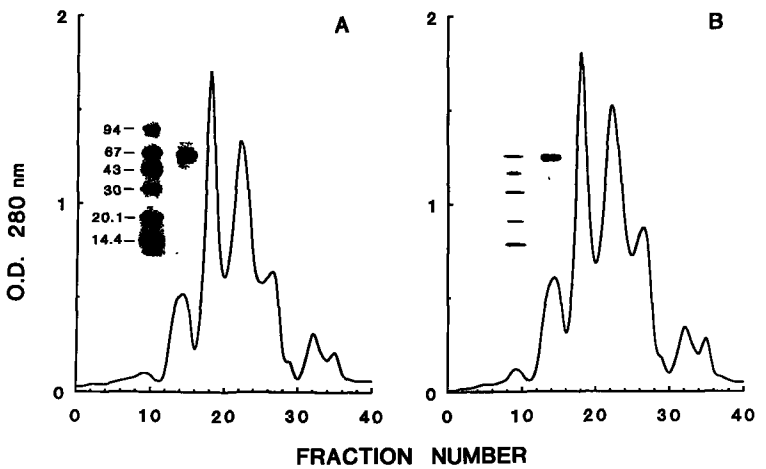


Figure 1. Elution profiles of FPLC purification of NDSK from CNBr digest of fibrinogen and SDS-PAGE analysis of Pooled fractions 12 -15. (A) normal fibrinogen (B) fibrinogen Naples II.3

Release of fibrinopeptides from fibrinogen and NDSK

The rate of FpA and FpB release was determined at 37°C with 100 μl of fibrinogen solution (4.0 mg/ml) or NDSK solution (0.7 mg/ml) and 10 μl of either 1 NIH unit/ml human α-thrombin or 1:30 diluted Reptilase^R. The reactions were stopped at various times by placing the samples in a boiling water bath for 2 min. The samples were centrifuged at 12,000 xg for 10 min and 50 μl of the supernatant was analyzed by HPLC (LKB, Bromma, Sweden) on a C-18 reversed phase column (Chrompack, Middelburg, The Netherlands) (24,32). The amount of FpA and FpB released was determined by measuring the peak area.

Preparation of ^{14}C-DFP α-thrombin

One ml of human α-thrombin, dissolved at 1 mg/ml in 10 mM Tris/HCl, pH 8.3, containing 0.75 M NaCl, was incubated with 100 μl of ^{14}C-DFP (108 mCi/mMol, 1.9 μM/ml, New England Nuclear, s'Hertogenbosch, The Netherlands) for 90 min at 25 °C. Subsequently 10 μl of a 0.1 M DFP solution in dry isopropanol was added, and incubated for 30 min. The sample was dialysed against 50 mM Tris/HCl, pH 7.5, 100 mM NaCl for 24 hours at 4 °C. Protein concentration was measured spectrophotometrically at 280 nm ($A^{1\%,1cm}$ = 18.0). The amidolytic activity of inhibited thrombin, determined using the synthetic substrate S-2238 (KabiVitrum, Stockholm, Sweden) according to the manufacturers instructions, was less than 0.05% of the original activity. The relative α, β and γ thrombin content was determined by SDS-PAGE (31) followed by autoradiography. The amount of α-thrombin was higher than 95% (Fig. 2). To prevent nonspecific adsorption, gelatine (final concentration 0.1% w/v) was added to the thrombin solution, and this solution was stored at -70 °C.

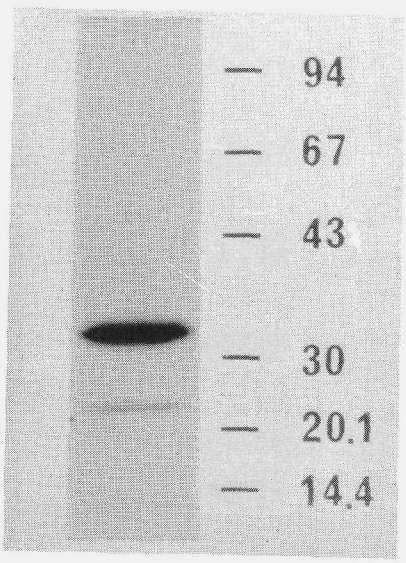


Figure 2. Autoradiogram of ^{14}C-DFP α-thrombin after SDS-PAGE under reducing conditions.

^{14}C-DFP α-thrombin binding to fibrin

Fibrinogen was dialysed against 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, and diluted to 0.5 mg/ml. Part of this fibrinogen solution was radiolabelled with ^{125}I (Amersham, Buckinghamshire, England) as described (33). To 0.15 ml of fibrinogen, 50 μl of solutions containing different amounts of ^{14}C-DFP inhibited α-thrombin diluted in 50 mM Tris/HCl pH 7.5, 100 mM NaCl, 0.1 % (w/v) gelatine were added. Subsequently, fibrin formation was induced by incubating with 10 μl undiluted Reptilase^{R} solution for 30 min. Fibrin was collected by centrifugation for 10 min at 12,000 xg. The pellet was washed three times with 0.5 ml 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, and dissolved in 0.2 ml

50 mM acetic acid. The sample was mixed with 3.0 ml scintillation fluid (Ultima Gold, Packard, Downers Grove, IL, USA) and ^{14}C was counted (Tricarb 1900 CA, Packard). The amount of fibrin formed was determined in parallel experiments containing trace amounts of ^{125}I-fibrinogen. After dissolving the fibrin in acetic acid, the amount of radioactivity was counted in a gamma counter (Cobra, Packard). Under these conditions normal fibrinogen, fibrinogen Naples II.3 and II.4 were 93-96% clottable.

Polymerase chain reaction

Genomic DNA was isolated from blood cells as described (34). Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer (Foster City, CA, USA). Oligonucleotides α1a (5'GCAGATAGTGGTGAAGGTGAC 3') and α1b (5'GTTATTG GCTGAGGAAAAATCGCC 3') were used to amplify the part of the α-chain gene, coding for amino acids 1 to 95. Oligonucleotides β1a (5'GGTGTTGGAATAGTTACA TTCC 3') and β1b (5'GGTGTGTGAGTTCTTCTGGA 3') were used to amplify the part of the Bβ-chain gene, coding for amino acid 9 to 209. β2a (5'GCCTCTAAGGTTGTAG GAATTCTTCAG 3') and β2b (5'ATCAGTGCACCCACCAAGTCTGGG 3') were used to amplify the β-gene segment coding for amino acids 9 to 72. Oligonucleotides γ1a (5'GCTCTTCACAAAACGTTGTTTAAAATGGAATTCTGG 3') and γ1b (5'CAGTCT TGCAGAGCAAATTAAAACAAAAATCCTTAC 3') were used to amplify the part of the γ-chain gene coding for amino acids 1 to 108. Amplification by PCR (26) was performed in a 100 μl reaction volume containing 1 μg genomic DNA, 0.2 mM of each dNTP (Pharmacia), 0.2 μM of each primer in 1X reaction buffer (10 mM Tris/HCl pH 8.3 at 25°C, 50 mM KCl, 3.0 mM $MgCl_2$ and 0.001% (w/v) gelatine). The DNA was denatured at 95°C for 8 min and 2.0 units Taq DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA, USA) were added. Cycles consisted of a 1 min 95°C, 0.5 min 60°C and 3 min 70°C incubation. After 30 cycles 5 μl of the sample was analyzed on a 1.0 % agarose gel (A-6013, Sigma, St.Louis, USA).

Direct sequencing PCR fragments

PCR samples were run on a 1% (w/v) ultra low gelling agarose gel (A-5030, Sigma). The band with the appropriate size, as predicted by the genomic sequence of the Aα (16), Bβ (16,35) and γ chain genes (36), was cut out of the gel and heated to 65°C. 1 μl of the melted agarose, containing approximately 10 ng DNA, was mixed with 1 μl of the appropriate PCR primer (60 ng), and 2 μl 5X sequence buffer (T7 sequence kit, Promega, Madison, WI, USA), the volume was brought up to 10 μl with distilled water. The mixture was heated to 95°C for 3 min and immediately put on ice. Labelling and termination reactions were performed using the T7 DNA sequence kit (Promega) according to the manufacturer's instruction.

Southern blot analysis of family members and normal population

Amplified fragments of the Bβ-chain gene containing the sequence coding for amino acids 9 to 72 (approximatey 50 ng) were run on a 2% (w/v) agarose gel (A-6013, Sigma). The gel was washed (2 x 15 min) with 0.5 M NaOH, 1.5 M NaCl and the denatured DNA was transferred to a nylon membrane (Hybond N, Amersham, Buckinghamshire, UK) using the vacugene blotting system (LKB). 100 ng of sequence specific oligonucleotides (β-Normal: 5'TGTCTTCACGCTGACCCAG 3'and β-Naples:5'TGTCTTCACACTGACCC AG 3') were radiolabeled with T4 Polynucleotide kinase (GIBCO BRL, Breda, The Netherlands) according to the manufacturer's instructions, using $^{32}P\gamma$-ATP (3000 Ci/mmol, Amersham). The blots were washed with 2x SSC (1X SSC contains 0.15 M NaCl and 0.015 M $Na_3C_6H_5O_7$) and hybridized with the labelled oligonucleotides in 40 ml of 7% SDS, 0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4 and 10 mM EDTA for 4 hours at 42°C. Blots were washed three times for 30 min at 58°C. with 6X SSC containing 0.5% (w/v) SDS, and exposed to X-ray film (X-AR, Kodak, Rochester, NY, USA) for 16 hours.

Table 1. Plasma coagulation studies.

	Plasma clotting time(s)		Plasma Fibrinogen (mg/ml)	
	Human α-Thrombin	Reptilase^R	Functional	Immunological
normal	19.8	22.1	2-4	2-4
I.1	21.3	22.3	2.1	2.5
I.2	20.7	22.6	2.6	2.8
II.1	>180	21.9	1.6	2.7
II.2	>180	22.4	1.4	2.4
II.3	>180	21.8	1.7	3.0
II.4	21.0	22.9	2.3	2.5

RESULTS

Coagulation studies on plasma

Table 1 shows the results of the thrombin and Reptilase^R clotting time assays and of the immunological and functional fibrinogen assays performed on plasma of normal individuals, the propositus (II.3) and his family members. The propositus and two of his siblings (II.1, II.2) showed a strongly delayed thrombin clotting time, but a normal Reptilase^R time. They also showed a discrepancy between the immunologically and the

functionally-determined fibrinogen concentration. Three other family members (father, mother and one other sibling: I.1, I.2, II.4) showed normal thrombin and Reptilase^{R} clotting times and no significant difference between the immunologically and functionally-determined fibrinogen concentration.

Fibrinopeptide release of fibrinogen

Figure 3 shows the thrombin catalyzed release of the FpA and FpB from normal fibrinogen, fibrinogen Naples II.3 (propositus with prolonged thrombin clotting time) and Naples II.4 (sibling with normal thrombin clotting time). FpA release (Fig. 3A) from both fibrinogens Naples II.3 and II.4 is strongly delayed as compared with normal fibrinogen. There is a large difference in the amount of FpA released after 60 min from normal fibrinogen (100%), fibrinogens Naples II.4 (63%) and Naples II.3 (24%). Fibrinogen Naples II.3 also showed a lag of about 10 min before any FpA could be detected; fibrinogen Naples II.4 and normal fibrinogen did not show this lag period. The release of FpB (Fig. 3B) from fibrinogens Naples II.3 and II.4 was also strongly delayed as compared with normal fibrinogen. After 60 min, normal fibrinogen released 92% of FpB, fibrinogen Naples II.4 released 30% of FpB and Naples II.3 only released 10% of FpB. With normal fibrinogen and fibrinogen Naples II.4, FpB was detected after a lag period of 2.5 min; with fibrinogen Naples II.3 this lag period was 25 min. Reptilase^{R} catalyzed release of FpA was the same for all three fibrinogens (data not shown).

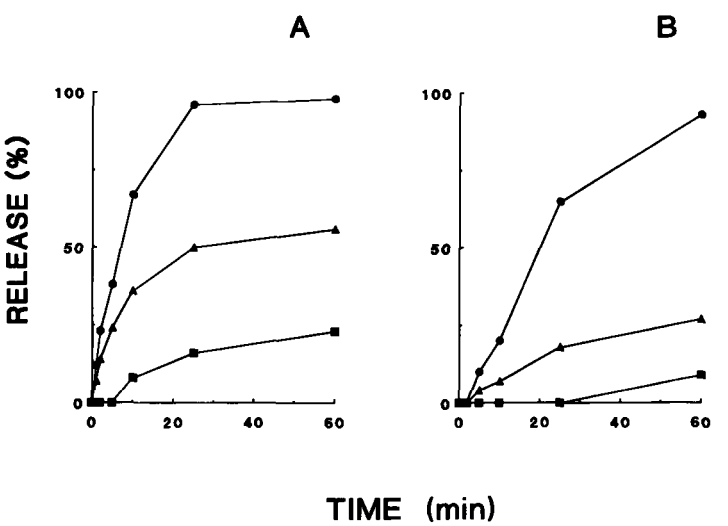


Figure 3. Release of fibrinopeptides from intact fibrinogen by human α-thrombin, determined by HPLC. Panels (A) FpA and (B) FpB release from (●) Normal fibrinogen (▲) fibrinogen Naples II.4 and (■) fibrinogen Naples II.3.

Fibrinopeptide release of NDSK

Figure 4 shows the release of FpA and FpB by thrombin from NDSK purified from normal fibrinogen and fibrinogen Naples II.3. As was found with intact fibrinogens, the release of FpA from NDSK Naples II.3 was strongly delayed as compared with that of normal NDSK. The release of FpA from both normal and Naples II.3 NDSK was identical to the release from intact normal and Naples II.3 fibrinogen, respectively (compare Fig. 4 with Fig. 3A).

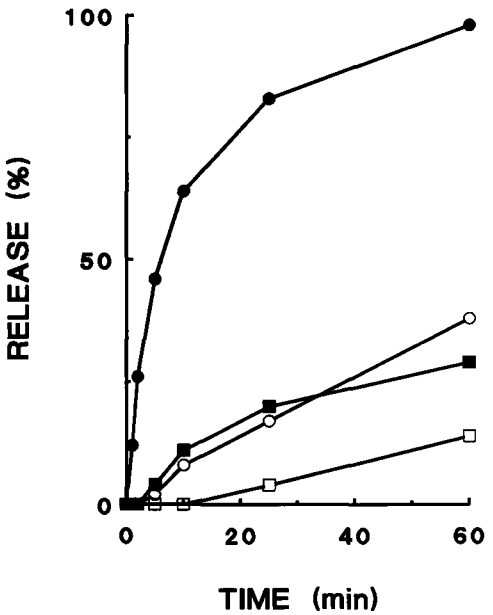


Figure 4. Release of fibrinopeptides from purified NDSK by human α-thrombin determined by HPLC. (●) FpA, (○) FpB form Normal NDSK and (■) FpA, (□) FpB from NDSK Naples II.3.

The release of FpB from NDSK Naples II.3 was delayed as compared with normal NDSK. In contrast to the FpA release, the FpB release from normal NDSK is slower than the FpB release from intact normal fibrinogen (compare Fig. 4 with Fig. 3B). However, the FpB release of NDSK Naples II.3 was approximately the same as the FpB release of intact fibrinogen Naples II.3 (compare Fig. 4 with Fig. 3B).

The release of FpA by Reptilase^{R} from NDSK Naples II.3 was the same as that from normal NDSK (data not shown).

Binding of active-site inhibited human α-thrombin to fibrin

Figure 5 shows that the binding of ^{14}C-DFP inhibited α-thrombin to Reptilase^{R}-induced fibrin clots from normal fibrinogen, fibrinogen Naples II.3 and II.4. With normal fibrin, thrombin binding increased with increasing thrombin concentration, reaching saturation at approximately one mole of inhibited α-thrombin per mole of fibrin. With fibrin Naples II.4 the shape of the binding curve was similar, but saturation was achieved at approximately 0.5 moles of thrombin per mole of fibrin. Thrombin bound poorly to fibrin Naples II.3 with less than 0.1 moles of thrombin bound/mole of fibrin at the highest thrombin concentration tested. Scatchard analysis of the binding data for normal fibrinogen and Naples II.4 (inset, Fig. 5) indicated one class of binding sites (10,13,14). The maximal molar binding ratio (thrombin/fibrin) for normal fibrin was 1.1 ± 0.25 with K_a = 12.7 ± 3.0 x $10^5 M^{-1}$, for fibrin Naples II.4 this ratio was 0.70 ± 0.15 with K_a = 13.8 ± 3.1 x 10^5 M^{-1}. No Scatchard plot could be constructed for the binding data obtained with fibrin Naples II.3, because of low binding under these conditions.

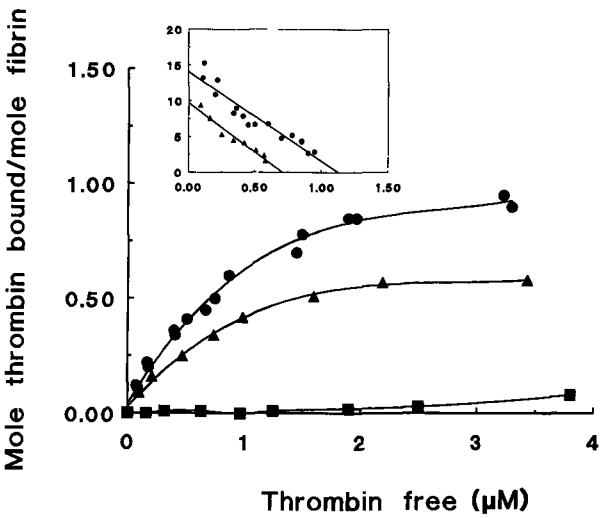


Figure 5. Binding of ^{14}C-DFP α-thrombin to Reptilase^{R} induced fibrin clots. (●) normal fibrinogens (▲) fibrinogen Naples II.4 and (■) fibrinogen Naples II.3. Inset: Scatchard plot of binding data from panel A (normal fibrinogen and fibrinogen Naples II.4).

Amplification and direct sequencing of genomic DNA fragments

Based on the evidence of fibrinopeptide release by thrombin, which showed that the defect was located in the NDSK part of fibrinogen Naples, we amplified the genomic DNA which codes for NDSK, specifically amino acids Aα 1-51, Bβ 1-118 and γ 1-79 of the fibrinogen molecule. After amplification, fragments with the sizes predicted from the genomic DNA sequences for the human fibrinogen Aα, Bβ and γ chain genes (16,35,36)

were sequenced. The fragments containing the Aα or γ gene sequence were completely normal, while the Bβ fragment of Naples II.3 had a single base substitution (Fig. 6) in the codon normally coding for Alanine at position 68. This mutation changed the codon GCT (Alanine) to ACT which codes for Threonine. The normal sequence was completely missing in Naples II.3, indicating that the propositus was homozygous for this mutation.

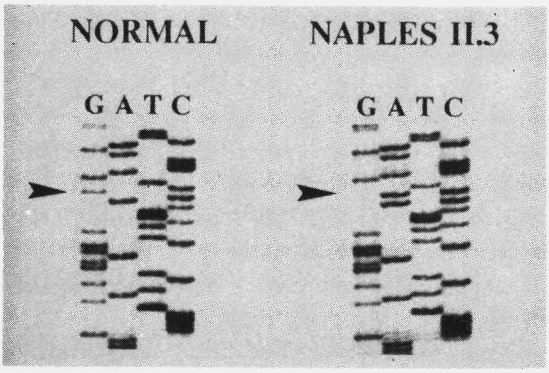


Figure 6. DNA sequence of Bβ-chain gene fragment after amplification coding for amino acids 9 to 209 (arrows indicate the mutation).

Detection of mutation in family members and normal individuals

The amplified Bβ fragments of the family members were hybridized with two synthetic oligonucleotides, one with the normal sequence (β-normal) and the other with the sequence found in fibrinogen Naples (β-Naples). Fig. 7 shows that the amplified Bβ

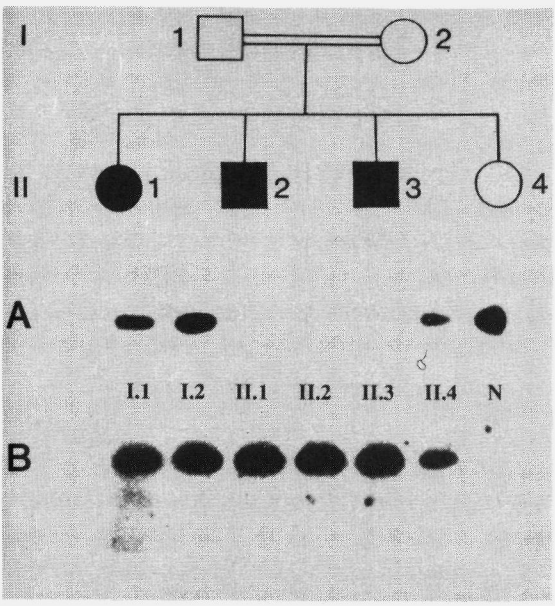


Figure 7. (Top) Pedigree of the family. The propositus (II.3) with symptoms of venous thromboembolism, his sister (II.1) and brother (II.2) with symptoms of arterial thrombosis, whereas the parents I.1 and I.2 (first cousins) and the brother II.4 are asymptomatic.

(Bottom) Southern blot analysis of the Bβ-chain gene fragment of normal and Naples family using sequence specific oligonucleotides (A) normal sequence (B) Naples sequence.

fragments of Naples I.1, I.2 and II.4 hybridize with both oligonucleotides, indicating that the asymptomatic family members are heterozygous for the mutation found in the Bβ fragment. Amplified Bβ fragments of Naples II.1, II.2 and II.3 hybridized only with the β-Naples oligonucleotide, showing that the symptomatic family members were homozygous for the mutation. The corresponding fragment of 120 normal individuals hybridized only with the β-normal oligonucleotide (data not shown), indicating that the mutation was not a common polymorphism.

CONCLUSION AND DISCUSSION

The mutation in fibrinogen Naples is associated with a defective release of FpA and FpB by thrombin. As this defect was present in the purified NDSK fragment of fibrinogen Naples, we amplified and sequenced the genomic DNA segments encoding the NDSK fragments Aα, Bβ and γ chains. Sequence analysis of the amplified products demonstrated that the propositus (II.3) was homozygous for a single base substitution in the codon for Bβ Ala 68 (GCT) resulting in a Thr (ACT) at this position. Southern blot analysis using sequence specific oligonucleotides showed that all three asymptomatic family members were heterozygous for the mutation while the three symptomatic members (including the propositus) were homozygous. This indicates that the homozygous mutation (Bβ 68 Ala - Thr) is associated with thrombophilia, and that this type of dysfibrinogenemia is clinically recessive.

Using fibrinogen purified from the homozygous propositus, α-thrombin catalyzed FpA release was delayed relative to normal fibrinogen. The rate of FpA release from fibrinogen isolated from a heterozygous family member (II.4) was approximately half that of normal, indicating that heterozygous individuals have both normal and abnormal molecules. Human α-thrombin catalyzed release of FpA from the purified NDSK fragment of normal fibrinogen was identical to that of intact fibrinogen. As previously reported (2,3), this provides evidence that the NDSK fragment contains all the essential information for an effective interaction of thrombin with fibrinogen. Analogously, NDSK from the homozygous fibrinogen Naples II.3 had the same decreased rate of FpA release by α-thrombin as intact fibrinogen Naples II.3, indicating that the defective domain is located in the NDSK part of the fibrinogen Naples molecule. The release of FpA by Reptilase^{R} from fibrinogen Naples and NDSK Naples II.3 was normal, indicating that the mutation did not affect the substrate cleavage site of FpA in the Aα-chain.

The rate of FpB release from homozygous fibrinogen Naples II.3 was also strongly delayed as compared to normal fibrinogen. The FpB release from NDSK Naples II.3 was approximately the same as from intact fibrinogen Naples II.3, which is in contrast with the slower release of FpB from normal NDSK as compared with normal fibrinogen. The

reduced release of FpB from normal NDSK as compared with normal intact fibrinogen, can be explained by the accelerating effect of fibrin polymerization on FpB release (37-40), which is absent when using NDSK. The similar release of FpB from NDSK Naples II.3 and intact fibrinogen Naples II.3 suggests that this accelerating effect of fibrin polymerization is absent in fibrin Naples.

Active site inhibited thrombin binds to fibrin by a site independent of the catalytic site designated the anion-binding exosite (7-12). Active site inhibited human α-thrombin bound to normal fibrin formed by Reptilase^{R}. In contrast, thrombin binding to fibrin Naples II.3 was very low while thrombin binding to fibrin Naples II.4 was about half that of normal fibrin. Scatchard analysis of the binding data indicated a single class of binding sites with maximal binding of 1.1 moles of thrombin per mole of fibrin and K_a = 1.3 x 10^6 M^{-1}. This approximates the strongest binding (K_a = 5.8 x 10^5 M^{-1}) determined by Liu et al. (7), who reported that fibrin contains two classes of binding sites. Our data determined under different experimental conditions indicate only one class of binding sites. Irrespective of these differences, many studies indicate that α-thrombin binds to fibrin by a site distinct from its catalytic centre and that the binding site on fibrin is located in the NDSK part of the fibrinogen molecule (13-15). The results with fibrinogen Naples indicate that the Bβ Ala 68 → Thr substitution fully disrupts thrombin binding at this site.

Fibrinogen New York I (23) also showed a decreased release of FpA and FpB, and a defective binding of thrombin (21). In contrast to fibrinogen Naples, the defect in fibrinogen New York I is extensive, since amino acids Bβ 9-72 are deleted. This large deletion is likely to have multiple effects on the defective fibrinogen structure, particularly because Bβ Cys 65, which normally forms a disulphide bond with Aα Cys 36, is missing. Without information about the three-dimensional structure of NDSK, it is difficult to determine the exact influence of the Bβ 68 Ala → Thr substitution on the binding to thrombin. One possible explanation is, that Bβ 68 alanine participates in a non-polar interaction with thrombin and that this is disrupted by threonine. Alternatively, the slightly larger side chain of threonine could lead to incorrect folding or disulphide bond formation of this part of the fibrinogen molecule, preventing thrombin binding.

It has been suggested that thrombin binding to fibrin is a mechanism that prevents active thrombin from existing free in the circulation (7). In the absence of this binding, free active thrombin in the circulation results in excessive coagulation and/or platelet aggregation which in its turn can lead to thrombosis. From the dramatic history of thrombophilia in the family with fibrinogen Naples and the genetic analysis of the family it is clear that the occurrence of thrombosis is related to the defect in fibrinogen Naples only in homozygous family members. The mutation in fibrinogen Naples (Bβ 68 Ala → Thr) prevents thrombin binding to fibrin and is correlated with thrombophilia. These results demonstrate that thrombin binding to fibrin is an important in vivo mechanism

to limit the presence of free active thrombin in circulation and to prevent excessive coagulation and/or platelet activation leading to thrombosis.

ACKNOWLEDGEMENT

We thank Dr. Dana Fowlkes for synthesizing the oligonucleotides. This work was supported by NIH grant no HL 31048 (STL), NATO grant no CRG 860110 and PF Ingegneria Genetica CNR (PMM).

REFERENCES

1. Doolittle, R. F. 1984. Fibrinogen and fibrin. *Ann. Rev. Biochem.* 53:195-229.
2. Hogg, D. H., and B. Blombäck. 1978. The mechanism of the fibrinogen-thrombin reaction. *Thromb. Res.* 12:953-964.
3. Marsh, H. C., Y. C. Meinwald, T. W. Thannhauser, and H. A. Scheraga. 1983. Mechanism of action of thrombin on fibrinogen. Kenetic evidence for involvement of aspartic acid at position P_{10}. *Biochemistry* 22:4170-4174.
4. Ni, F., Y. Konishi, R. B. Frazier, H. A. Scheraga, and S. T. Lord. 1989. High-resolution NMR studies of fibrinogen-like peptides in solution: Interaction of thrombin with residues 1-23 of the Aα chain of human fibrinogen. *Biochemistry* 28:3082-3094.
5. Ni, F., Y. C. Meinwald, M. Vásquez, and H. A. Scheraga. 1989. High-resolution NMR studies of fibrinogen-like peptides in solution: Structure of a thrombin-bound peptide corresponding to residues 7-16 of the Aα chain of human fibrinogen. *Biochemistry* 28:3094-3105.
6. Lord, S. T., P. A. Byrd, K. L. Hede, C. Wei, and T. J. Colby. 1990. Analysis of fibrinogen Aα-fusion proteins: mutants which inhibit thrombin equivalently are not equally good substrates. *J. Biol. Chem.* 265:838-843.
7. Liu, C. Y., H. L. Nossel, and K. L. Kaplan. 1979. The binding of thrombin by fibrin. *J. Biol. Chem.* 254:10421-10425.
8. Fenton II, J. W., M. P. Zabinski, K. Hsieh, and G. D. Wilner. 1981. Thrombin noncovalent-protein binding and fibrin(ogen) recognition. *Thromb. Haemostas.* 46:177.
9. Wilner, G. W., M. P. Danitz., S. Mudd, K. H. Hsieh, and J. W. Fenton II. 1981. Selective immobilization of α-thrombin by surface-bound fibrin. *J. Lab. Clin. Med.* 97:403-411.
10. Kaminski, M., and J. McDonagh. 1983. Studies on the mechanism of thrombin. *J. Biol. Chem.* 258:10530-10535.
11. Berliner, L. J., and Y. Sugawara. 1985. Human α-thrombin binding to nonpolymerized fibrin-sepharose: evidence for an anionic binding region. *Biochemistry* 24:7005-7009.
12. Kaminski, M., and J. McDonagh. 1987. Inhibited thrombins. Interactions with fibrinogen and fibrin. *Biochem. J.* 242:881-887.
13. Fenton II, J. W., T. A. Olsen, M. P. Zabinski, and G. D. Wilner. 1988.Anion-binding exosite of human α-thrombin and fibrin(ogen) recognition. *Biochemistry* 27:7106-7112.
14. Vali, Z., and H. A. Scheraga. 1988. Localization of the binding site on fibrin for the secondary binding site of thrombin. *Biochemistry* 27:1956-1963.
15. Kaczmarek, E., and J. McDonagh. 1988. Thrombin binding to the Aα-, Bβ- and γ-chains of fibrinogen and to their remnants contained in fragment E. *J. Biol. Chem.* 263:13896-13900.

16. Ebert, R. 1990. Index of variant human fibrinogens, 1990 Edition. R. F. Ebert, editor. Ebert, R. F., Rockville, P.O. Box 5811, Maryland, USA.
17. Southan, C., D. A. Lane, W. Bode, and A. Henschen. 1985. Thrombin induced fibrinopeptide release from a fibrinogen variant (fibrinogen Sydney I) with an Aα Arg 16→ His substitution. *Eur. J. Biochem.* 147:593-600.
18. Kaudewitz, H., A. Henschen, C. Soria, J. Soria, O. Bertrand, and D. Heaton. 1986. The molecular defect of the genetically abnormal fibrinogen Christchurch II. In *In* Fibrinogen and its derivatives. Biochemistry, Physiology and Pathophysiology. G. Müller-Berghaus, U. Scheefers-Borchel, E. Selmayr, and A. Henschen, editors. Elsevier, Amsterdam. 31-36.
19. Pirkle, H., H. Kaudewitz, A. Henschen, I. Theodor, and G. Simmons. 1987. Substitution of Bβ-14 Arginine by Cyst(e)ine in fibrinogen Seattle I. *In* Fibrinogen 2. Biochemistry, Physiology and Clinical Relevance. G. D. O. Lowe, J. T. Douglas, C. D. Forbes, and A. Henschen, editors. Elsevier, Amsterdam. 49-52.
20. Koopman, J., and F. Haverkate. 1990. Structure-function relationships in abnormal fibrinogens. Defects at the DNA level. *In* Fibrinogen 4. Current basic and clinical aspects. M. Matsuda, S. Iwanaga, A. Takada, and A. Henschen, editors. Elsevier, Amsterdam. 179-183.
21. Liu, C.Y., P. Wallen, and D. A. Handley. 1986. Fibrinogen New York I: The structural, functional, and genetic defects and a hypothesis of the role of fibrin in the regulation of coagulation and fibrinolysis. *In* Fibrinogen - fibrin formation and fibrinolysis. D. A. Lane, A. Henschen, and M. K. Jasani, editors. Walter de Gruyter, Berlin. 79-90.
22. Liu, C. Y., J. A. Koehn, and F. J. Morgan. 1985. Characterization of fibrinogen New York I. *J. Biol. Chem.* 260:4390-4396.
23. Al-Mondhiry, H. A. B., S. B. Bilezikian, and H. L. Nossel. 1975. Fibrinogen "New York"- an abnormal fibrinogen associated with thromboembolism: Functional evaluation. *Blood* 45:607-619.
24. Haverkate, F., J. Koopman, C. Kluft, A. D'Angelo, M. Cattaneo, and P. M. Mannucci. 1986. Fibrinogen Milano II: A congenital dysfibrinogenaemia associated with juvenile arterial and venous thrombosis. *Thromb. Haemostas.* 55:131-135.
25. Haverkate, F., J. Koopman, C. Kluft, A. D'Angelo, M. Cattaneo, and P. M. Mannucci. 1987. Fibrinogens "Milano II" - "Naples". *Thromb. Haemostas.* 57: 375.
26. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 39:487-491.
27. Clauss, A. 1957. Gerinnungsphysiologische schnellmethode zur bestimmung des fibrinogens. *Acta Haematol.* 17:237-246.
28. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochem.* 2:235-254.
29. Van Ruijven-Vermeer, I. A. M., and W. Nieuwenhuizen. 1978. Purification of rat fibrinogen and its constituent chains. *Biochem. J.* 169:653-658.
30. Blombäck, B., B. Hessel, S. Iwanaga, J. Reuterby, and M. Blombäck. 1972. Primary structure of human fibrinogen and fibrin. *J. Biol. Chem.* 247:1496-1512.
31. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 227:680-685.
32. Kehl, M., F. Lottspeich, and A. Henschen. 1981. Analysis of human fibrinopeptide by high performance liquid chromatography. *Hoppe Seyler's Z. Physiol. Chem.* 362:1661-1664.
33. Nieuwenhuizen, W., J. J. Emeis, A. Vermond, P. Kurver, and D. van der Heide. 1980. Studies on the catabolism and distribution of fibrinogen in rats. Application of the iodogen® labelling technique. *Biochem. Biophys. Res. Commun.* 97:49-55.
34. Gustafson, S., J. A. Proper, E. J. W. Bowie, and S. S. Sommer. 1987. Parameters affecting the yield of DNA from human blood. *Anal. Biochem.* 165:294-299.

35. Chung, C. W., B. G. Que, M. W. Rixon, M. Mace, Jr., and E. W. Davie. 1983. Characterization of complementary deoxyribonucleic acid and genomic deoxyribonucleic acid for the β chain of human fibrinogen. *Biochemistry* 22:3244-3250.
36. Rixon, M. W., D. W. Chung, and E. W. Davie. 1985. Nucleotide sequence of the gene for the γ-chain of human fibrinogen. *Biochemistry* 24:2077-2086.
37. Blombäck, B., B. Hessel, D. Hogg, and L. Therkildsen. 1978. A two-step fibrinogen-fibrin transition in blood coagulation. *Nature* 275:501-505.
38. Hurlet-Jensen, A., H. Z. Cummins, H.L. Nossel, and C. Y. Liu. 1982. Fibrin polymerization and release of fibrinopeptide B by thrombin. *Thromb. Res.* 27:419-427.
39. Ruf, W., A. Bender, D. A. Lane, K. T. Preissner, E. Selmayr, and G. Müller-Berghaus. 1988. Thrombin-induced fibrinopeptide B release from normal and variant fibrinogens: influence of inhibitors of fibrin polymerization. *Biochim. Biophys. Acta* 965:169-175.
40. Naski, M. C., and J. A. Shafer. 1990. α-Thrombin-catalyzed hydrolysis of fibrin I. *J. Biol. Chem.* 265:1401-1407.

CHAPTER 8

THE MOLECULAR BASIS FOR FIBRINOGEN DUSART (Aα 554 Arg → Cys) AND ITS ASSOCIATION WITH ABNORMAL FIBRIN POLYMERIZATION AND THROMBOPHILIA

Jaap Koopman[1,2], Frits Haverkate[2], Jos Grimbergen[1], Susan T. Lord[3], Michael W. Mosesson[4], James P. DiOrio[4], Kevin S. Siebenlist[4], Chantal Legrand[5], Jeanette Soria[5], Claudine Soria[5], Jacques P. Caen[6].

[1] Department of Hematology, University Hospital, Leiden
[2] Gaubius Laboratory, IVVO-TNO, Leiden, The Netherlands.
[3] Department of Pathology, University of North Carolina, Chapel Hill, NC 27514.
[4] University of Wisconsin Medical School, Sinai Samaritan Medical Center, Milwaukee, WI 53233, USA
[5] Inserm U. 150, Hôpital Lariboisière, Paris
[6] Institut des Vaisseaux et du Sang, Hôpital Lariboisière, 75010 Paris, France.

SUMMARY

The molecular defect in the abnormal fibrinogen Dusart (Paris V) associated with thrombophilia, was determined by sequence analysis of genomic DNA that had been amplified using the polymerase chain reaction. The propositus was heterozygous for a single base change (C → T) in the Aα-chain gene, resulting in the amino acid substitution Aα 554 Arg → Cys. Restriction analysis of the amplified DNA derived from the family members showed that his father and his two sons were also heterozygous. Electron microscopic studies on fibrin formed from purified fibrinogen Dusart, demonstrated fibers that were much thinner than in normal fibrin. In contrast to the previously observed defective plasminogen binding, the binding of thrombospondin to immobilized fibrinogen Dusart was similar to that of normal. Immunoblot analysis of plasma fibrinogen demonstrated that a substantial part of the fibrinogen Dusart molecules were disulfide-linked to albumin. The plasma of the affected family members also contained fibrinogen-albumin complexes. Furthermore, small amounts of high molecular weight complexes containing fibrinogen were detected in all the heterozygous individuals. These data indicate that the molecular abnormality in fibrinogen Dusart (Aα 554 Arg → Cys) results in defective lateral association of the fibrin fibers, disulfide-linked complex formation with albumin and is associated with a family history of recurrent thrombosis in the affected individuals.

INTRODUCTION

Release of FpA and FpB from fibrinogen exposes two types of polymerization sites, designated "A" and "B", in the amino-terminal portion of the fibrin molecule (1) that appear to function cooperatively in the fibrin self-assembly process (2,3). Complementary polymerization sites, designated "a" and "b", respectively, are located in the carboxyl-terminal regions of the molecule (1). Fibrin assembly commences with formation of double-stranded fibrils (2-6), which then branch to form a three-dimensional matrix, concomitant with lateral association of fibrils, that results in increased fiber thickness (7-9). Fibril formation is predominantly due to interaction between the "A" and "a" sites (4); interaction between "B" and "b" sites contributes to lateral fibril association and augment thick fiber formation (4,9). It has recently been suggested that the carboxyl-terminal region of the Aα chain constitutes an important component of the "b" polymerization site in fibrinogen (10).

Congenital abnormal fibrinogens not only provide tools for studying the structure-function relationship in fibrinogen, they also offer us an opportunity to determine the

relationship between the molecular defect and the clinical symptoms of the affected individuals. More than 240 cases of inherited dysfibrinogenemia have been reported (11), of which 48 are associated with clinically significant thromboembolic disease. In one such case, fibrinogen Dusart, the functional defect is related to reduced plasminogen binding (12), impaired plasminogen activation by t-PA (13) and abnormal fibrin polymerization (12).

In this paper we report the structural defect of fibrinogen Dusart (Paris V), inferred from genetic analysis using the polymerase chain reaction (14). The presence of the mutation in the family members and the influence on fibrin polymerization was determined. The additional cysteine created by the mutation was involved in the formation of fibrinogen-albumin complexes in plasma. Furthermore, the family history of recurrent thrombosis and the analysis of the fibrinogen gene in the family members demonstrate a convincing association between the molecular defect and the thrombophilia.

METHODS

Coagulation studies on plasma

Blood was collected by venepuncture and anti-coagulated with 1/10 volume of 0.13 M trisodium citrate. Platelet poor plasma was prepared by centrifuging citrated blood at 2000 xg for 15 min at 15 °C. Thrombin and Reptilase^{R}-clotting times were performed as described before (15). Fibrinogen concentration was determined functionally according to Clauss (16) and immunologically according to Mancini (17). Antithrombin III activity was measured by a chromogenic assay (18), protein C activity by using synthetic substrate (Behring reagent) and protein S by immunoassay (STAGO).

Protein purification

Fibrinogen was purified from plasma of the propositus and a normal individual (19) and further analyzed as described (12). Thrombospondin was purified from the supernatant of thrombin-activated platelets by heparin-Sepharose affinity chromatography followed by Sepharose-4B gel filtration, essentially as described by Margossian et al. (20) and modified as described (21). The calcium concentration was maintained at 2 mM throughout the purification procedure to avoid structural modification of the thrombospondin molecule. Purified thrombospondin was radiolabeled with carrier-free ^{125}I using the chloramine-T procedure to a specific activity of approximately 0.2 μCi/μg protein. The radiolabeled thrombospondin had the same electrophoretic mobility as the unlabeled counterpart, and exhibited specific and saturable binding to thrombin-activated platelets (21). Protein concentrations were determined spectrophotometrically at 280 nm, the $A^{1\%,1cm}$ used for fibrinogen and thrombospondin were 15.0 and 10.4, respectively.

Electron microscopic studies on fibrin

Fibrin for critical point drying was prepared by addition of human α-thrombin (0.1 U/ml final concentration) to a solution of fibrinogen (50 μg/ml in 50 mM Tris/HCl, 100 mM NaCl, pH 7.4 buffer; μ, 0.14) followed by incubation for 60 min at room temperature. A specimen of the fibrin clot was picked up on a carbon-coated 200 mesh grid, fixed with glutaraldehyde/tannic acid, stained with uranyl acetate, dehydrated and then critical point dried. Electron Microscopy (EM) was carried out in a Philips 400 electron microscope at 120 kV.

Binding of thrombospondin to fibrinogen

Microtiter wells were coated in duplicate with 0.1 ml of a 10 μg/ml freshly prepared fibrinogen solution in 10 mM Tris/HCl, 150 mM NaCl, pH 7.4 (Tris-buffer) containing 2 mM $CaCl_2$, overnight at 22°C in a humid chamber. Wells were rinsed twice with Tris-buffer containing 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.05% (v/v) Tween 20 (Tris/Tween-buffer), and subsequently incubated for 1 hour with Tris/Tween-buffer containing 1.5% (w/v) BSA. The wells were rinsed four times and incubated with increasing concentrations of ^{125}I-thrombospondin in Tris/Tween-buffer for 3 hours. The wells were washed 4 times with Tris/Tween-buffer, cut out, and the radioactivity associated with each well was counted. Non specific binding was determined by measuring the binding of ^{125}I-thrombospondin to wells coated only with BSA.

Immunoblot analysis of plasma

SDS-PAGE was performed on 5-25% gradient gels according to Laemmli (22) or on 2-16% precast gradient gels (Pharmacia, Uppsala, Sweden) using an electrophoresis buffer containing 40 mM Tris, 20 mM sodium acetate, 2 mM disodium EDTA, 0.2% (w/v) SDS, pH 7.4 and a sample buffer containing 10 mM Tris/HCl, 1 mM disodium EDTA, 1% (w/v) SDS, pH 8.0. Molecular weights were estimated using a low mol wt calibration kit (Pharmacia) containing proteins with a mol wt ranging from 14.4 kD to 94 kD or using a mixture of purified fibrinogen (340 kD), fibronectin (450 kD) (purified from fibrinogen by affinity chromatography on gelatin-Sepharose) and purified mouse monoclonal IgM (900 kD) (a generous gift from Dr.R. Bos, Gaubius laboratory, The Netherlands).

Proteins from the SDS-PAGE gels were electroblotted onto nitrocellulose (23) for 16 hours at 400 mA and 10°C. The nitrocellulose sheets were blocked by incubating them in Tris/Tween-buffer, pH 7.4, containing 0.15 M NaCl, 0.5% (w/v) gelatin for two hours. After blocking, the blots were washed with Tris/Tween-buffer and incubated for two hours at room temperature with mouse monoclonal anti-fibrinogen Y18 (24) conjugated to horse radish peroxidase (HRP) diluted in Tris/Tween-buffer (kindly provided by Dr. W. Nieuwenhuizen, Gaubius Laboratory, The Netherlands). Identical blots were incubated for two hours at room temperature with goat anti-human albumin

conjugated to HRP (Nordic, Tilburg, The Netherlands) diluted in Tris/Tween-buffer. The protein bands reacting with the different immuno conjugates were visualized by incubation with a substrate solution containing 4-chloro-1-nahphtol (25).

DNA amplification and sequencing

Genomic DNA was isolated from blood cells as described (26). Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer (Foster City, CA). Oligonucleotides β2a(5'GCCTCTAAGGTTGTAGGAATTCTTCAG 3') and β2b (5'ATCAGTGCACCCACCAAGTCTGGG 3') were used to amplify the Bβ gene segment coding for amino acids 9 to 72. Oligonucleotides α6a (5'GGCACGCTGG-ATGGGTTC 3') and α6b (5'GGACTTACAGTCGACCACAAAAACAGACC 3') were used to amplify the part of the Aα-chain gene coding for amino acids 492 to 625 and a 121 bp non-translated 3' sequence. Amplification by PCR (14) was performed in a 100 μl reaction volume containing 1 μg genomic DNA, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia), 0.2 μM each of primer a and b in 1X reaction buffer (10 mM Tris/HCl pH 8.3 at 25°C, 50 mM KCl, 3.0 mM $MgCl_2$ and 0.001% (w/v) gelatin). The DNA was denatured at 94°C for 4 min and 2.0 units Taq DNA polymerase (Perkin Elmer-Cetus) was added. Cycles consisted of a 1 min 94°C, 0.5 min 58°C and 1.5 min 72°C incubation. After 30 cycles, the amplified DNA was precipitated with ethanol, dried, dissolved in 20 μl of distilled water, and run on a 1.0 % (w/v) ultra low gelling agarose gel (A-5030, Sigma). The bands with the appropriate size, as predicted from the DNA sequence of the Aα (27) and Bβ (28) chain genes were cut out of the gel in a volume of approximately 20 μl and heated to 55°C. 1 μl of the melted agarose, containing approximately 10 ng DNA, was mixed with 1 μl of the appropriate amplification primer (60 ng), and 2 μl 5X annealing buffer (T7 sequence kit, Pharmacia), the volume was brought up to 14 μl with distilled water. The mixture was heated to 95°C for 3 min and immediately after this put on ice, labeling (5 min) and termination (10 min) reactions were performed using the T7 DNA sequence kit according to the manufacturer's instructions.

Detection of mutation in family members by restriction analysis

PCR samples containing the Aα fragment from the family members were incubated with the restriction endonuclease BSAJ I (New England Biolabs) according to the manufacturer's instructions. After incubation the restriction digests were analyzed on a 2% (w/v) agarose gel (A-6013, Sigma) and stained with ethidium bromide. DNA molecular size markers were prepared by Rsa I (New England Biolabs) digestion of M13mp18 DNA.

RESULTS

Case report and laboratory data

The propositus (II.2) is a male caucasian who developed spontaneous phlebitis at the age of 37, which was treated by heparin followed by oral anticoagulant. At the age of 41, he suffered from pulmonary embolism and deep vein thrombosis of the left leg. The thrombin clotting time of the propositus was prolonged (35 s, control 20 s) as well as the Reptilase^R clotting time (29.5 s, control 20 s). His father (I.1) developed three episodes of spontaneous deep vein thrombosis at the age of 46. At the age of 52 he had an episode of pulmonary embolism, and between the age of 54 and 70 he developed three episodes of thrombotic disorder (deep vein thrombosis and superficial thrombosis). At the age of 76 he suffered a pulmonary embolus confirmed by angiography without accompanying evidence of deep venous thrombosis. The thrombin and Reptilase^R clotting times of the father were also prolonged. Two brothers (II.1 and II.3) of the propositus died at the age of 20 and 30 respectively, from pulmonary embolism after several episodes of thrombophlebitis. Brother II.3 showed a prolonged thrombin clotting time; brother II.1 was not investigated. The two sons (III.1 and III.2) of the propositus born in 1962 and 1964, both had prolonged thrombin and Reptilase^R clotting times. They were treated prophylactically with oral anticoagulants from 1981 and no history of thrombosis was reported. Two nieces (III.3 and III.4) had normal thrombin and Reptilase^R clotting times and no history of thrombotic disorders (see also Table 1). Antithrombin III, protein C and protein S levels, determined in the plasma of the propositus (II.2) and his father (I.1) during an interruption of oral anticoagulant therapy, were all within the normal range.

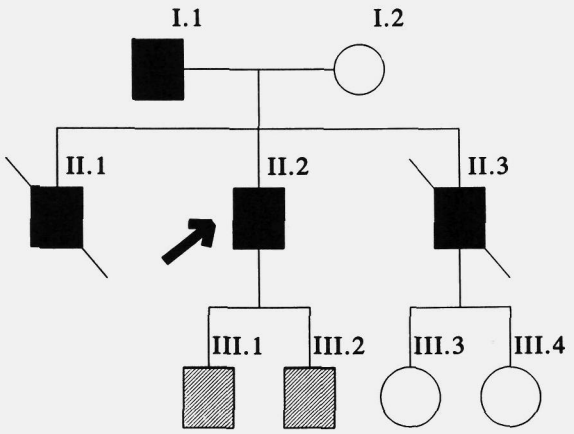


Figure 1. Pedigree of the Dusart family. The arrow indicates the propositus. Symbols used: (□) male, (○) female, (■) thrombotic disorder, (◥■) fatal thrombotic disorder, (▨) treated prophilactically with oral anticoagulant.

Amplification and direct sequencing of genomic DNA fragments

Based on the evidence suggesting that lateral association of fibrin fibrils was impaired (see below), we amplified the genomic DNA coding for the amino-terminus of the Bβ chain (amino acid 9-72) and the carboxy-terminus of the Aα chain (amino acid 492-625) of the fibrinogen molecule. After amplification, fragments with the size predicted from the DNA sequence for the Aα and Bβ chain genes (27,28) were sequenced. The fragments containing the Bβ gene sequence were completely normal, while the Aα fragment of Dusart II.2 had a single base substitution (Fig. 2) in the codon normally coding for arginine at position 554. This mutation changed the codon CGT (arginine) to TGT which codes for cysteine. The normal sequence was also present in Dusart II.2, indicating that the propositus was heterozygous for this mutation.

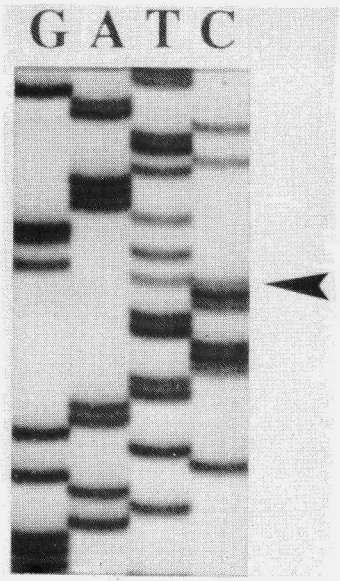


Figure 2. Part of the DNA sequence of the amplified Aα-chain gene fragment coding for amino acids 492 to 610. Direct sequence analysis of the amplified fragment demonstrated a heterozygous mutation at the position indicated by the arrow.

Fibrin structure determined by electron microscopy

The fibrin fibers formed from fibrinogen Dusart at physiological pH and ionic strength were much thinner than the fibers present in normal fibrin (Fig. 3), indicating impaired lateral association of the fibrin fibrils formed from fibrinogen Dusart. These results are consistent with previous observations that showed low turbidity of polymerized fibrin Dusart compared with normal fibrin (12).

Binding of thrombospondin to fibrinogen

Thrombospondin binds to the carboxyl-terminal part of the fibrinogen Aα- and Bβ-chain (29,30), and is thought to be an inhibitor of complex formation between fibrin, plasminogen and t-PA (31). As the mutation in fibrinogen Dusart is located in the

carboxyl-terminus of the Aα-chain, the binding of thrombospondin to immobilized fibrinogen Dusart was compared with binding to normal fibrinogen. ^{125}I-thrombospondin bound to normal fibrinogen and fibrinogen Dusart in a concentration-dependent manner (Fig. 4). The specificity of the binding to fibrinogen was demonstrated by the fact that wells coated with only BSA retained negligible amounts of ^{125}I-thrombospondin. The binding of ^{125}I-thrombospondin to fibrinogen Dusart was similar to that of normal fibrinogen in terms of maximal amounts of thrombospondin bound and apparent dissociation constants (K_d = 1.3 x 10^{-8} M, Fig. 3), indicating that the mutation in the Aα-chain did not influence thrombospondin binding.

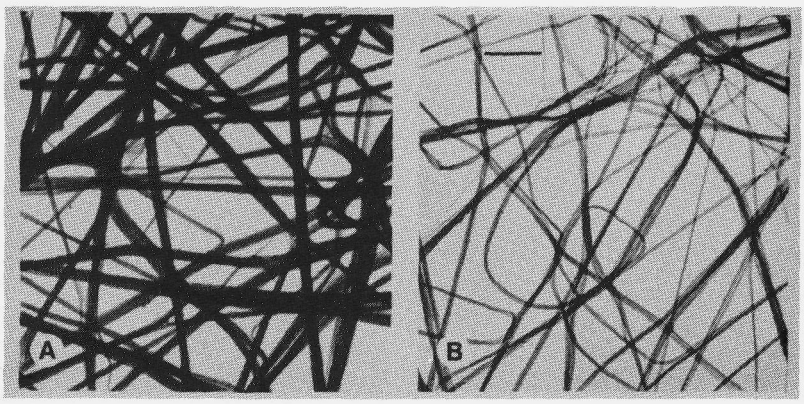


Figure 3. Electron microscopic images of fibrin formed by thrombin from normal fibrinogen (panel A) and fibrinogen Dusart (panel B), (bar = 500 nm; 27,245 X).

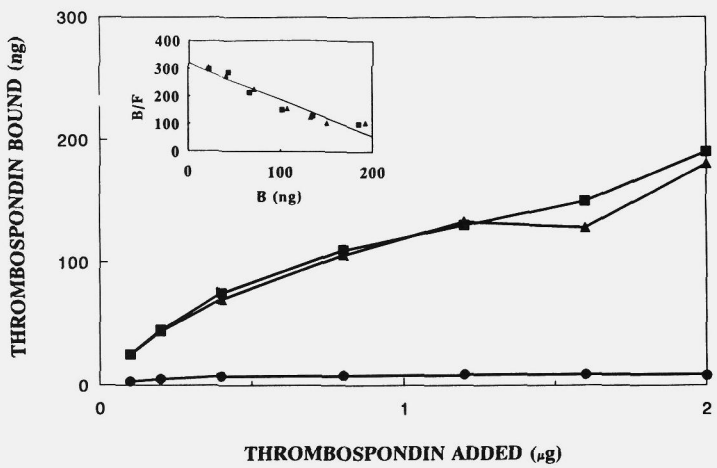


Figure 4. Binding of ^{125}I-thrombospondinto immobilized normal fibrinogen (■), fibrinogen Dusart (▲) and BSA (●). The data are expressed as the total amount of the relevant protein, and the total amount bound to the well. Inset: Scatchard plot of binding data.

Detection of the mutation in family members

The mutation found in the Aα gene fragment of fibrinogen Dusart, abolishes the recognition sequence (CCNNGG) for the restriction endonuclease BSAJ I, which is found in the normal Aα gene fragment. The loss of the restriction site at this position results in the formation of a 275 bp band for the Aα fragment of Dusart in place of the 251 bp band for normal Aα fragment. Restriction analysis of the amplified Aα gene fragment from a normal individual and the family members of the propositus (Fig. 5) showed that the propositus (II.2), his father (I.1) and his two sons (III.1 and III.2) contained both the normal fragment (251 bp) and the abnormal fragment (275 bp). These results indicate that the father and the two sons of the propositus are also heterozygous for the mutation found in the propositus. The normal individual and the two nieces (III.3 and III.4) contained only the normal fragment (251 bp), indicating that they do not contain the mutation in the Aα gene fragment. The DNA of the two brothers (II.1 and II.3) of the propositus was not studied. However, one of them (II.3) showed a prolonged thrombin clotting time, indicating that he probably contained the molecular defect (see also Table 1).

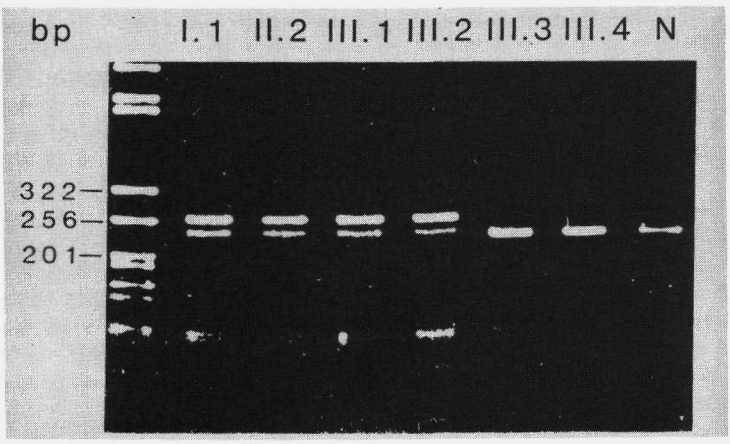


Figure 5. EtBr stained gel of restriction digests by BSAJ I of amplified Aα-chain gene fragment from the propositus (II.2), his siblings (I.1, III.1, III.2, III.3 and III.4) and a normal individual (N). DNA molecular size marker: M13mp18 Rsa I digest.

Sulfhydryl content

To determine whether the cysteine residue at position Aα 554 in fibrinogen Dusart had been oxidized to disulfides, we evaluated the titratable sulfhydryl content using Ellman's reagent (37). The analysis was performed in the presence of 8 M urea to denature the protein, because sulfhydryl groups can be present in native proteins in a non-titratable

state (38). Calculation of the number of free sulfhydryl groups showed that both normal fibrinogen and fibrinogen Dusart did not contain any detectable free sulfhydryl groups (< 0.05 mol/mol).

Immunoblot analysis of plasma

To determine whether the additional cysteine created by the mutation in fibrinogen Dusart (Aα 554 Arg → Cys) is involved in disulfide linked complex formation with other proteins (32,33,34), plasma of the propositus, his siblings and normal plasma was analyzed by immunoblotting following separation of plasma proteins on 2-16% gradient SDS-PAGE gels. The blots were incubated with a mouse monoclonal anti-fibrinogen antibody (Y18) and a goat anti-human albumin antibody, both conjugated with HRP. After reaction with Y18/HRP (Fig. 6A), normal plasma and the plasma of Dusart III.3 and III.4 showed two intense bands with M_r's of approximately 340,000 and 300,000, corresponding to the HMW and LMW form of fibrinogen (35,36). Plasma of the four other family members of Dusart (I.1, II.2, III.1 and III.2) contained the same two bands as normal plasma, and an additional intense band with a M_r of approximately 400,000.

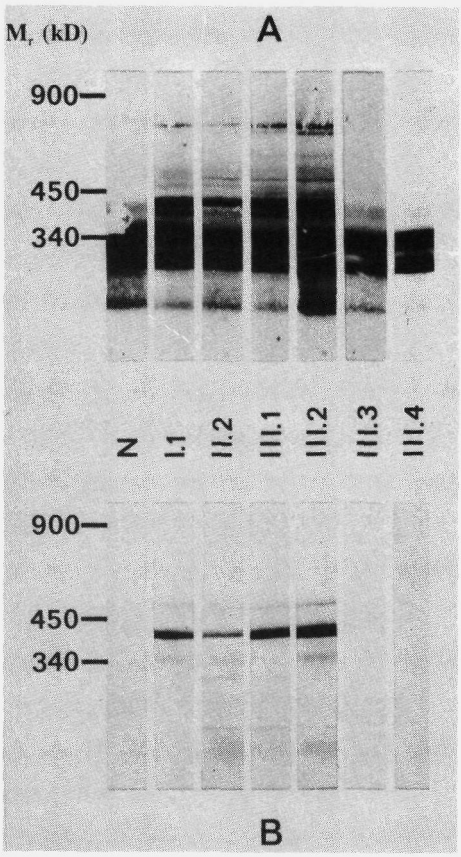


Figure 6. Immunoblot of plasma of the propositus (II.2), his siblings (I.1, III.1, III.2, III.3 and III.4) and a normal individual (N) after separation of the plasma proteins on a 2-16% gradient SDS-PAGE gel, using Y18/HRP, a monoclonal antibody specific for fibrinogen (panel A) and goat anti-human albumin/HRP (panel B).

Several other faint bands were also present in the plasma of these individuals (I.2, II.2, III.1 and III.2). After reaction with goat anti-human albumin/HRP (Fig. 6B) normal plasma and plasma from Dusart III.3 and III.4 showed no bands which reacted with this antibody. Plasma samples from Dusart I.1, II.2, III.1 and III.2 showed one intense band with a M_r of 400,000 after reaction with the goat anti-human albumin/HRP conjugate. As indicated by the intense band with M_r 400,000 which reacted with anti-fibrinogen and anti-albumin antibodies the mutation in fibrinogen Dusart results in the formation of covalent complexes between the mutated fibrinogen and plasma albumin. Immunoblot analysis of reduced plasma samples with Y18/HRP (data not shown), did not show any high molecular weight complex, indicating that the complex formation involves disulfide bridges between the different proteins.

In summary, the presence of the mutation in the Aα-chain of fibrinogen Dusart is accompanied by a prolonged thrombin clotting time and the presence of disulfide-linked fibrinogen-albumin complexes in plasma of the affected family members (Table 1).

Table 1. Summary of laboratory data of the propositus with fibrinogen Dusart (II.2) and his family members.

	I.1	I.2	II.1	II.2	II.3	III.1	III.2	III.3	III.4
Heterozygous CGT (Aα 554 Arg) → TGT (Cys) mutation in genomic DNA	Yes	N.D.	N.D.	Yes	N.D.	Yes	Yes	No	No
Prolonged thrombin clotting time in plasma	Yes	No	N.D.	Yes	Yes	Yes	Yes	No	No
Disulfide-linked fibrinogen-albumin complexes in plasma	Yes	N.D.	N.D.	Yes	N.D.	Yes	Yes	No	No

DISCUSSION

Using the polymerase chain reaction, we identified a single base substitution in the gene coding for the Aα chain of the abnormal fibrinogen Dusart. The mutation in fibrinogen Dusart is associated with abnormal fibrin polymerization (12), reduced binding of Lys-plasminogen (12), and defective t-PA induced plasminogen activation (13). Since the defect was found to affect fibrin polymerization, genomic DNA segments coding for the carboxy-terminus of the Aα chain and the amino-terminus of the Bβ chain were amplified. Sequence analysis of the amplified products, demonstrated that the propositus (II.2) was heterozygous for a single base substitution in the codon for Aα Arg 554 (CGT)

resulting in a Cys (TGT) at this position. The absence of titratable sufhydryl groups in fibrinogen Dusart, indicated that the additional cysteine residue at position Aα 554 had been oxidized. Immunoblot analysis of plasma from the propositus and his family members demonstrated that in all four heterozygous family members fibrinogen was linked to other proteins by disulfide bonds. The predominant complex was identified as a fibrinogen-albumin complex. The occurrence of a similar complex was first described for the variant antithrombin III molecule Northwick Park (32,33) and recently for two abnormal fibrinogens with an Arg → Cys substitution in the amino-terminus of the fibrinogen Bβ-chain (34). The other faint bands present on the blot, could mean that a small amount of fibrinogen Dusart is linked to other proteins as well.

It has been known for many years (39) that fibrin polymers formed from fibrinogen lacking carboxyl-terminal regions of Aα chains develop less turbid clots, suggesting that fibers in the clot matrix are reduced in thickness. Recent studies by Hasegawa and Sasaki (10) provided direct evidence that this region of the Aα chain plays a critical role in the process of lateral fibril association, and constitutes all or part of the so-called "b" polymerization site in the fibrin molecule. Electron microscopic images of the fibrin formed from fibrinogen Dusart showed a marked reduction in the width of fibrin fibers in the clot matrix. These observations support the conclusion that the carboxyl-terminal region of the Aα chain plays an important role in lateral fibril association. Whether the substitution of cysteine at Aα 554, per se, or whether the albumin molecules bound to the fibrinogen at this position cause the defective function, cannot yet be deduced.

Previous reports on fibrinogen Dusart demonstrated that fibrin formed from it had a reduced binding of lys-plasminogen (12) and a reduced accelerating effect on t-PA induced plasminogen activation (13). An explanation for these effects could be that Aα Arg 554 is part of a plasminogen binding site in normal fibrin, or that the presence of albumin, linked to Aα Cys 554, masks this binding site in fibrin Dusart. However, thrombospondin, thought to bind to the carboxyl-terminal end of the fibrinogen Aα-chain (30), bound normally to fibrinogen Dusart. Another explanation is that the functional defects related to fibrinolysis are the result of the impaired lateral association of fibrin fibrils in fibrin Dusart. The latter explanation is in agreement with the observation that inhibition of fibrin polymerization reduces the acceleratory effect of fibrin on t-PA induced plasminogen activation (40-42). Further evidence for this hypothesis is presented by Mirshahi et al. (43) who showed that fibrin formed from LMW fibrinogen, which results in reduced lateral association (10), is more resistant to fibrinolysis induced by t-PA than fibrin prepared from HMW fibrinogen.

Restriction analysis of the Aα gene fragments demonstrated that the three family members with a prolonged thrombin clotting time (I.1, III.1 and III.2) were also heterozygous for the defect, whereas the two family members with a normal thrombin clotting time (III.3 and III.4) did not contain the mutated DNA sequence. From the

family history of recurrent and massive thrombosis and the genetic analysis of the family members, the association of the clinical symptoms with the defect in fibrinogen Dusart is convincing. The mechanism responsible for the recurrent thrombosis in this family is most likely related to the decreased plasminogen binding to fibrin and the reduced acceleratory effect of fibrin on t-PA induced plasminogen activation. This would indicate that the co-factor function of fibrin in regulating fibrinolysis is an important in vivo mechanism for inducing efficient thrombolysis and for preventing the occurrence of thrombosis.

ACKNOWLEDGEMENTS

We like to thank the patients, who gave their consent for frequent blood examinations, and Dr. Dana Fowlkes for synthesizing the oligonucleotides. This work was supported by NIH Grant Nr. HL 31048 (STL) HL 28444 (MWM), Nato Grant Nr. CR6 860110 and a grant of the Dutch Thrombosis Foundation.

REFERENCES

1. Kudryk, B.J., D. Collen, K.R. Woods, and B. Blombäck. 1974. Evidence for localization of polymerization sites in fibrinogen. J.Biol. Chem. 249:3322-3325
2. Shainoff, J.R., and B.N. Dardik. 1979. Fibrinopeptide B and aggregation of fibrinogen. Science 204:200-202
3. Shainoff, J.R., and B.N. Dardik. 1983. Fibrinopeptide B in fibrin assembly and metabolism: physiologic significance in delayed release of the peptide. Ann. N.Y. Acad. Sci. 408:254-268
4. Olexa, S.A., and A.Z. Budzynski. 1980. Evidence for four different polymerization sites involved in human fibrin formation. Proc. Natl. Acad. Sci. USA 77:1374-1378
5. Olexa, S.A., A.Z. Budzynski, R.F. Doolittle, B.A. Cottrell, and T.C. Greene. 1981. Structure of fragment E species from human cross-linked fibrin. Biochemistry 21:6139-6145
6. Budzynski, A.Z., S.A. Olexa, and B.V. Pandya. 1983. Fibrin polymerization sites in fibrinogen and fibrin fragments. Ann. N.Y. Acad. Sci. 408:301-314
7. Carr, M.E. Jr., L.L. Shen, and J. Hermans. 1977. Mass-lenght ratio of fibrin fibers from gel permeation and light scattering. Biopolymers 16:1-15
8. Hantgan, R.R., and J. Hermans. 1979. Assembly of fibrin: a light scattering study. J.Biol.Chem. 254:11272-11281
9. Mosesson, M.W. 1990. Fibrin polymerization and its regulatory role in hemostasis. J. Lab. Clin. Med. 116:8-17
10. Hasegawa, N., and S. Sasaki. 1990. Location of the binding site "b" for lateral polymerization of fibrin. Thromb. Res. 57:183-195
11. Ebert, R.F. 1991. Index of variant human fibrinogens, 1991 Edition. R.F. Ebert, editor. CRC Press, Boca Raton, Florida
12. Soria, J., C. Soria, and J.P. Caen. 1983. A new type of congenital dysfibrinogenaemia with defective fibrin lysis-Dusard syndrome: possible relation to thrombosis. Br. J. Haematol. 53:575-586

13. Lijnen, H.R., J. Soria, C. Soria, D. Collen, and J.P. Caen. 1984. Dysfibrinogenemia (fibrinogen Dusard) associated with impaired fibrin-enhanced plasminogen activation. Thromb. Haemost. 51:108-109
14. Saiki, R.K., D.H. Gelfand, S.Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 39:487-491.
15. Caen, J., M.J. Larrieu, and M. Samama. 1975. L'Hémostase, méthode d'exploration et de diagnostic pratique. L'Expansion Scientifique Française, Paris.
16. Clauss, A. 1957. Gerinnungsphysiologische schnellmethode zur bestimmung des fibrinogens. Acta. Haematol. 17:237-246
17. Mancini, G., A.O. Carbonara, and J. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochem. 2:235-254
18. Abilgaard, U., O.R. Odengard, and M. Lie. 1975. Heparin cofactor activity measured with an amidolytic method. Thromb. Diath. Haemorrh. 6:287-297
19. Blombäck, B., and M. Blombäck. 1956. Purification of human and bovine fibrinogen. Ark. Kem. 10:415-443
20. Margossian, S.S., J.W. Lawler, and H.S. Slayter. 1981. Physical characterization of platelet thrombospondin. J. Biol. Chem. 256:7495-7500
21. Dubernard, V., and C. Legrand. 1991. Characterization of the binding of thrombospondin to human platelets and its association with the platelet cytoskeleton. J. Lab. Clin. Med. 118:446-457
22. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680-685
23. Towbin, H., T. Staehelin, and J. Gordon. 1979.Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354
24. Koppert, P.W., C.M.G. Huijsmans, and W. Nieuwenhuizen. 1985. A monoclonal antibody, specific for human fibrinogen, fibrinopeptide A-containing fragments and not reacting with free fibrinopeptide A. Blood 66:503-507
25. Hong, C.S., B.M. Stadler, M. Wälti, and A.L. De Weck. 1986. Dot-immunobinding assay with monoclonal anti-IgE antibodies for the detection and quantitation of human IgE. J. Immunol. Methods 95:195-202
26. Gustafson, S., J.A. Proper, E.J.W. Bowie, and S.S. Sommer. 1987. Parameters affecting the yield of DNA from human blood. Anal. Biochem. 165:294-299
27. Kant, J.A., S.T. Lord, and G.R. Crabtree. 1983. Partial mRNA sequences from human $A\alpha$, $B\beta$, and γ fibrinogen chains: Evolutionary and functional implications. Proc. Natl. Acad. Sci. USA 80:3953-3957
28. Chung, D.W., B.G. Que, M.W. Rixon, M. Mace, Jr., and E.W. Davie. 1983. Characterization of complementary deoxyribonucleic acid and genomic deoxyribonucleic acid for the β chain of human fibrinogen. Biochemistry 22:3244-3250
29. Bacon-Baguley, T., B.J. Kudryk, and D.A. Walz. 1987. Thrombospondin interaction with fibrinogen. J. Biol. Chem. 262:1927-1930
30. Tuszynski, G.R., S. Srivastava, H.I. Switalska, J.C. Holt, C.S. Cierniewski, and S. Niewiarowski. 1985. The interaction of human platelet thrombospondin with fibrinogen. Thrombospondin purification and specificity of interaction. J. Biol. Chem. 260: 12240-12245.
31. Silverstein, R.L., L.L.K. Leung, P.C. Harpel, and R.L. Nachman. 1984. Complex formation of platelet thrombospondin with plasminogen: modulation of activation by tissue activator. J. Clin. Invest. 74:1625-1633
32. Erdjument, H., D.A. Lane, H. Ireland, M. Panico, V. Di Marzo, I. Blench, and H.R. Morris. 1987. Formation of a covalent disulfide-linked antithrombin-albumin complex by an antithrombin variant, antithrombin "Northwick Park". J. Biol. Chem. 262:13381-13384
33. Erdjument, H., D.A. Lane, M. Panico, V. Di Marzo, and H.R. Morris. 1988. Single amino acid substitution in the reactive site of antithrombin leading to thrombosis. J. Biol. Chem. 263:5589-5593

34. Koopman, J., F. Haverkate, J. Grimbergen, L. Engesser, I. Nováková, A. Kerst, and S.T. Lord. 1991. Formation of fibrinogen-albumin and fibrinogen-fibrinogen complexes by disulphide bridges in fibrinogen IJmuiden (Bβ Arg 14 → Cys) and Nijmegen (Bβ Arg 44 → Cys). Thromb. Haemostas. 65:822
35. Mosesson, M.W., D.K. Galanakis, and J.S. Finlayson. 1974. Comparison of human plasma fibrinogen subfractions and early plasmic fibrinogen derivatives. J. Biol. Chem. 249:4656-4664
36. Lipinska, I., B. Lipinski, and V. Gurewich. 1974. Fibrinogen hetrogeneity in human plasma. Electrophoretic demonstration and characterization of two major fibrinogen components. J. Lab. Clin. Med. 84:509-516
37. Ellman, G.L. 1959. Tissue sulfhydryl groups. Arch. Biochem.Biophys. 82:70-77
38. Takahashi, N., and M. Hirose. 1990. Determination of sulfhydryl groups and disulfide bonds in a protein by polyacrylamide gel electrophoresis. Anal. Biochem. 188:359-365
39. Mosesson M.W., N. Alkjaersig, B. Sweet, and S. Sherry. 1967. Human fibrinogen of relatively high solubility. Comparative biophysical, biochemical, and biological studies with fibrinogen of lower solubility. Biochemistry. 6:3279-3287
40. Suenson, E., and L.C. Petersen. 1986. Fibrin and plasminogen structures essential to stimulation of plasmin formation by tissue-type plasminogen activator. Biochim. Biophys. Acta. 870:510-519
41. Koopman, J., L. Engesser, M. Nieveen, F. Haverkate, and E.J.P. Brommer. 1986. Fibrin polymerization associated with tissue-type plasminogen activator (t-PA) induced Glu-plasminogen activation. In Fibrinogen and its derivatives Biochemistry, Physiology and Pathophysiology. G. Muller-Berghaus, U.Scheefers-Borchel, E.Selmayr, and A. Henschen, editors. Elsevier, Amsterdam. 315-318
42. Soria, J., C. Soria, O. Bertrand, M. Mirshahi, F.W. Dunn, E. Svenson, P. Desvignes, P. Bonnet, M. Samama, and J.P. Caen. 1986. Role of fibrin polymerization in fibrinolysis: comparison between polymerisable and unpolymerisable fibrins in the potentiating effect of plasminogen activation by tissue-type plasminogen activator. In: Fibrinogen - Fibrin formation and fibrinolysis Vol. 4. D. Lane, A. Henschen, and M.K. Jasani, editors. Walter de Gruyter & Co., Berlin. 153-158
43. Mirshahi, M., J. Soria, and C. Soria. 1990. Fibrinogen populations in plasma. Differences in their degradability. In Fibrinogen 4. Current basic and clinical aspects. M.Matsuda, S.Iwanaga, A.Takada, and A.Henschen, editors. Elsevier, Amsterdam. 123-130.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUDING REMARKS

GENERAL DISCUSSION

The studies reported in this thesis were aimed at elucidating structure-function relationships in variant fibrinogens associated with thrombophilia. Furthermore, the relationship between the observed defects in the fibrinogen molecule and the clinical symptoms of the patients were investigated. The work focused on variant fibrinogens associated with thrombophilia, because of the intriguing discrepancy between the slow and impaired clotting of the variant fibrinogen and the clinical symptoms related to thrombosis. In addition to this, it became apparent from the literature that variant fibrinogens associated with bleeding or without any clinical symptoms, predominantly contained a defect in the first 20 amino acids of the Aα-chain, affecting the thrombin cleavage site for fibrinopeptide A (chapter 2). Approximately 60% of the variant fibrinogens of which the molecular defect is known, have a mutation in the FpA part of the molecule. These mutations are readily identified using HPLC methods and protein sequence analysis. The structural defects in the variant fibrinogens associated with thrombosis were expected to reside in the fibrin part of the molecule, making protein sequence analysis a time consuming and laborious method. To overcome this problem, the structural defects were analyzed at the DNA level by using the recently-developed polymerase chain reaction (Saiki et al., 1988), followed by DNA sequence analysis. An additional advantage of this approach was that the presence of the molecular defect in family members of the propositus could rapidly be determined, which is essential information for associating dysfibrinogenemia with clinical symptoms, making family studies possible to link the defect with the clinical symptoms. Furthermore, hetero- and homozygosity could be established unambiguously at the DNA level. To analyze the functional defect, several variant fibrinogens were purified from the plasma of the propositus and compared with fibrinogen purified from the plasma of normal individuals.

The six-base deletion in exon VIII of the fibrinogen Vlissingen γ-chain gene resulted in the deletion of the amino acids γ Asn 319 and Asp 320 (chapter 3). This deletion reduced the number of high-affinity calcium binding sites in the D domains of fibrinogen Vlissingen. Previously, this binding site was localized in the region γ 303-336 (Dang et al., 1985a) and was believed to involve γ Asp-316, Asp-318, Asp-320, Phe-322, Gly-324, and Glu-328. Since amino acids Asn-319 and Asp-320 are missing in fibrinogen Vlissingen, our results are consistent with this hypothesis. The impaired fibrin polymerization of fibrinogen Vlissingen induced by Reptilase^R in the absence of calcium ions, indicates that the carboxyl-terminal "a" polymerization site is defective, and that this is independent of the reduced calcium binding. This is in conjunction with the data of Laudano and Doolittle 1981, who demonstrated that the interaction between the "a" polymerization site and a peptide analog of the amino-terminal "A" polymerization site

is not influenced by calcium ions. In conclusion, the data indicate that the Asn-319 and Asp-320 are crucial for maintaining the integrity of the carboxyl-terminal polymerization sites and calcium binding domain in fibrinogen.

The identification of the structural defect in the heterozygous variant fibrinogens IJmuiden (Bβ 14 Arg → Cys) and Nijmegen (Bβ 44 Arg → Cys) has demonstrated that these variants contain an additional cysteine (chapter 4). The presence of this additional cysteine results in the formation of disulfide-linked complexes between fibrinogen and other proteins, predominantly albumin. It is not yet known whether fibrinogen is linked to albumin during or after synthesis of the molecules. The decreased release of FpB by thrombin from fibrinogen IJmuiden, is most likely due to the substitution of the arginine at position 14 in the Bβ-chain, because this amino acid is part of the thrombin cleavage site in normal fibrinogen. Fibrin polymerization induced by Reptilase^R was impaired for fibrinogens IJmuiden and Nijmegen when compared with normal fibrinogen. The reason for this impairment could be that the arginines at position 14 and 44 in the Bβ-chain are part of the polymerization site in normal fibrinogen, which becomes activated by removal of FpA. This explanation is consistent with the data of Siebenlist et al. (1990), who reported that fibrinogen lacking Bβ 1-42 clotted more slowly than did intact fibrinogen after FpA cleavage by Reptilase^R. However, it cannot be ruled out, that the complex formation between albumin and the variant fibrinogens had some effect on fibrin polymerization. Also the previously-described defective t-PA binding to fibrin Nijmegen (Engesser et al., 1987) could be explained by the substitution of an amino acid (Bβ Arg 44) essential for t-PA binding, or by the formation of several types of sulfhydryl complexes. In summary, studies on the variant fibrinogens IJmuiden (Bβ 14 Arg → Cys) and Nijmegen (Bβ 44 Arg → Cys), have indicated that the presence of an additional cysteine can result in the formation of disulfide linked complexes with other proteins, predominantly albumin. These findings severely complicate structure-function analysis in terms of associating a particular amino acid substitution with the altered functions of the fibrinogen molecule.

Fibrinogen Marburg (chapter 5) was identified as a homozygous variant with a single-base substitution in the codon for Aα Lys 461 (AAA) resulting in a stop codon (TAA) at this position. As a result of this mutation, the amino acid stretch Aα 461-610 in the carboxyl-terminal end of the Aα-chain is lacking in fibrinogen Marburg. Coagulation studies on purified fibrinogen Marburg indicate that the deleted Aα segment contains residues that are critical for fibrin polymerization. This is consistent with the report of Hasegawa and Sasaki 1990, who demonstrated that reaggregation of fibrin monomers prepared from low molecular weight fibrinogen was impaired, as compared with high molecular weight fibrinogen. Cheresh et al., 1989, demonstrated that the Arg-Gly-Asp containing site, Aα residues 572-574, is essential for endothelial cell binding. Since fibrinogen Marburg lacks this Arg-Gly-Asp containing domain, the loss of endothelial cell

adhesion to this variant fibrinogen confirms the previous findings. However, the structure-function analysis of fibrinogen Marburg is hampered by the presence of fibrinogen-albumin complexes, most likely due to disulfide bond formation between the free SH group of albumin and the unpaired cysteine residue at position 442 in the Aα-chain of fibrinogen Marburg, which is unpaired by lacking its counterpart Aα 472 Cys. Five of the family members of the propositus were heterozygous for the defect. The plasma of these individuals contained variant fibrinogen at a concentration which was less than 20% of the total plasma fibrinogen concentration. The plasma fibrinogen concentration of the homozygous propositus was also very low (0.6 mg/ml). These low values of fibrinogen Marburg indicate that the synthesis, assembly, secretion and/or plasma lifetime of the fibrinogen Marburg variant is decreased. The clinical symptoms of the propositus consisted of severe haemorrhage after delivery followed by repeated thrombotic events. The severe haemorrhage may be explained by the low fibrinogen concentration and the impaired clotting of fibrinogen Marburg. A mechanism to explain the repeated thrombotic events, could be related to the impaired fibrin polymerization, which is found to reduce the stimulatory effect of fibrin on t-PA induced plasminogen activation (Suenson and Petersen 1986; Koopman et al., 1986). The heterozygous family members show no clinical symptoms, probably due to the relatively low concentration of variant fibrinogen in the plasma of these individuals. From these results we conclude that fibrinogen Marburg (Aα 461 Lys → Stop) is a clinically recessive hypo-dysfibrinogenemia.

Fibrinogen Naples (chapters 6 and 7) was detected in a patient suffering from recurrent episodes of severe deep-vein thrombosis. The propositus was homozygous for a single-base substitution in the codon for Bβ Ala 68 (GCT) resulting in a Thr (ACT) at this position. The delayed release of FpA and FpB by thrombin, in contrast to the normal release of FpA by Reptilase^{R}, indicated that the mutation specifically affected the interaction between thrombin and fibrin(ogen) Naples. The binding of α-thrombin, by a site independent of the catalytic site, to fibrin Naples was less than 10% of that to normal fibrin. This indicates that the Bβ 68 Ala → Thr substitution fully disrupts thrombin binding at this site and results in a reduced release of FpA and FpB. Since there is little information about the three-dimensional structure of the amino-terminal part of fibrinogen, it is difficult to determine the exact influence of the Bβ 68 Ala → Thr substitution on the binding of thrombin. One possible explanation is that Bβ 68 alanine participates in a non-polar interaction with thrombin and that this is disrupted by threonine. Alternatively, the slightly larger side chain of threonine could lead to incorrect folding or disulfide bond formation of this part of the fibrinogen molecule, preventing thrombin binding. Two family members of the propositus were also homozygous for the mutation, while three other family members were heterozygous. All three homozygous individuals (including the propositus) demonstrated an extensive history of both venous or arterial thrombosis, while the heterozygous individuals showed no clinical symptoms.

It has been suggested that thrombin binding to fibrin is a mechanism that prevents active thrombin from existing free in circulation (Liu et al., 1979). In the absence of this binding, free active thrombin in the circulation results in excessive coagulation and/or platelet aggregation which in turn can lead to thrombosis. From the dramatic history of thrombophilia in the family with fibrinogen Naples and the genetic analysis of the family it is clear that the occurrence of thrombosis is only related to the defect in fibrinogen Naples in homozygous family members. In summary, the mutation in fibrinogen Naples (Bβ 68 Ala → Thr) prevents thrombin from binding to fibrin and is correlated with thrombophilia. These results demonstrate that thrombin binding to fibrin is an important in vivo mechanism for limiting the presence of free active thrombin in circulation and for preventing excessive coagulation and/or platelet activation leading to thrombosis.

Fibrinogen Dusart, associated with impaired fibrin polymerization, reduced binding of lys-plasminogen (Soria et al., 1983) and defective t-PA induced plasminogen activation (Lijnen et al., 1984) was identified as a heterozygous Aα 554 Arg → Cys substitution (chapter 8). As reported for fibrinogens IJmuiden and Nijmegen (chapter 4), fibrinogen Dusart was also linked to albumin by disulfide bonds. The reduced fiber thickness observed in fibrin Dusart, indicates that the lateral association step in fibrin formation is affected by the mutation in fibrinogen Dusart. This confirms the observations of Hasegawa and Sasaki (1990), who presented evidence that the carboxyl-terminal domain of the Aα-chain constitutes all or part of the so-called "b" polymerization site, which is involved in lateral fibril association. Whether the substitution of cysteine at Aα 554, per se, or whether the albumin molecules bound to the fibrinogen at this position cause the defective function, cannot yet be deduced. The defective lys-plasminogen binding to fibrin Dusart, suggests that Aα 554 Arg is part of a plasminogen binding site in normal fibrin, or that the presence of albumin, linked to Aα 554 Cys, masks this binding site in fibrin Dusart. Another explanation is that the reduced plasminogen binding and stimulatory effect on t-PA induced plasminogen activation of fibrin Dusart are the result of the impaired fibrin polymerization. The latter explanation is consistent with the observation that inhibition of fibrin polymerization reduces the stimulatory effect of fibrin on t-PA induced plasminogen activation (Suenson and Petersen 1986; Koopman et al., 1986). Three family members of the propositus were also heterozygous for the defect, whereas two family members were normal. From the family history of recurrent and massive thrombosis and the genetic analysis of the family members, association of the clinical symptoms with the defect in fibrinogen Dusart seems convincing. The mechanism responsible for the recurrent thrombosis in this family is most likely related to defective fibrinolysis, because of the decreased plasminogen binding to fibrin and the reduced stimulatory effect of fibrin on t-PA induced plasminogen activation. This would indicate that the co-factor function of fibrin in regulating fibrinolysis is an important in vivo mechanism for inducing efficient thrombolysis and for preventing the occurrence of

thrombosis.

In summary, the results in this thesis clearly demonstrate the effectiveness of gene analysis, using the polymerase chain reaction, in determining the molecular defect in variant fibrinogens at the DNA level. In all cases homo-or heterozygosity for the mutation could be established unambiguously by sequence analysis of the amplified genomic DNA from the propositus. The presence of the variant protein in circulation was confirmed by analysis of purified fibrinogen or plasma. Furthermore, the presence of the mutation in the family members of the propositi with fibrinogens Marburg, Naples, and Dusart was determined by restriction analysis or Southern blotting of the amplified genomic DNA segment containing the mutation. The results presented in this thesis demonstrate that structure-function analysis of variant fibrinogens in vitro can provide direct evidence for the localization of functional domains in fibrinogen as shown by the studies on fibrinogens Vlissingen and Naples. The presence of disulfide-linked fibrinogen-albumin complexes in the variants IJmuiden, Nijmegen, Marburg and Dusart, severely complicates the structure-function analysis in these variants. It also emphasizes the importance of complementing gene analysis by extensive protein studies to assess the complete impact of the mutation on the variant proteins and the observed functional abnormalities. Furthermore, a convincing association between the structural abnormalities and the observed thrombosis in the affected individuals was demonstrated in fibrinogens Naples and Dusart, indicating that these types of dysfibrinogenemia are risk factors for thrombosis, and that fibrin plays an important role in regulating coagulation and fibrinolysis in vivo.

CONCLUDING REMARKS

Variant fibrinogens have proved to be valuable tools for elucidating structure-function relationships in fibrinogen and fibrin. More than 250 variants have been reported (Ebert, 1991), of which the vast majority were detected by a prolonged plasma thrombin clotting time. Consequently, the variant fibrinogens studied so far all show a defective clotting due to impaired release of fibrinopeptides or fibrin polymerization. However, it is conceivable that variant fibrinogens exist which exhibit normal clotting but have a defect in one of the other functions of fibrinogen or fibrin. As this type of variant fibrinogen is of potential interest, new assays which can be performed routinely on plasma samples need to be developed. The presence of disulfide fibrinogen-albumin complexes in variant fibrinogens with additional or unpaired cysteine residues could provide the opportunity to develop such assays, as these complexes can be detected in plasma by enzyme immunoassays. However, structure-function studies in these type of variants are hampered by these complexes. To determine whether the effective function in these

variant fibrinogens is due to the mutation per se or to complex formation with albumin, similar mutants should be constructed and expressed in vitro. Recently, intact fibrinogen has been expressed in eukaryotic cells by transfection of the Aα, Bβ, and γ chain cDNA (Hartwig and Danishefsky, 1991; Farrell et al., 1991; Roy et al., 1991). These type of experiments will undoubtedly have a great impact on structure-function analysis of fibrinogen in the future, and can be used to complement and authenticate the information obtained from studying variant fibrinogens.

Variant fibrinogens not only provide tools for studying the structure-function relationships in fibrinogen, they also offer us an opportunity to determine the relationship between the molecular defect and the clinical symptoms of the affected individuals. The clinical symptoms of patients with congenital dysfibrinogenemia vary from mild bleeding disorders to severe thrombotic tendencies. Over the last 15 years, the number of reported cases of dysfibrinogenemias associated with thrombosis has grown to approximately 20% of the total number. However, these include only a few well-documented cases, which demonstrate a convincing relationship between the molecular defect and the observed thrombophilia. The mechanisms involved are related to thrombin binding to fibrin (fibrinogen Naples) and to the stimulatory effect of fibrin on t-PA induced plasminogen activation (fibrinogen Dusart). Whether defects in other regulatory functions of fibrinogen or fibrin can lead to thrombophilia needs to be determined. Therefore, future research on abnormal fibrinogen associated with thrombophilia, should focus on the relation between the structural and functional defects on the one hand and the clinical symptoms on the other. These type of studies can reveal which in vitro functions of fibrin and fibrinogen are essential to the role these proteins play in regulating coagulation and fibrinolysis in vivo.

REFERENCES

Cheresh DA, Berliner SA, Vicente V, Rugger ZM: Recognition of distinct adhesive sites on fibrinogen by related integrins on platelets and endothelial cells. Cell 58:945, 1989

Dang CV, Bell WR, Ebert RF, Starksen NF: Protective effect of divalent cations in the plasmin degradation of fibrinogen. Arch Biochem Biophys 238:452, 1985a

Engesser L, Koopman J, De Munk G, Haverkate F, Nováková I, Verheijen JH, Briët E, Brommer EJP: Fibrinogen Nijmegen: Congenital dysfibrinogenemia associated with impaired t-PA mediated plasminogen activation and decreased binding of t-PA. Thromb Haemostas 60:113, 1988

Farrell DH, Mulvihill ER, Huang S, Chung DW, Davie EW: Recombinant human fibrinogen and sulfation of the γ' chain. Biochemistry 30:9414, 1991

Hasegawa N, Sasaki S: Location of the binding site "b" for lateral polymerization of fibrin. Thromb Res 57:183, 1990

Hartwig R, Danishefsky KJ: Studies on the assembly and secretion of fibrinogen. J Biol Chem 266:6578, 1991

Koopman J, Engesser L, Nieveen M, Haverkate F, Brommer EJP: Fibrin polymerization associated with tissue-type plasminogen activator (t-PA) induced Glu-plasminogen activation, in Muller-Berghaus G, Scheefers-Borchel U, Selmayer E, Henschen A (eds): Fibrinogen and its derivatives. Biochemistry, Physiology and Pathophysiology. Amsterdam, Elsevier, 1986, p 315

Lijnen HR, Soria J, Soria C, Collen D, Caen JP: Dysfibrinogenaemia (fibrinogen Dusard) associated with impaired fibrin-enhancing plasminogen activation. Thromb Haemostas 51:108, 1984

Liu CY, Nossel HL, Kaplan KL: The binding of thrombin by fibrin. J Biol Chem 254:10421, 1979

Roy SN, Procyk R, Kudryk BJ, Redman CM: Assembly and secretion of recombinant human fibrinogen. J Biol Chem 266:4758, 1991

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA: Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Sience 39:487, 1988

Siebenlist KR, DiOrio JP, Budzynski AZ, Mosesson MW: The polymerization and thrombin-binding properties of Des-(Bβ 1-42)-fibrin. J Biol Chem 265:18650, 1990

Soria J, Soria C, Caen JP: A new type of congenital dysfibrinogenaemia with defective fibrin lysis-Dusard syndrome:possible relation to thrombosis. Br J Haematol 53:575, 1983

Suenson E, Petersen LC: Fibrin and plasminogen structures essential to stimulation of plasmin formation by tissue-type plasminogen activator. Biochim Biophys Acta 870:510, 1986

SUMMARY

In this thesis, the structure-function relationship in variant fibrinogens associated with thrombosis was analyzed. Furthermore, the relation between the structural defect in the variant fibrinogen and the clinical symptoms in the patient was investigated. The first variant fibrinogen of which the structural defect was identified solely by gene analysis, was designated fibrinogen Vlissingen (Chapter 3). Fibrinogen Vlissingen was found to be heterozygous for a six-base deletion in exon VIII of the γ-chain, coding for amino acids Asp 319 and Asn 320 in normal fibrinogen. These amino acids are thought to be part of the putative high-affinity calcium binding site in the D domain of human fibrinogen. The number of high-affinity calcium binding sites in fibrinogen Vlissingen was reduced to 1.1 at pH 5.8 and 2.4 at pH 7.4, whereas normal fibrinogen contained 2.0 and 3.1 calcium binding sites, respectively. The loss of the high-affinity calcium binding site resulted in a decreased protective effect of calcium ions on the plasmin degradation of the carboxyl-terminus of the γ-chain. Also, the fibrin polymerization of fibrinogen Vlissingen was impaired after clotting was induced by thrombin or Reptilase^R, both in the presence and absence of calcium ions. These results demonstrate that amino acids γ Asp 319 and Asn 320 are crucial in maintaining the high affinity calcium binding site and the polymerization site, both located in the carboxyl-terminus of fibrinogen.

Fibrinogens IJmuiden and Nijmegen were identified as heterozygous variant fibrinogens with a single-base substitution in exon II of the Bβ-chain gene (chapter 4). The mutation in fibrinogen IJmuiden resulted in a Bβ 14 Arg → Cys substitution and in the case of fibrinogen Nijmegen in a Bβ 44 Arg → Cys substitution. Fibrinopeptide release by thrombin and Reptilase^R demonstrated that only half of the fibrinopeptide B was released from fibrinogen IJmuiden by thrombin, all other release rates were normal. Clotting induced by thrombin and Reptilase^R was impaired in both variant fibrinogens, indicating a defective fibrin polymerization. SDS-PAGE followed by immunoblotting demonstrated that part of the variant fibrinogens were linked by disulfide bonds to albumin. Also high molecular weight complexes with M_r's between 600,000 and 700,000, which reacted with the fibrinogen specific monoclonal antibody Y18, were present in both variant fibrinogens. Quantitative analysis demonstrated that, of the total plasma fibrinogen concentration of fibrinogen IJmuiden, approximately 20% was linked to albumin and 5% was present as high molecular weight complex. Plasma Nijmegen contained approximately 13% fibrinogen-albumin complexes and 7% high molecular weight complexes. Furthermore, free sulfhydryl groups were detected in fibrinogen IJmuiden (0.18 mol SH/mol) and fibrinogen Nijmegen (0.13 mol SH/mol) whereas normal fibrinogen contained less than 0.05 mol SH/mol fibrinogen. These results indicate that the presence of an additional cysteine in fibrinogens IJmuiden (Bβ 14 Arg → Cys)

and Nijmegen (Bβ 44 Arg → Cys), can result in the formation of disulfide-linked complexes with other proteins, predominantly albumin.

Fibrinogen Marburg (chapter 5) was homozygous for a single-base substitution in the Aα-chain gene, which changed the codon Aα 461 AAA (lys) to TAA (Stop). The stop codon at position Aα 461 results in the deletion of the carboxyl-terminal segment Aα 461-610. The Aα-chain of purified fibrinogen Marburg demonstrated a relative molecular weight of approximately 47,000, as predicted by the DNA sequence. The release of FpA by thrombin and Reptilase^R was normal, whereas the fibrin polymerization was impaired, both in the absence and presence of calcium ions. The number of human endothelial cells bound to fibrinogen Marburg was less than 10% of that bound to normal fibrinogen. The amino acid segment (Aα 461-610) deleted in fibrinogen Marburg contains a cysteine residue at position 472 which normally forms an intra-molecular disulfide bridge with Aα Cys 442. As demonstrated for fibrinogens IJmuiden and Nijmegen (chapter 4), fibrinogen Marburg contained fibrinogen-albumin complexes, most likely due to disulfide bond formation between the free SH group in albumin and the unpaired Cys at position 442 in the Aα-chain(s) of fibrinogen Marburg. The plasma fibrinogen concentration of the propositus was measured by three different methods: a functional method (less than 0.25 mg/ml), an immunological method using polyclonal antibodies (0.6 mg/ml) and an immunological method based on two monoclonal antibodies specific for the amino-terminus and carboxyl-terminus of the Aα-chain (<0.05 mg/ml). Family studies demonstrated that the father and five siblings were heterozygous for the mutation, whereas three other siblings were normal. Using the two immunological methods, it appeared that only 10-15% of the plasma fibrinogen of the heterozygous siblings was abnormal.

Fibrinogen Naples (previously called Milano II) was detected in a patient suffering from severe deep-vein thrombosis (chapter 6). The FpA and FpB release by thrombin were strongly delayed, whereas the FpA release by Reptilase^R was normal. The fibrin polymerization, the interaction with platelets and the interaction between fibrin Naples and the fibrinolytic system were all normal. A decreased binding of bovine thrombin to fibrin Naples was detected. Analysis of the genomic DNA of the propositus demonstrated a single-base substitution (G→A) in the Bβ-chain gene that results in an amino acid substitution of alanine by threonine at position 68 in the Bβ-chain of fibrinogen Naples (chapter 7). The propositus and two siblings were found to be homozygous for the mutation, whereas the parents and another sibling were heterozygous. Quantitative analysis of thrombin binding to fibrin demonstrated that fibrin Naples bound less than 10% of active site inhibited human α-thrombin, as compared with normal fibrin. The fibrin derived from a heterozygous sibling bound approximately 50% of α-thrombin as compared with normal fibrin. All three homozygous individuals had a history of both severe arterial and venous thrombosis, while the heterozygous individuals had no clinical

symptoms.

The molecular defect in fibrinogen Dusart associated with thrombophilia, was identified as a heterozygous single-base change (C→T) in the Aα-chain gene, resulting in the amino acid substitution Aα 554 Arg → Cys. Restriction analysis of the amplified DNA derived from the family members of the propositus showed that his father and his two sons were also heterozygous. Electron microscopic studies on fibrin formed from purified fibrinogen Dusart, demonstrated fibers that were much thinner than in normal fibrin. Immunoblot analysis of plasma fibrinogen of the heterozygous individuals, showed that a substantial part of the fibrinogen Dusart molecules were disulfide-linked to albumin. All heterozygous individuals, except the ones treated prophylactically with oral anticoagulants, demonstrated a history of recurrent thrombosis.

SAMENVATTING

Het werk beschreven in dit proefschrift was gericht op de bestudering van de structuur-functie relatie in erfelijk abnormale fibrinogenen geassocieerd met trombophilie. Ook werd de relatie tussen het structurele defect in het abnormale fibrinogeen en de klinische symptomen in de patiënt bestudeerd. De belangrijkste reden om alleen abnormale fibrinogenen geassocieerd met trombose te bestuderen, was de intrigerende tegenstelling tussen de langzame en onvolledige stolling van deze fibrinogenen en de klinische symptomen van trombose. De structurele defecten in de abnormale fibrinogenen, beschreven in de hoofdstukken 3 t/m 8, zijn bepaald m.b.v. de polymerase ketting reactie gevolgd door DNA sequentie analyse.

In hoofdstuk 1 wordt een overzicht gegeven van de verschillende functies die fibrinogeen en het hiervan afgeleide fibrine vervullen in de bloedstolling en de fibrinolyse.

Hoofdstuk 2 geeft een overzicht van de functionele defecten en klinische symptomen van de abnormale fibrinogenen waarvan de structurele afwijkingen zijn opgehelderd. Er blijkt een relatie te bestaan tussen de aanwezigheid van structurele defecten in de amino-terminus van de Aα-keten van fibrinogeen en de vertraagde afsplitsing van FpA door trombine. De klinische symptomen van patiënten met dit type dysfibrinogenemie suggereren dat mutaties in dit gedeelte van het fibrinogeen molecuul aanleiding geven tot een verhoogde kans op bloeding. Een verhoogde kans op trombophilie werd voornamelijk gesignaleerd in patiënten met een defect in de carboxyl-terminus van de Aα- en γ-ketens en de amino-terminus van de Bβ keten.

De structuur-functie analyse van fibrinogeen Vlissingen wordt beschreven in hoofdstuk 3. Het structurele defect in fibrinogeen Vlissingen is een deletie van 6 basen in exon VIII van het γ-keten gen, dat in normaal fibrinogeen codeert voor de aminozuren Asp 319 en Asn 320. De propositus was heterozygoot voor deze mutatie op DNA en eiwit niveau. De twee ontbrekende aminozuren in fibrinogeen Vlissingen worden verondersteld deel uit te maken van de plaats in het D-domein van fibrinogeen met een hoge affiniteit voor calcium ionen. Het aantal calcium bindingsplaatsen in fibrinogeen Vlissingen is kleiner dan dat van normaal fibrinogeen. Ook het beschermende effect van calcium ionen op de afbraak van de carboxyl-terminus van de γ keten door plasmine was gereduceerd. De fibrine polymerizatie van fibrinogeen Vlissingen verloopt langzamer bij stolling door trombine of Reptilase^{R}, zowel in de aanwezigheid als afwezigheid van calcium. Deze resultaten tonen aan dat de aminozuren γ Asp 319 en Asn 320 belangrijk zijn voor behoud van de calcium bindingsplaats en de polymerizatie functie, beide gelocaliseerd in de carboxyl-terminus van fibrinogeen.

Fibrinogeen IJmuiden en fibrinogeen Nijmegen werden geïdentificeerd als abnormale

fibrinogenen met een substitutie van één base in exon II van het Bβ-keten gen (hoofdstuk 4). Beide proposita zijn heterozygoot voor de mutaties, die in fibrinogeen IJmuiden leiden tot een Bβ 14 Arg→Cys substitutie en in fibrinogeen Nijmegen tot een Bβ 44 Arg→Cys substitutie. De afsplitsing van FpA door zowel trombine als Reptilase^R was voor beide abnormale fibrinogenen normaal. Van fibrinogeen IJmuiden werd slechts de helft van het FpB afgesplitst door trombine, terwijl de FpB afsplitsing van fibrinogeen Nijmegen normaal was. De stolling van beide abnormale fibrinogenen, geïnduceerd door trombine of Reptilase^R, was vertraagd, wat duidt op een verstoorde fibrine polymerizatie. SDS-PAGE gevolgd door immunoblotting, toonde aan dat een gedeelte van beide abnormale fibrinogenen een complex had gevormd met albumine d.m.v. disulfide bindingen. Beide abnormale fibrinogenen bevatten ook complexen met een M_r tussen 600.000 en 700.000, die kruisreactie vertoonden met het fibrinogeen specifieke monoclonale antilichaam Y18. Quantitatieve analyse toonde aan, dat van de totale plasma fibrinogeenconcentratie van fibrinogeen IJmuiden ongeveer 20% als fibrinogeen-albumine complex aanwezig was en 5% als complex met een M_r groter dan 600.000. Het fibrinogeen Nijmegen bevatte 13% fibrinogeen-albumine complexen en 7% hoog-moleculair fibrinogeen complexen in plasma. Beide abnormale fibrinogenen bleken ook vrije SH-groepen te bevatten, dit in tegenstelling tot normaal fibrinogeen. De resultaten van de structuur-functie analyse van fibrinogeen IJmuiden en fibrinogeen Nijmegen tonen aan dat een extra cysteine residue in fibrinogeen aanleiding kan geven tot de vorming van verschillende disulfide complexen. De aanwezigheid van deze complexen is een ernstige belemmering voor een specifieke interpretatie van de structuur-functie relatie in deze abnormale fibrinogenen.

Fibrinogeen Marburg (hoofdstuk 5) werd geïdentificeerd als een homozygoot abnormaal fibrinogeen met een substitutie van één-base in het Aα-keten gen. Deze substitutie veranderde het codon Aα 461 AAA (Lys) in TAA (stop) en resulteerde in de deletie van het carboxyl-terminale segment Aα 461-610 in fibrinogeen Marburg. De afsplitsing van FpA door trombine en Reptilase^R was normaal, in tegenstelling tot de vertraagde fibrine polymerizatie. Het aantal humane endotheelcellen dat bond aan fibrinogeen Marburg was kleiner dan 10% van het aantal bij binding aan normaal fibrinogeen. Het peptidesegment (Aα 461-610) dat ontbreekt in fibrinogeen Marburg, bevat een cysteïne residue op positie 472 die in normaal fibrinogeen een intra-moleculaire disulfide brug vormt met Aα Cys 442. Zoals aangetoond voor de abnormale fibrinogenen IJmuiden en Nijmegen (hoofdstuk 4), bevat fibrinogeen Marburg ook fibrinogeen-albumine complexen, die waarschijnlijk het gevolg zijn van disulfide brugvorming tussen de vrije SH groep in albumine en de niet-gepaarde cysteïne op positie 442 in de Aα-keten. Familiestudies toonden aan dat de vader, de zoon, drie zusters en één broer heterozygoot waren voor de mutatie, terwijl drie andere familieleden alleen de normale DNA sequentie bevatten. Met behulp van

immunologische fibrinogeenbepalingen werd aangetoond dat in de plasma's van de heterozygote familieleden slechts 10-15% van het abnormale fibrinogeen aanwezig was. De resultaten van het structuur-functie onderzoek suggereren een belangrijke rol voor de carboxyl-terminus van de Aα-keten in fibrine polymerizatie en binding van endotheelcellen aan fibrinogeen, hoewel de aanwezigheid van de fibrinogeen-albumine complexen de interpretatie van deze resultaten bemoeilijkt.

Fibrinogeen Napels werd ontdekt in een patiënt met ernstige diep-veneuze trombose (hoofdstuk 6). De afsplitsing van FpA en FpB door trombine was sterk vertraagd, daarentegen was de afsplitsing van FpA door Reptilase^{R} normaal. De fibrine polymerizatie, en de interactie met bloedplaatjes en het fibrinolytische systeem waren normaal. De analyse van het genomische DNA van de propositus bracht een homozygote substitutie aan het licht van één base (G → A) in het Bβ-keten gen (hoofdstuk 7). Deze mutatie resulteerde in de substitutie van alanine door threonine op positie 68 in de Bβ-keten van fibrinogeen Napels. Ook een broer en zuster van de propositus waren homozygoot voor deze mutatie, terwijl de vader, moeder en een andere zuster heterozygoot waren. Uit quantitatieve analyse van de binding van inactief humaan α-trombine aan fibrine bleek dat fibrine Napels minder dan 10% van de hoeveelheid trombine bond in vergelijking met normaal fibrine. Het fibrine van de drie heterozygote familieleden bond ongeveer 50% α-trombine. De drie homozygote familieleden hadden een anamnese van ernstige arteriële en veneuze trombose, daarentegen waren de heterozygote familieleden asymptomatisch. Samenvattend, de mutatie in fibrinogeen Napels (Bβ 68 Ala →Thr) voorkomt binding van trombine aan fibrine en is gecorreleerd met thrombophilie. Deze resultaten tonen aan dat trombinebinding aan fibrine een belangrijk mechanisme is in vivo, om de hoeveelheid vrij, actief trombine in de circulatie te beperken en overmatige stolling en/of plaatjesaggregatie te voorkomen.

Het moleculaire defect in fibrinogeen Dusart, geassociëerd met trombophilie, werd geïdentificeerd als een heterozygote substitutie (C → T) van één-base in het Aα-keten gen, die resulteert in de aminozuur substitutie Aα 554 Arg→Cys (hoofdstuk 8). Restrictieanalyse van het geamplificeerde DNA van de familieleden toonde aan dat de vader en de twee broers ook heterozygoot waren voor de mutatie. Uit electronen-microscopische studies aan het fibrine gevormd uit zuiver fibrinogeen Dusart bleek dat de gevormde fibrinedraden veel dunner waren dan die van normaal fibrine. Immunoblot analyse van de plasma's van de heterozygote familieleden, toonde aan dat een deel van de fibrinogeen Dusart moleculen via disulfide bindingen gecomplexeerd was met albumine. Alle heterozygote familieleden, behalve diegenen, die profilactisch behandeld werden met orale antistollingsmiddelen, hadden een anamnese van ernstige trombose. De structuur-functie studies van fibrinogeen Dusart laten zien dat de substitutie Aα 554 Arg → Cys de laterale associatie van de fibrine protofibrillen verstoort. Of dit het gevolg is van de mutatie op zich, of van de albumine moleculen gebonden aan fibrinogeen

Dusart op deze positie, kan niet worden uitgemaakt. De eerder gerapporteerde reductie van lys-plasminogeen binding aan fibrine Dusart suggereert dat de carboxyl-terminus van de Aα-keten onderdeel is van een plasminogeen bindingsplaats in normaal fibrinogeen. Ook is het mogelijk dat het gebonden albumine deze bindingdsplaats maskeert. Uit de familieanamnese van ernstige en herhaalde trombose en de genetische analyse van de familieleden, komt een overtuigende associatie tussen de klinische symptomen en de mutatie in fibrinogeen Dusart naar voren. Het mechanisme verantwoordelijk voor de trombose in deze familie is waarschijnlijk gerelateerd aan een verlaagde fibrinolyse, omdat het stimulerende effect van fibrine Dusart op de t-PA geïnduceerde plasminogeen activatie lager is dan dat van normaal fibrine. Dit zou aangeven dat de co-factorfunctie van fibrine een belangrijk mechanisme is voor het induceren van trombolyse en het voorkomen van trombose in vivo.

De resultaten beschreven in dit proefschrift tonen duidelijk aan dat gen analyse, met behulp van de polymerase ketting reactie, een effectieve manier is om het moleculaire defect in abnormale fibrinogenen op te sporen. De structuur-functie analyse van abnormale fibrinogenen kan leiden tot de nauwkeurige localizatie van functionele domeinen in het fibrinogeen molecuul, zoals in fibrinogeen Vlissingen en fibrinogeen Napels. De aanwezigheid van disulfide fibrinogeen-albumine complexen in de abnormale fibrinogenen IJmuiden, Nijmegen, Marburg en Dusart, compliceert de structuur-functie analyse in ernstige mate. Een overtuigende associatie bestaat tussen het structurele defect en de waargenomen trombose in de familieleden met de mutatie in het geval van fibrinogeen Napels en Dusart. Hieruit blijkt dat deze typen dysfibrinogenemie risicofactoren zijn voor trombose en dat normaal fibrine een belangrijke rol speelt in de regulatie van stolling en fibrinolyse in vivo.

ABBREVIATIONS

ASA	acetylsalicylic acid
Bap	bacterial alkaline phosphatase
BSA	bovine serum albumin
CNBr	cyanogen bromide
D_1	plasminogen fragment of fibrinogen, formed in the presence of calcium ions
D_3	plasminogen fragment of fibrinogen, formed in the presence of EDTA
DDAVP	desamino D-arginine-vasopressin
DFP	diisopropylfluorophosphate
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EDTA	ethylene diamine tetra acetic acid
EF	euglobulin fraction
EM	electron microscopy
Fbg	fibrinogen
FpA	fibrinopeptide A
FpB	fibrinopeptide B
FPLC	fast protein liquid chromatography
HRP	horse radish peroxidase
HMW	high molecular weight
K_a	association constant
K_d	dissociation constant
kD	kilo Dalton
KIU	kallikrein inactivating unit
LMW	low molecular weight
Mab	monoclonal antibody
M_r	relative molecular weight
MW	molecular weight
NDSK	amino-terminal disulfide knot
NIH	national institute of health
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PNGB	p-nitrophenyl-p'-guanidobenzhoate-HCL
PRP	platelet rich plasma
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
t-PA	tissue-type plasminogen activator

NAWOORD

Aan de totstandkoming van dit proefschrift hebben veel mensen op zeer uiteenlopende wijzen bijgedragen. Daarom wil ik graag de mensen die een belangrijke bijdrage hebben geleverd met name noemen:

Frits Haverkate, voor zijn begeleiding, inspirerende discussies en de prettige samenwerking gedurende 10 jaar.
Susan Lord, for her hospitality during my stay in her lab, the many fruitful discussions and for all her help with the writing of this thesis.
Jos Grimbergen voor zijn uitzonderlijke technische hulp zowel in als buiten het lab, zijn tomeloze inzet en goed humeur.
Ria van den Hoogen voor haar hulp bij het maken van foto's en dia's, de celkweek experimenten en de talloze gezellige uurtjes na vijven.
Marijke Voskuilen, Rogier Bos, Kees van Leuven, Leny de Pagter, Ton Vermond, Mick Welling, Bep Hoegee, Mervyn Bregonje, Paul Quax, Hans Princen en alle andere medewerkers van het Gaubius die meegedacht, meegewerkt en meegeleefd hebben.
Marisa Horsting en Clara Horsting voor al het voortreffelijke typewerk en het verzorgen van de lay-out.
Josephine "Nightmare Editing" Bosman-Say for her corrections of my poor english and her special sense of humour.
Marijn Snoep voor het ontwerpen van de kaft.

Mijn ouders, voor de mogelijkheid om "door te leren" toen dit niet van zelfsprekend was en voor de onvoorwaardelijke steun tijdens het gehele promotie onderzoek.
Janneke, je geduld, begrip en relativeringsvermogen hebben meer aan dit boekje bijgedragen dan je denkt.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 15 augustus 1959 geboren te Sint Maartensdijk in Zeeland. Na een grillige middelbare school opleiding, volgde hij de 4-jarige H.N.W.O. opleiding aan het Dr. Struycken Instituut in Etten Leur, waarvan hij in 1981 het diploma behaalde. In augustus van dat zelfde jaar trad hij in dienst bij het Gaubius Instituut, T.N.O. in Leiden als research analist. Onder de leiding van Dr. F. Haverkate werkte hij aan verschillende research projecten op het gebied van de bloedstolling en fibrinolyse. In het kader van deze werkzaamheden was hij in 1985 een half jaar werkzaam op het laboratorium van Dr. S.T.Lord aan de University of North Carolina in Chapel Hill, USA. Van medio 1987 tot eind 1990 was hij als A.I.O. in dienst bij het Academisch Ziekenhuis in Leiden en gedetacheerd op het Gaubius Instituut. In deze periode werd het grootste deel van het in dit proefschrift beschreven onderzoek verricht bij de afdeling fibrinolyse van het Gaubius Instituut. Per 1 juli 1991 is hij werkzaam als post-doc op de afdeling tumor biologie van het Nederlands Kanker Instituut.