

The Molecular Pathogenesis of Hemophilia A

Habart D.

Institute of Hematology and Blood Transfusion, Prague, Czech Republic

SUMMARY

Hemophilia A is one of the most common inherited bleeding disorders. The bleeding phenotype is caused by mutations in the factor VIII gene, which result in factor VIII deficiency in plasma. Various genetic defects have been described as affecting factor VIII transcription, translation or its amino acid composition. The application of a 3D factor VIII model helped to identify amino acid substitutions that adversely affect protein folding and those that disturb various factor VIII interactions. Genetic defects were stratified on the basis of the relative risk of factor VIII inhibitor formation, and mechanisms were described by which antibodies inhibit the factor VIII function. The findings have been applied in current diagnostic practice and in the development of future therapeutic strategies.

Key words: hemophilia A, factor VIII, factor VIIIa, factor VIII gene, inhibitor, mutation, inversion, protein folding, von Willebrand factor, factor IXa, tenase.

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INTRODUCTION

The hereditary nature of a bleeding disorder that has been called hemophilia ever since Schoenlein's time was described in Babylonian Talmud 1800 years ago. Fifty years ago the development in diagnostic methods made it possible to distinguish between classical hemophilia A and the less common hemophilia B. Later it became known that hemophilia could be considered a disorder of the tenase enzyme complex, either of its catalytic (factor IX, hemophilia B) or cofactor (factor VIII, hemophilia A) component.

DEFINITION AND CLASSIFICATION OF HEMOPHILIA A

Hemophilia A (OMIM 306700) is caused by defects in the gene coding the coagulation factor VIII. A total absence or partial reduction of factor VIII coagulation activity is observed in the plasma of patients. Hemophilia is classified as severe (<1%), moderate (1-5%) or mild (5-40%), depending on the scale of the deficit. The severity of bleeding symptoms correlates quite accurately with the scale of the deficit. Patients with the severe form of hemophilia typically bleed several times a month, often spontaneously, especially into weight-bearing joints and muscles. Patients with milder forms of the disease have excessive bleeding during small traumatic injuries or during surgical or dental procedures.

For research purposes, the coagulation activity in plasma (FVIII:C) can be compared with plasmatic level of the antigen (FVIII:Ag, CRM - cross-reactive material). This allows to distinguish the CRM-negative phenotype, i. e. the total absence of factor VIII in plasma, the CRM-positive phenotype, i. e. the presence of dysfunctional factor VIII in plasma (defined as a FVIII:C/FVIII:Ag ratio of <0.3), and hemophilia A with reduced CRM, when plasma levels of functional factor VIII are reduced.

The most serious complication of replacement therapy in hemophilia A is the creation of factor VIII inhibiting antibodies. This occurs in 10-35% of patients with the severe form of the disease. An inhibitor of the activity of one Bethesda unit reduces factor VIII activity in normal plasma by 50 per cent.

ROLE OF FACTOR VIII

The interconnected system of specific proteolytic reactions of plasma coagulation leads to regulated, temporarily and spatially limited formation of thrombin and fibrin at the site of the damaged vascular wall. Three key enzyme complexes form on membrane surfaces of certain types of cells (Fig. 1). Factor VIII is a component of the tenase complex in which it acts as a cofactor. Inadequate function of the tenase complex leads to reduced generation of thrombin. The consequence is formation of a defective coagulum, along with inadequate inhibition of fibrinolysis. These mechanisms result in bleeding diathesis observed in the clinical picture, which, depending on the severity of the hemophilia, fluctuates between complete failure to achieve hemostasis and late hemorrhage that occurs, within a week of the injury.

FACTOR VIII GENE

The factor VIII gene (Fig. 2a), length 186 kb, was localized at the distal end of the long arm of the X chromosome (Xq28), in the telomere direction from the factor IX gene. The gene consisting of 26 exons sizing 70 bp-3 kb (1-3), is transcribed from the telomere to the centromere. So far, more than 10 intronic and exonic polymorphisms and short tandem repetitions have been identified, of which a part have been utilized in clinical diagnostics. The extensive intron 22 contains a CpG island and two pseudogenes, F8A and F8B. A repetitive int22h-

Address for correspondence:

David Habart, MD., Ph.D.

Institute of Hematology and Blood Transfusion

128 02 Prague 2, U Nemocnice 1

Czech Republic

E-mail: dh@uhkt.cz

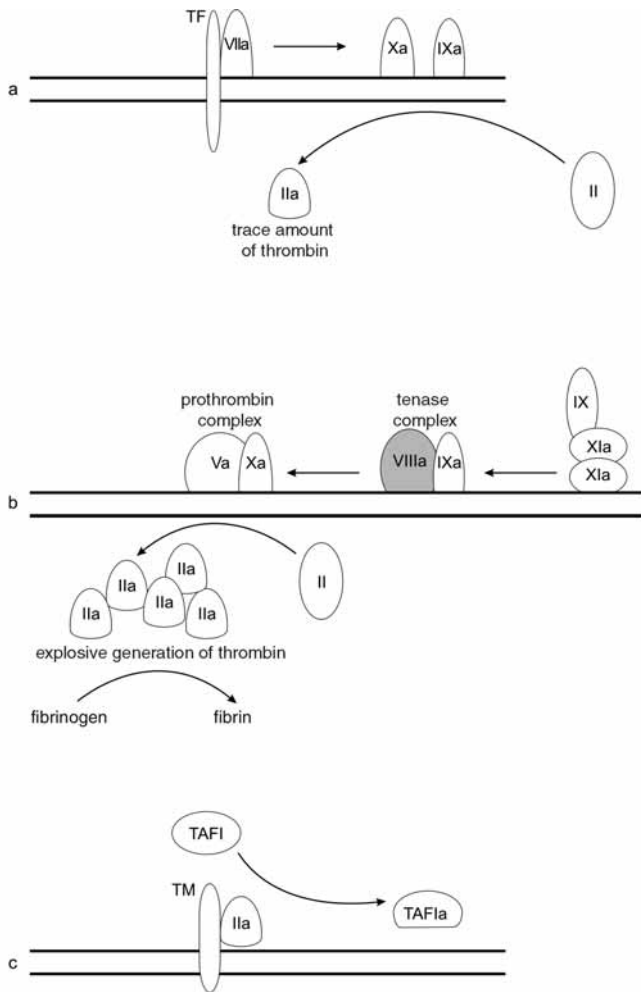


Fig. 1. Role of factor VIII in plasma coagulation

a) The coagulation process is initiated by the assembly of tissue factor (TF) with factor VIIa on the surface of exposed subendothelial fibroblasts, activated endothelium or micro-particles originating from activated monocytes. Resulting activation of factors X and IX leads to the generation of trace amounts of thrombin, whose role seems rather regulatory than hemostatic – it activates platelets and cofactors (FVIII and FV) of specific serin proteases, the factors IX and X. b) The tenase complex (FIXa/FVIIIa) builds up on the surface of the activated platelets, allowing, by very effective activation of factor X, the emergence of the prothrombinase complex (FXa/FVa) on the same phospholipid membrane. After activation of platelets, factor IX can be activated by factor XIa. The prothrombinase complex is a key enzyme in the explosive production of thrombin, which seems necessary for hemostatically effective generation of fibrin. c) Thrombin complexed with thrombomodulin (TM) changes its substrate specificity towards protein C and also activates TAFI (Thrombin-Activable Fibrinolysis Inhibitor), thus inhibiting the fibrinolysis.

FACTOR VIII STRUCTURE

The repetitive structure of factor VIII (2332 AA, Mr 330 kD) is formed by three types of domains A, B and three short ar1-3 acidic amino acid-rich regions (4). The linear order of the domains is A1-ar1-A2-ar2-B-ar3-A3-C1-C2 (Fig. 3). The three-dimensional model of factor VIII consisting of five main domains has been constructed

recently. The model contains neither the regions ar1-3 nor the factor VIII specific B domain. The dimensions of the lipid membrane bound molecule twisted into a spiral shape were estimated to 8 x 7 x 10 nm (Fig. 4) (5). The C2 domain structure was determined using x-ray diffraction (6). Domains A1-3 were modeled based on the homologous structure of ceruloplasmin. Domain C1 was modeled based on the structure of C2 domain; both belonging to the discoidin family (7).

BIOSYNTHESIS OF FACTOR VIII

The factor VIII messenger RNA (~9kb) has been detected in a number of tissues including the liver, kidney and spleen (8). The physiological significance of liver production of factor VIII has been confirmed by permanent normalization of its level in hemophiliacs after liver transplantation (9). A transient alleviation of the symptoms of hemophilia A has been observed after spleen transplantation (10). The exact cell type in which physiologically significant synthesis of factor VIII takes place has not yet been determined, and the question of whether binding of the factor VIII to von Willebrand factor occurs intracellularly or only in the plasma has not been clarified.

The signal peptide guides the nascent factor VIII polypeptide into the endoplasmic reticulum, where it is folded into the correct conformation. This process depends on the gradual interaction of factor VIII polypeptide with molecular chaperones. Chaperone BiP binds the protein component of the forming A1 domain (Fig. 3a). The lectine type chaperones, calnexin and calreticulin, bind the monoglucosylated residues carried by the B domain (11). The quality control system utilizes system of deglycosylation and glycosylation to mark the correctly and incorrectly folded factor VIII molecules. Aberrantly folded molecules are transported into the cytoplasm and degraded. The transport of correctly folded FVIII into the Golgi system is mediated by specialized ERGIC (Endoplasmic Reticule - Golgi Intermediate Compartment) vesicles. The key role is played by the MCFD2 and LMAN1 (OMIM 601567 and 607788) protein complex, which binds mannose residues present on the B domain (Fig. 3a), thus serving in the ERGIC membrane as a lectine-type receptor for factor VIII (and factor V) (12).

Post-translation modifications of factor VIII protein (Fig. 3a) include formation of eight disulphide bonds, incorporation of a copper atom, N-bound glycosylation of some 23 amino acid residues predominantly on the B domain, sulphatation of six tyrosine residues in the regions ar1-3, phosphorylation of further amino acid residues in these regions and proteolytic cleavage of factor VIII receptor by furin (Fig. 3a). Mature factor VIII is secreted by the cell in the form of a heterodimeric glycoprotein, composed of the light (80 kD) and heavy (200 kD) chains (Fig. 3b).

FACTOR VIII FUNCTION INTERACTION

Stabilization of factor VIII

Factor VIII circulates in plasma in the form of a heterodimer bound to von Willebrand factor via domains ar1, C1 and C2 (Fig. 3b) (13, 14). This bond lengthens the plasma half-life of factor VIII from 2 to 12 hours, thus significantly affecting its plasma levels.

Activation of factor VIII

Factor VIII is activated in plasma by the effect of trace amounts of thrombin generated in the initial coagulation phase (Fig. 1a). Thrombin (FIIa) binds to domain C2 and cleaves factor VIII heterodimer at three sites behind specific arginines at the edges of the regions ar1, ar2 and ar3 (Fig. 3b). Thus the B domain is released and a heterotrimer of the active factor VIIIa is formed, which no longer binds von Willebrand factor, giving room to C2 domain affinity to

phospholipids. Domain A2 is connected to the remaining heterotrimer only by weak noncovalent bonds. Under physiological conditions, its dissociation governs the termination of factor VIIIa activity.

Tenase complex

The build-up of the tenase complex (Fig. 1b and 4) requires the presence of a lipid bilayer rich in phosphatidylserine, the activated factors VIIIa and IXa and calcium ions. A particularly suitable surface is a membrane of the activated platelet, in which phosphatidylserine has been translocated from the interior into the exterior lipid layer. Factor VIIIa binds to the lipid membrane via domain C2. Its distal end is formed by a cluster of hydrophobic amino acids inserted into the hydrophobic intermediary layer of the lipid membrane. Positively charged amino acids forming a crown around of the hydrophobic end of the C2 domain, bind electrostatically to the negatively charged heads of phosphatidylserine on the surface of the external membrane layer (15). Factor VIIIa affinity to phospholipids is orders of magnitude higher than the affinity of FIXa (Fig. 4). The A3 domain of factor VIII serves as a receptor for factor IXa (it binds through the domain EGF1; Fig. 4). The A2 domain interacts directly with the catalytic domain of factor IXa. The contact amino acids on both proteins have been identified to a considerable degree (16). The A1 domain binds the substrate of the tenase complex, the factor X. The cofactor function of factor VIIIa manifests as catalytic activity of the tenase complex, enhanced by approximately three orders of magnitude as compared to the factor IXa alone.

Clearance and degradation of factor VIII

Factor VIII is taken up from the blood stream by endothelial cells via a receptor mechanism (Fig. 2b). Clathrin-mediated internalization of the factor VIII occurs after LPR receptor (LDL-receptor related protein) binds the domains A1 and A2 (17). The internalization is further strengthened by the interaction between factor VIII and HSPG (heparan sulphate proteoglycan), which is present on the surface of the endothelium. The proteolytic degradation of factor VIIIa occurs in

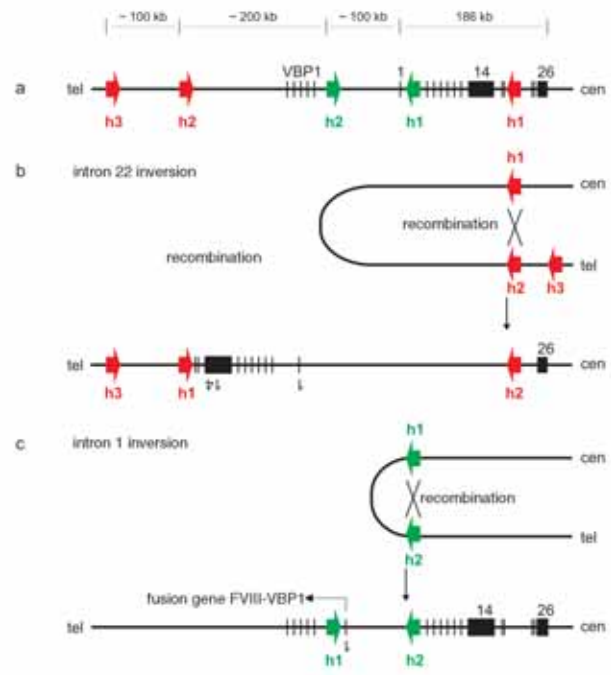


Fig. 2. Factor VIII gene

a) The diagram illustrates distribution of exons (black boxes) and introns of the factor VIII gene. Repetitive homologous sequences are indicated by arrows showing their mutual orientation. The intron 1 and intron 22 homologous regions are shown in green and red, respectively. The orientation of int22h2 has recently been challenged (20a*) and novel mechanism of the intron 22 inversion involving polymorphism of the orientation has been suggested (20b*). Localization of the VBP1 gene is also shown. b) Recombination between the homologous regions int22h1 and int22h2 and the resulting factor VIII gene re-arrangement (the proximal or type 2 inversion). Recombination of int1h regions and resulting re-arrangement of the factor VIII and VBP1 genes in the case of intron 1 inversion.

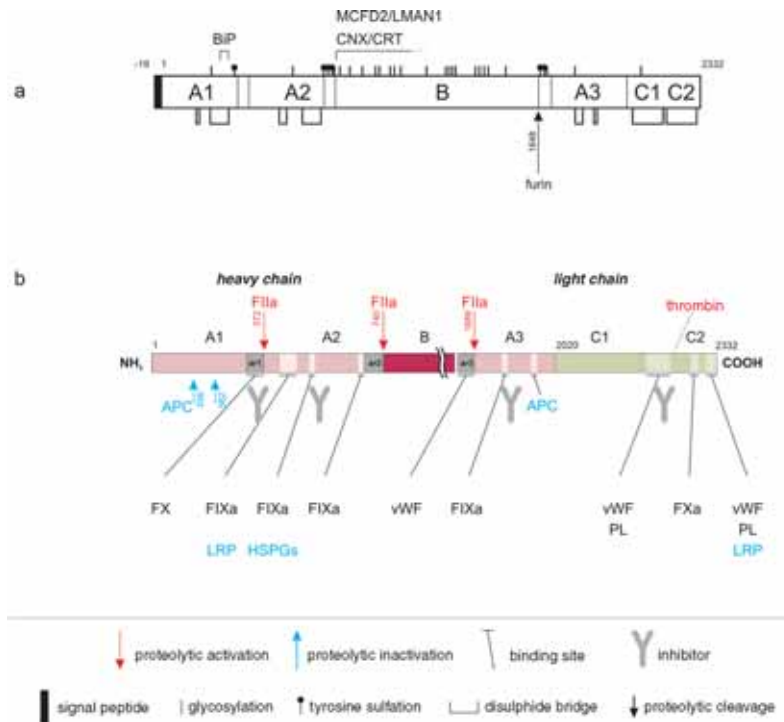


Fig. 3. Domain structure and function interaction of factor VIII

a) Arrangement of repetitive domains of factor VIII with chaperone binding sites, localization of individual post-translation modifications and ERGIC receptor binding sites. b) Heavy and light chain of factor VIII with activation and cleavage sites and the binding sites for vWF, FIXa, phospholipids (PL), LRP and HSPG. Interactions leading to factor VIII activation are indicated in red, inactivating ones are blue. Epitopes on factor VIII molecule of the most common inhibitory antibodies overlap partly with important functional sites. Numbering of amino acid residues in the mature chain is used.

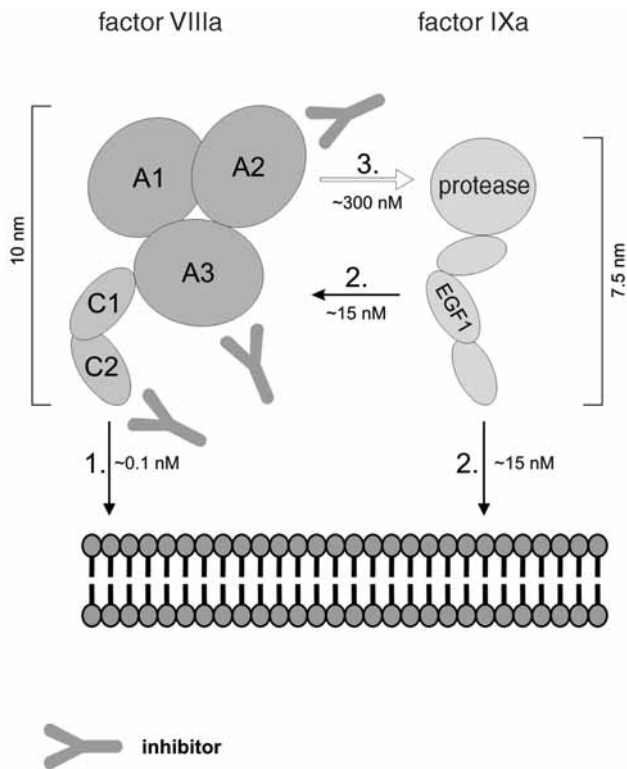


Fig. 4. Tenase complex

The arrangement of domains of factors VIIIa and IXa and their mutual adjustment on the surface of the phospholipid membrane (40) and (5). The assembly of the tenase complex is probably gradual (sequentially numbered arrows) according to the kinetics of individual interactions, as illustrated by dissociation constants.

plasma by activated protein C (APC), which cleaves domains A1 and A2. As long as factor VIII (VIIIa) is bound within the macromolecular complex to von Willebrand factor or the tenase, it seem to be protected against these mechanisms.

MOLECULAR GENETIC PRINCIPLE OF HEMOPHILIA A

Factor VIII deficiency is caused by a broad spectrum mutations, which occur along the entire length of the factor VIII gene. The mutations lead to defect at the level of transcription or translation or to changes of individual amino acids in factor VIII protein. With the exception of inversions, the majority of mutations are unique for a given family. The relative representation of various types of genetic defects in a set of 953 unique mutations listed in the international database is shown in Fig. 5 (18). Severe hemophilia is typically caused by inversions, insertions, deletions and nonsense mutations and also by missense mutations. Milder forms of hemophilia are usually caused by missense mutations while single nucleotide deletions or splicing errors may occur.

Factor VIII gene inversion

Inversions cause about half of the cases of severe hemophilia A. They seem to arise during the meiotic phase of spermatogenesis in a healthy father of a future female carrier, the mother of the first hemophiliac in a family. Approximately 45% of hemophiliacs with severe form of hemophilia are affected by the inversion with a break point within intron 22, which develops as a consequence of homologous recombination between the region int22h1 and one of its extragenous copies (19, 20) (Fig. 2a, 2b). According to the distance of the extragenous copy involved in the recombination, it

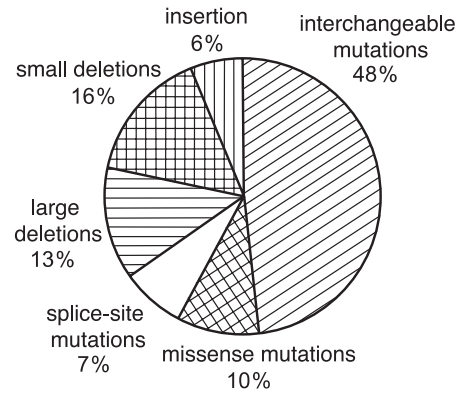


Fig. 5. Frequency of unique mutations in hemophilia A. The diagram is based on data of the international HAMSTeRS database (18), which, as of December 2004 (953 unique mutations).

is possible to distinguish distal (type 1), proximal (type 2) inversion and rarer variants (21). The mechanism leading to the inversion with the break point within intron 1 is similar (Fig. 2a, 2c); however, the consequence is not only the disintegration of the factor VIII gene but also the emergence of a new fusion gene, whose significance remains unknown (22). The prevalence of intron 1 inversion among severe hemophiliacs varies between 1-5% (22, 23).

Insertions and deletions

Insertions and deletions that cause severe hemophilia A are classified into large (more than 50bp) and small which usually span one or several nucleotides. Various types of repetitive sequences, including SINES and LINES, which are present in the factor VIII gene, may be involved. Insertions and deletions of a single nucleotide cause a shift of the reading frame and thus, usually, a severe form of the disease. An interesting phenomenon relates to single-nucleotide deletions in continuous rows of 6-9 adenosines in exon 14. They occur during replication as the consequence of erroneous incorporation of adenosine by DNA-polymerase. It has been observed that at these sites RNA-polymerase is prone to similar errors (insertion of missing adenosine). During transcription of the damaged gene the RNA-polymerase thus synthesizes some molecules with correct sequence (24). A minute, nonetheless immunologically significant amount of normal factor VIII is formed. Hemophilia in such patients is somewhere on the border between the severe and moderate forms.

CONSEQUENCES OF AMINO ACID SUBSTITUTIONS

Amino acid substitutions cause the absolute majority of moderate and mild forms of hemophilia A. The mechanisms, by which missense mutations lead to decreased factor VIII activity in plasma, depend on the localization and nature of substituted amino-acid residues.

Protein instability

Amino-acid substitutions affecting the hydrophobic core of one of the factor VIII domains cause aberrant folding of the domain and degradation of the mutant factor VIII in cytoplasm. Such mutations have been described in domains A1, A2, A3 and C2 (25-27). They are usually associated with the moderate or mild CRM-reduced hemophilia A.

Abnormal interaction with von Willebrand factor and phospholipids

Certain amino acid substitutions in the region ar3 and domains C1

and C2 may become an obstacle to the normal binding of factor VIII to von Willebrand factor. The result is shortening of the mutant factor VIII plasma half-life and mild or moderate CRM-reduced hemophilia A. This can be exemplified by a substitution His2155Asp, which is associated with a change of a surface charge of the C1 domain and marked reduction of factor VIII binding to vWF (28) or by substitutions studied in (14). Another example is a substitution of sulphated Tyr1680 in the region ar3, which points to the importance of post-translation modification for functional interactions of factor VIII (29). The 3D model revealed that in C2 domain mutations are localized in the region involved in binding both the von Willebrand factor and the phospholipid membrane (30). So far, however, only one mutation (amino acid deletion) has been found to alter the interaction between factor VIII and the phospholipid membrane (explained by redundancy of the binding mechanism; see the tenase complex).

Thrombin activation defect

Two types of mutations lead to insufficient thrombin activation of factor VIII. The consequence is usually moderate or mild, CRM-positive hemophilia with dysfunctional factor VIII in plasma. One type of mutations alter amino acids within the thrombin cleavage sites. In addition, substitutions outside the cleavage sites have been described, e. g. substitution of sulphated Tyr349 in domain A1 – yet another example of the significance of post-translation modifications (31).

Accelerated A2 domain dissociation

An interesting group of missense mutations involves amino acids forming the interfaces between domains A1 and A2 and domains A2 and A3. Substitutions of these residues cause destabilization of the noncovalent interaction of the A2 domain within heterotrimer of the activated factor VIIIa and thus lead to premature termination of its activity. In such patients with mild or moderate hemophilia, lower FVIII:C activity is detected when determined using the two-stage assay (longer incubation period) as compared to the one stage assay (32).

Abnormal interaction with factor IXa

Interaction between factors VIIIa and IXa on the surface of the phospholipid membrane can be disturbed by missense mutations in the A3 domain, which serves the receptor site for factor IXa. Amino acid substitutions within the A2 domain may affect, on the one hand residues which are in direct contact with the catalytic domain of factor IXa, and on the other hand the more distant residues, where an effect on the conformation of the contact surface can be presumed (33). The consequence of these mutations is a decrease of factor VIIIa cofactor activity resulting in CRM-positive hemophilia of severity ranging from severe to mild.

FACTOR VIII INHIBITOR

Factor VIII inhibiting antibodies in some patients with severe form of hemophilia, usually develop during childhood following the first doses of replacement therapy. The antibodies usually belong to the IgG₄ class, they do not bind the complement, and their formation is subject to T-lymphocyte regulation. Certain B-cell and T-cell epitopes have been successfully characterized in recent years. Interestingly, non-inhibiting antibodies binding factor VIII occur commonly among healthy blood donors (34).

The inhibition of FVIII is usually by mechanism of sterical hindrance resulting from the antibody binding to particular epitope on factor VIII protein. The epitopes of inhibitory antibodies partly overlap with functional domains on the factor VIII surface (Fig. 3b and 4) (35). The inhibitory antibodies most often adversely affect the

tenase complex activity by targeting the domains A2, A3, and C2. Binding of the inhibitory antibody to the A2 domain prevents interaction between factor VIII and the catalytic domain of factor IXa. The antibody binding to the A3 domain of factor VIII blocks interaction with domain EGF1 of factor IXa, thus preventing the assembly of the tenase complex. The antibodies directed towards the C2 domain usually recognize an epitope extending to the cluster of hydrophobic amino acid residues, thus preventing factor VIIIa binding to the lipid membrane and assembly of the tenase complex. An interesting inhibitor has been characterized, which slows thrombin-cleaved factor VIIIa release from the bond to von Willebrand factor (36). It is presumed that the consequence of this deceleration is the dissociation of the A2 domain even before the assembly of the tenase complex. An inhibitor with an epitope in the ar1 region overlapping with the binding site of factor VIIIa for factor X has been described. In some patients catalytic antibodies with specific hydrolytic activity towards factor VIII have been detected (37).

Studies on twins and other family studies have pointed to the existence of a genetic component in the risk of inhibitor development. Types of mutations of the factor VIII gene have been stratified according to their relative risk of inhibitor development, ranging between 2-68% (38). The highest risk has been associated with large deletions of several domains (68% of patients with a mutation have an inhibitor), nonsense mutations in the light chain (50%) and inversions (35%). Certain amino acid substitutions seem to be associated with an increased risk, e. g. 5 out of 14 hemophilic patients with Arg2150His mutation developed an inhibitor. Low risk of inhibitor development (2-6%) is associated with splice site defects, missense mutations in the heavy chain, and with single nucleotide deletions and insertions in series of rows of adenines. The association of the HLA alleles with the risk of inhibitor is not very significant (39).

CONCLUSION

The knowledge of the molecular pathogenesis of hemophilia A has a practical application in diagnostics and therapeutic practice. Understanding the close relationship between factor VIII and von Willebrand factor has been applied in differential diagnosis of hemophilia and von Willebrand's disease. The identification of causal mutations allows reliable determination of female carriers and prenatal diagnosis. Detailed knowledge of the factor VIII gene and protein has enabled for the industrial production and therapeutic utilisation of concentrates of recombinant factor VIII, independent on plasma from voluntary donors, as well as for the early clinical trials of therapeutic gene transfer. Understanding the relation between the structure and function of factor VIII and its functional interactions facilitates the design of new improved factor VIII molecules and opens the road to the development of peptide mimetics.

Abbreviations

BiP	- immunoglobulin binding protein
CNX	- calnexin
CRM	- cross-reactive material
CRT	- calreticulin
ERGIC	- Endoplasmic Reticulum - Golgi Intermediate Compartment
FIIa	- thrombin
FVIII:Ag	- factor VIII antigen
FVIII:C	- factor VIII coagulation activity
HSPG	- heparan sulphate proteoglycan
LINES	- long period interspersed sequences
LMAN1	- lectin, mannose-binding 1
LPR - LDL	- receptor related protein
MCFD2	- multiple coagulation factor deficiency 2
OMIM	- Online Mendelian Inheritance in Man
SINES	- short period interspersed sequences

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