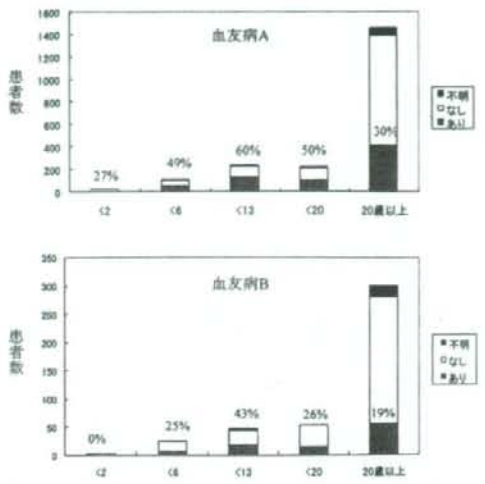

図4　わが国における定期補充療法の年齢別の実施率[9]

学会血友病部会で企画され，同学会で2004年11月に承認され，現在進行中である．乳幼児の重症型血友病患者に対して定期補充療法を行い，関節症の進展予防効果，出血予防効果，日常生活活動度（activity of daily living; ADL）の改善効果を検討し，さらに安全性および利便性に対する検討を加え，定期補充療法の至適な開始年齢を明らかにする．また，定期補充療法開始前の出血歴および凝固因子の精密測定（0.2～1.0%）を行い，重症型の中でも定期補充療法をより早期に開始する必要のある患者が存在するかどうか，また定期補充療法での適切なトラフレベルについても検討する．対象は，過去に定期補充療法を行ったことがない8歳未満の重症型血友病で，定期補充療法の開始年齢により4群（0歳から2歳未満：P1群，2歳から4歳未満：P2群，4歳から6歳未満：P3群，6歳から8歳未満：P4群）に分け，各群を経時的に追跡比較する．各群とも25例を予定しているが，2007年11月現在の登録（予定を含む）患者数はそれぞれ，P1群9例，P2群12例，P3群6例，P4群1例である．内容の詳細は，日本小児血液学会のウェブサイト（http://www.med.hokudai.ac.jp/~ped-w/JSPH-hemo.htm）に掲載している．本研究は，多施設，オープン試験で実施中であり，皆様方の積極的な参加をお願いする次第である．内容の詳細あるいは参加希望の施設は，下記事務局にFAXあるいはE-mailで連絡していただきたい．

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VIII. 今後の課題

定期補充療法（とくに一次定期補充療法）に関して今後解決すべきいくつかの課題が挙げられている．そのおもなものは，開始時期，中止時期，対象患者，用量用法，評価方法，対費用効果，副反応などである．また，患者の精神発達への影響や親子関係への影響[10]，患者および保護者のQOLへの影響ついても，今後の重要な研究課題であろう．

疾病の最終的なゴールは治癒であり，血友病においては遺伝子治療に期待が寄せられている．しかし，まだ臨床応用までには克服すべき多くの課題が残されている．一次定期補充療法は，新たな発想から生まれた治療法であり，適切に実施することにより治癒に匹敵するQOLをもたらす可能性が期待される．

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トピックス

血友病患者インヒビターの産生と制御

瀧　正志[1)]

〔KEYWORDS〕 血友病，インヒビター，免疫寛容導入療法

1. はじめに

血友病の止血治療は20世紀後半大きく進歩し，重篤な出血の止血管理および大手術も可能となった．しかし，21世紀に残された課題もまだ多く，その一つは根治療法である．その方略の一つとして遺伝子治療に大きな期待が寄せられているが，臨床応用までには解決すべき問題が多い．もう一つの大きな課題は，インヒビターに関連することで，インヒビター発生時の止血治療，インヒビターの消失をはかる治療法，さらにインヒビターを発生させない治療法の確立である．インヒビターに関していまだ不明な点が多く，本稿ではインヒビターの産生と制御について，最近の知見を中心に概説したい．

2. 血友病のインヒビター

血友病患者に発生するインヒビターとは，先天性血友病A，B患者に発生する凝固第Ⅷあるいは第Ⅸ因子に対する同種抗体(インヒビター)であり，治療薬である凝固因子製剤の投与に起因するものである．一方，非血友病患者に突然発生する抗凝固第Ⅷ因子抗体は自己免疫や免疫寛容の破綻の結果として産生される自己抗体であり種類が異なる．

1）インヒビターの発生頻度

一般的に重症型血友病Aで10～20%，血友病Bで1～5%といわれる．わが国の患者のインヒビター発生率に関する資料はないが，保有率は厚生労働省委託研究事業の血液凝固異常症全国調査の平成17年度報告書によると，血友病Aでは6.1%，(インヒビター不明例を除く1,689例中103例)，血友病Bでは4.4%(338例中15例)であった[1)]．

2）発見される契機

凝固因子の補充効果が見られない時に発見されることが多いが，定期的な検査で偶然発見される場合もある．抗第Ⅸ因子抗体の場合，製剤投与時のアナフィラキシー症状を契機に発見されることもある．インヒビターの発生時期としては，凝固因子製剤の使用延べ日数(exposure days)が10～20日の時点に集中している．

3）インヒビターの検査

Bethesda法[2)]が汎用されている．第Ⅷ因子インヒビターの検査は，等量の正常プール血漿と患者血漿を混和し，これを対照とともに37℃で2時間孵置する．対照は正常プール血漿とイミダゾール緩衝液の等量混合液である．両方の反応混合液に残存する第Ⅷ因子活性を測定し，対照混合液の残存第Ⅷ因子活性を100%基準値として用いる．この基準値に対して50%の第Ⅷ因子活性を示す希釈被検混合液を1Bethesda単位(BU)/mlと定義する．インヒビター力価のカットオフ値に関しては一般的に0.5以下を陰性とするが，未だ定まったものでなく施設により一定ではない．その他の検査法としてNijmegen変法[3)]，Oxford法[4)]などがある．現在広く用いられているBethesda法は低力価域における精度など問題も多く[5)]，今日，国際血栓止血学会/学術標準化委員

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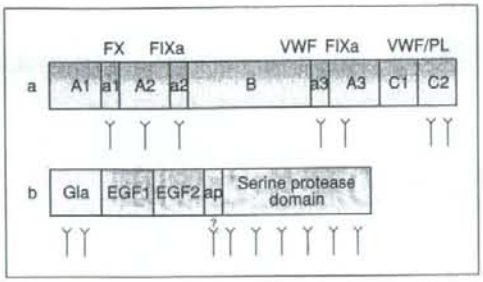

図1　凝固第Ⅷ因子(a)および第Ⅸ因子(b)に対するインヒビターのエピトープ
FX：第X因子，FIXa：活性型第Ⅸ因子，VWF：von Willebrand因子，Gla：γカルボキシグルタミン酸ドメイン，EGF：上皮増殖因子．
〔文献16)より改変して転載〕

会ではNijmegen変法を推奨[3]している．また，酵素免疫測定(enzyme-linked immunosorbent assay：ELISA)法による抗体検出法もあり，この測定法では第Ⅷ因子に対する阻害抗体と非阻害抗体の両者を検出する．後者の抗体の意義は不明な点もあるが第Ⅷ因子のクリアランスおよび半減期に関係することが示唆されている[6]．

4）インヒビターの免疫学的特性

第Ⅷ因子に対するインヒビター，第Ⅸ因子に対するインヒビターはともに主としてIgG_4サブクラスに属する．また，第Ⅸ因子に対するインヒビターはほかにIgG_1も認められ，アナフィラキシーとの関連[7]が指摘されている．

5）ハイレスポンダー(high responder；HR)とローレスポンダー(low responder；LR)

インヒビター患者は通常HRとLRに分類され，前者はインヒビター値が5 Bethesda units/ml(BU/ml)以上の患者および当該凝固因子製剤の注射後に既往応答で5 BU/ml以上に上昇する患者，後者はインヒビター力価が当該凝固因子製剤の注射後においても絶えず5 BU/ml未満の患者と定義される．既往応答は通常，凝固因子製剤投与後5～7日後に生じる．

6）type Ⅰインヒビター，タイプⅡインヒビター

インヒビターの濃度依存性に凝固因子を抑制するものをtype Ⅰ，そうでないものをtype Ⅱと呼ぶ．先天性の血友病患者に発生するインヒビターは主として前者，非血友病患者に突然発生するインヒビターは主として後者にそれぞれ属する．

7）インヒビターのエピトープ

第Ⅷ因子に対して凝固阻害活性を持つインヒビターIgGの第Ⅷ因子蛋白への結合部位は，重鎖A2ドメイン，軽鎖A3-C1ドメイン，さらに軽鎖C2ドメインに存在する[8,9]**(図a)**．個々の患者にみられるインヒビターは，これら多数のエピトープに反応するポリクローナルな抗体である．インヒビターの阻害機序は，インヒビターが認識するエピトープにより異なる．抗A2抗体は第X因子活性化複合体の触媒作用を低下させる[10]ことにより，抗A3抗体は第Ⅸa因子と第Ⅷa因子の相互作用を阻害する[8,9]ことにより，抗C2抗体の大半は第Ⅷ因子がリン脂質およびvon Willebrand因子(VWF)に結合するのを阻害する[11,12]ことにより第Ⅷ因子の凝固活性を阻害する．C1またはC2ドメインに結合しVWFへの第Ⅷ因子結合を安定化させ，これにより第Ⅷ因子がリン脂質と結合するのを阻害する機序[13]も報告されている．軽症型や中等症の患者に発生するインヒビターや過去に治療歴のある患者に対して特定の第Ⅷ因子製剤の投与により出現するインヒビターはtype Ⅱの特性をもち，第Ⅷ因子の軽鎖C2ドメインにエピトープ特異性を示す．このインヒビターによる部分的な第Ⅷ因子の不活化の機序は，インヒビターが第Ⅷ因子と結合する際にVWFと競合するため[14]と考えられている．また，蛋白分解により不活化機序[15]を示す抗体もある．一方，第Ⅸ因子インヒビターのエピトープはγカルボキシグルタミン酸(Gla)領域のN末およびセリンプロテアーゼドメインに局在し[16]**(図b)**，Xase複合体による第Ⅷ因子依存性第X因子の活性化を阻害，あるいは，リン脂質および第Ⅷ因子のGlaドメインへの結合を阻害する．

3. インヒビターの発生要因

インヒビターの発生要因は患者要因と治療要因に大別される(表)．患者要因では，重症度，第Ⅷ因子あるいは第Ⅸ因子の遺伝子変異の種類，インヒビターの家族歴，人種，HLA，IL10遺伝子の遺伝子多型，ワクチン接種，感染症，母乳など多岐にわたる．遺伝子変異に関して，大欠失，ナンセンス変異，イントロン22逆位は重度の分子欠損を示すが，これらの患者ではインヒビター保有

表　インヒビター発生に関与する因子

患者要因	治療要因
重症度 遺伝子変異 インヒビターの家族歴 人種 IL10 遺伝子の遺伝子多型 免疫学的応答 ワクチン接種 感染症 母乳 その他	凝固因子製剤の種類(純度，ウイルス不活化法，遺伝子組換え製剤か血漿由来製剤か，VWF の関与など) 初回注射の年齢 投与パターン(持続投与) 治療の強度(短期間の集中治療) 定期補充療法か出血時治療か 異なる製剤の投与 その他

率は 20～40% と高率である．ミスセンス変異およびスプライス部位変異では蛋白が完全に消失することはなく，インヒビター保有率は 10% 未満である．小欠失や挿入は血友病 A では中等度のリスクであるが血友病 B では高リスクとなる．また，大欠失の中でも複数のドメインが影響を受けた場合は単一のドメインの影響を受けた場合よりリスクがさらに高まる．イントロン 22 逆位は重症型血友病 A で最も頻度の高い変異であり，リスクは高い．ミスセンス変異は全体では最も頻度が高く，重症型以外の患者のほぼすべてを占める．この変異では蛋白の機能はないが，ある程度の蛋白合成があるため免疫寛容が誘導されインヒビターの保有率は低いと考えられている．血友病 A では全体で 5%，血友病 B の重症型では 0.5% である．また，第Ⅷ因子の C1 および C2 ドメインにおけるミスセンス変異はほかのドメインにおけるミスセンス変異よりも約 3 倍高いことより，特定の領域がその蛋白の抗原性に重要であると考えられている．

インヒビター発生の免疫応答遺伝子の関与については，人種に関する研究[17]，同族親族内での兄弟と遠縁親戚でのインヒビター発生率の比較研究[18]があり，これらの 2 つの研究により免疫応答に参加する遺伝子がインヒビター発生に影響することが間接的に示された．また，*MHC* クラス遺伝子の影響についても検討されている．クラスⅡ遺伝子の機能は細胞外抗原を患者に提示することであり興味が持たれる．細胞内抗原をプロセッシングするクラスⅠ遺伝子もインヒビター発生に重要な役割を果たす可能性がある．最近，IL-10 遺伝子の遺伝子多型とインヒビター発生との関連性[19]，制御性 T 細胞(Treg)がインヒビター発生に関与する可能性の報告[20～23]など今後の進展が期待される報告が相次いでいる．

治療要因もインヒビターの発生に重要な影響を及ぼす可能性がある．製剤の種類に関連したインヒビター発生の危険度に関して，純度の問題，加熱処理などのウイルス不活化の問題，遺伝子組み換え製剤か血漿由来製剤か[24,25]，第Ⅷ因子製剤では VWF との複合体製剤か否か，初回注射の年齢[26,27]，治療の強度[26]，定期補充療法[26]などが絶えず議論されているが，一定の見解を得るまでには至っていない．インヒビター発生に関して現時点で明らかにされていることはほんの一部であり，病因の大半は未解決のままで，インヒビター発生を予測することはできない．

4. インヒビターの制御に関する治療

血友病患者に発生した同種インヒビター抗体はステロイドなどの免疫抑制剤単独の効果は期待できない．インヒビターの低下あるいは消失を目的とする治療法で現在最も期待されているのは免疫寛容導入(immune tolerance induction：ITI)療法である．基本的には，インヒビター保有血友病 A に対して凝固第Ⅷ因子製剤を，インヒビター保有血友病 B に対して凝固第Ⅸ因子製剤を定期的に静注する．方法は，高用量の Bonn 方式[28,29]，低用量方式[30]，抗体吸着カラムや免疫抑制剤を併用する Malmö 方式[31,32]などが代表的である．ITI 療法の治療結果に影響する因子として，ITI 療法開始時のインヒビター値は最も重要[33,34]で，治療成功率，治療成功までの期間の両者に影響を及ぼす．用量[33,34]については意見が分かれている．この治療法は長期間の忍耐と高額な

医療を必要とするが，まだ最適なプロトコールが確立されていない．そこで現在，特にインヒビター保有血友病A患者に対する用量についての国際的な無作為化多施設共同臨床試験が行われておりわが国も参加している．1日でも早い安全で有効率の高いプロトコールの確立が望まれる．また，インヒビター保有血友病Aに対するITIの成功率は高いが，インヒビター保有血友病Bに対するITIの成功率は低くまたインヒビター保有血友病Aにはみられないアナフィラキシー[7,35]，ネフローゼ症候群[36]の発生がみられるため，免疫調節剤を含め新たな治療戦略の開発が望まれる．

5. おわりに

近年，血友病の止血治療は凝固因子製剤の改良，在宅自己注射療法の導入により大きく進歩した．また，関節障害発症前の乳幼児期から開始する一次定期補充療法が一般的な治療法として最近認識されるようになり，今後ますます血友病患者のQOLは改善されることが想定される．しかし，インヒビターが発生した場合止血治療は難渋することが多く，血友病治療に残された大きな課題である．現在，血友病の根治療法として大いなる期待が寄せられている遺伝子治療に関してもインヒビターの産生，制御を十分に理解し，進めていく必要があろう．

免疫寛容導入療法(immune tolerance induction therapy)

免疫寛容(immune tolerance)とは抗原により誘導される抗原特異的な免疫反応のない状態をさす．血友病患者に発生したインヒビターを駆逐する方法として考案された．具体的には，インヒビター保有血友病A患者には第Ⅷ因子製剤，インヒビター保有血友病B患者には第Ⅸ因子製剤をそれぞれ定期的に静注する．投与量，投与間隔などのレジメはまだ確立されていない．すべての患者のインヒビターを消失せしめることはできないが，現時点ではインヒビターを消失せしめる最も有効な方法である．

定期補充療法(regular replacement therapy)

出血時に欠乏する凝固第Ⅷあるいは Ⅸ因子製剤を補充する治療法を on demand 療法あるいは episodic 療法と呼び，出血後に止血を図る治療法である．しかしながら，この治療法では多くの重症患者の関節内出血の反復を阻止できず，加齢に伴い血友病性関節症を発症する．この治療法に対し，非出血時に欠乏する凝固因子を長期間にわたり定期的に補充する治療法が北欧を中心に行われ，関節障害の予防効果が示されていた．最近新たなエビデンスが加わり，現在では治療法の主流となろうとしている．この治療法を prophylaxis と欧米では呼ぶが，わが国では定期補充療法とよぶ．関節障害発症前に開始する一次定期補充療法と，関節障害発症後に開始する二次定期補充療法に大別される．

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Identification of a plasmin-interactive site within the A2 domain of the factor VIII heavy chain ☆

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Abstract

Factor VIII is activated and inactivated by plasmin by limited proteolysis. In our one-stage clotting assay, these plasmin-catalyzed reactions were inhibited by the addition of isolated factor VIII A2 subunits and by Glu-Gly-Arg-active-site modified factor IXa. SDS-PAGE analysis showed that an anti-A2 monoclonal antibody, recognizing the factor IXa-interactive site (residues 484–509), blocked the plasmin-catalyzed cleavage at Arg^{336} and Arg^{372} but not at Arg^{740}. Surface plasmon resonance-based assays and ELISA demonstrated that the A2 subunit bound to active-site modified anhydro-plasmin with high affinity (K_d: 21 nM). Both an anti-A2 monoclonal antibody and a peptide comprising of A2 residues 479–504 blocked A2 binding by ~80% and ~55%, respectively. Mutant A2 molecules where the basic residues in A2 were converted to alanine were evaluated for binding of anhydro-plasmin. Among the tested mutants, the R484A A2 mutant possessed ~250-fold lower affinity than the wild-type A2. The affinities of K377A, K466A, and R471A mutants were decreased by 10–20-fold. The inhibitory effect of R484A mutant on plasmin-catalyzed inactivation of factor VIIIa was ~20% of that of wild-type A2. In addition, the inactivation rate by plasmin of factor VIIIa reconstituted with R484A mutant was ~3-fold lower than that with wild-type A2. These findings demonstrate that Arg^{484} plays a key role within the A2 plasmin-binding site, responsible for plasmin-catalyzed factor VIII(a) inactivation.

Keywords: Factor VIII; A2 domain; Plasmin; Interactive-site; Inactivation; A2 mutant

1. Introduction

Factor VIII, a plasma protein deficient or defective in individuals with the severe congenital bleeding disorder, hemophilia A, functions as a cofactor in the tenase complex responsible for phospholipid surface-dependent conversion of factor X to Xa by factor IXa [1]. Factor VIII is synthesized as a multi-domain, single chain molecule (A1-A2-B-A3-C1-C2) consisting of 2,332 amino acid residues [2,3]. It is processed into a series of metal ion-dependent heterodimers by cleavage at the B-A3 junction, generating a heavy chain consisting of the A1 and A2 domains, plus heterogeneous fragments of a partially proteolyzed B domain, linked to a light chain consisting of the A3, C1, and C2 domains [2–4].

Factor VIII, circulating as a complex with von Willebrand factor, is converted into its active form, factor VIIIa, by limited proteolysis, catalyzed by either thrombin or factor Xa [5]. Cleavages at Arg^{372} and Arg^{740} of the heavy chain produce 50-kDa A1 and 40-kDa A2 subunits. Cleavage of the 80-kDa light chain at Arg^{1689} produces a 70-kDa A3C1C2 subunit. Proteolysis at Arg^{372} and Arg^{1689} is essential for generating factor VIIIa cofactor activity [6]. Cleavage at the former site exposes a functional factor IXa-

Abbreviations: APC, activated protein C; mAb, monoclonal antibody; EGR-factor IXa, Glu-Gly-Arg-active-site modified factor IXa; 6-AHA, 6-aminohexanoic acid; Ah-plasmin, anhydro-plasmin; wild-type A2, wt-A2; SPR-based assay, surface plasmon resonance-based assay; LRP, low-density lipoprotein receptor-related protein

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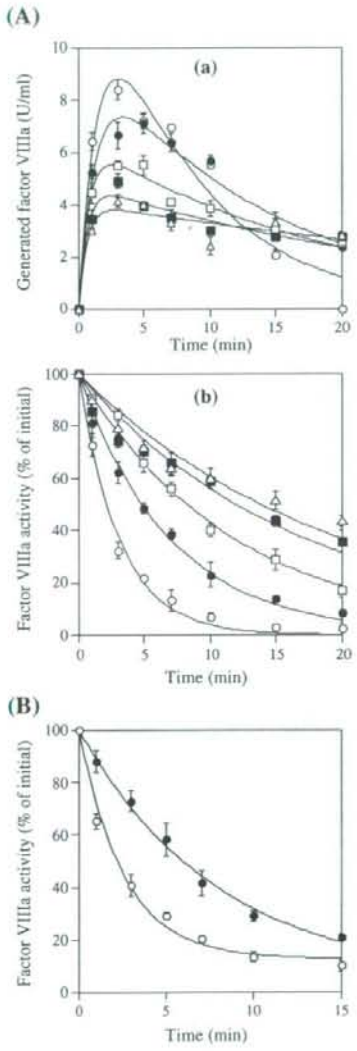
interactive site within the A2 domain that is cryptic in the non-activated factor VIII molecule [7]. Cleavage at the latter site liberates the cofactor from von Willebrand factor [8], and contributes to the overall specific activity of the cofactor [9,10]. Serine proteases including activated protein C (APC) [5], factor Xa [5], and factor IXa [11] inactivate factor VIII(a) by cleavage at Arg^{336} within the A1 subunit. This inactivation mechanism is related to altered interaction of the A2 subunit with the truncated A1 and an increase in the K_m for substrate factor X [12,13]; the latter reflecting the loss of factor X-interactive site within the A1 residues 337–372 [14].



Plasmin, the most potent fibrinolytic protease, is composed of a heavy chain consisting of five kringle domains and a light chain containing the catalytic domain. The enzyme reacts with numerous proteins, represented typically by fibrin, by mechanisms involving lysine-binding sites [15]. Recent studies have revealed that plasmin proteolytically inactivates several coagulation proteins including factors Va [16,17], factor IXa [18], and factor X [19]. The interactive sites in the coagulation factor substrates are not characterized, however. Recently, our laboratory has demonstrated that plasmin rapidly inactivates factor VIII by proteolytic cleavage at sites identical to those cleaved by factor Xa, in particular Arg^{336} [20]. Our data indicated that specific cleavage at Arg^{336} was selectively regulated following interaction of the protease with the A2 domain of factor VIII.

In the present study, we identify plasmin-interactive site(s) within the A2 domain using a combination of approaches employing isolated factor VIII subunits, synthetic peptides, antibodies, and recombinant factor VIII A2 mutants. Our results indicate that the A2 domain of factor VIII, in particular residue Arg^{484}, contributes to a unique plasmin-interactive site within the heavy chain that promotes plasmin docking during cofactor inactivation cleavage of the heavy chain.

2. Materials and methods

2.1. Proteins and reagents

Purified recombinant factor VIII preparations were generous gifts from Bayer Corp. Japan (Osaka, Japan). The light and heavy chains, A1/A3C1C2 dimer, A1, and A2 subunits of factor VIII were purified as previously described [12,13]. Factor VIIIa was isolated from thrombin-cleaved factor VIII by CM-Sepharose chromatography [21]. SDS-PAGE of the isolated subunits followed by staining with GelCode BlueStain Reagent (Pierce, Rockford, IL) showed >95% purity. An anti-factor VIII A1 monoclonal antibody (mAb) 58.12 was a generous gift from Bayer Corp. [22]. Anti-A2 mAb413 and anti-C2 mAbNMC-VIII/5 were produced as described [7,23]. A second anti-A2 mAbJR8 was obtained from JR Scientific Inc. (Woodland, CA). Human plasmin (Lys-plasmin), devoid of factor Xa and APC, and 6-aminohexanoic acid (6-AHA) was purchased from Sigma (St Louis, MO). Pefabloc (Roche, Basel, Switzerland) and horseradish peroxidase-labeled streptavidin (Chemicon, Australia) were purchased from the indicated vendors. Glu-Gly-Arg-active-site modified factor IXa (EGR-factor IXa) was obtained from Haematologic Technologies Inc. (Essex Junction, VT), and was shown to contain <0.5% factor IXa activity in a specific chromogenic assay. No unbound chloromethylketone was evident in this product (data not

Fig. 1. Plasmin-catalyzed activation or inactivation of factor VIII(a) — (A) Effect of the addition of exogenous A2 subunit: Factor VIII (panel a) or factor VIIIa (panel b) (25 nM) was incubated with plasmin (1 nM) and phospholipid (10 μM) in the presence of various concentrations of exogenous A2 (0 nM, open circles; 25 nM, closed circles; 50 nM, open squares; 100 nM, closed squares; 200 nM open triangles). Factor VIII(a) activity was measured at the indicated times using a one-stage clotting assay. The initial activities of factor VIII or factor VIIIa at time zero were ~10 U/ml and ~50 U/ml (100% level), respectively. The values of factor VIII(a) activity were plotted as a function of incubation time and the data in (panel a) and (panel b) were fitted using the formula Eqs. (2) and (1), respectively, as described in Materials and methods. (B) Effect of the addition of EGR-factor IXa: Factor VIIIa (25 nM) was incubated with plasmin (0.8 nM) and phospholipid (10 μM) in the absence (open circles) or presence (closed circles) of EGR-factor IXa (40 nM). Factor VIIIa activity was measured at the indicated times using a one-stage clotting assay. The initial activity of factor VIIIa (100% level) was ~50 U/ml. The values of factor VIIIa activity were plotted as a function of incubation time and the data were fitted using the formula Eq. (1).

Table 1
Kinetic parameters characterizing plasmin-catalyzed factor VIII(a) activation and inactivation in the presence of exogenous A2 subunit

A2 added	Factor VIII activation/inactivation		Factor VIIIa inactivation
	k_1	k_2	k
nM	min^{-1}		min^{-1}
0	1.01±0.19	0.160±0.032	0.325±0.016
25	0.78±0.10	0.081±0.006	0.144±0.005
50	0.54±0.08	0.052±0.007	0.084±0.003
100	0.26±0.04	0.036±0.002	0.058±0.005
200	0.24±0.04	0.035±0.004	0.050±0.004

Factor VIII(a) (25 nM) was incubated with plasmin (1 nM) and phospholipid (10 μM) in the presence of A2 subunit as described under "Materials and methods". Rate constant values were calculated from the data shown in Fig. 1A. All experiments were performed at least three separate times, and average values and standard deviations are shown.

shown). Phospholipid vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine (Sigma) were prepared using *N*-octylglucoside [24]. The mAb IgG preparations were biotinylated using *N*-hydroxysuccinimido-biotin (Pierce). $F(ab')_2$ fragments of mAb413 IgG were prepared using immobilized pepsin-Sepharose (Pierce). Synthetic peptides corresponding to factor VIII A2 residues 479–504, 484–509, and 489–514 were prepared by BioSynthesis (Lewisville, TX).

2.2. Preparation of anhydro-plasmin (Ah-plasmin)

Ah-plasmin, a catalytically inactive derivative of plasmin, was prepared as previously described for the preparation of Ah-factor Xa [25]. Briefly, plasmin was chemically modified with phenylmethylsulfonyl fluoride. Then, to convert phenylmethylsulfonyl-residues of the modified plasmin to dehydroalanine, the product was diluted with 50 mM NaOH, incubated for 10 min at 0 °C, and the pH was adjusted to 7.5. After dialysis against 50 mM Tris–HCl, pH 7.5 containing 1 M NaCl, Ah-plasmin was purified by benzamidine-Sepharose 4B column chromatography. The Ah-plasmin demonstrated <1% plasmin activity, and its molecular weight was similar to that of native plasmin.

2.3. Recombinant factor VIII A2 molecules

Recombinant wild-type and mutants of the A2 domain were constructed and expressed using the Bac-to-Bac baculovirus systems [26]. The mutations predominantly targeted positively charged residues located on the surface of the A2 domain, Lys^{376}, Lys^{377}, His^{378}, Lys^{380}, Lys^{466}, Arg^{471}, Arg^{484}, Tyr^{487}, Ser^{488}, Arg^{489}, Arg^{490}, Leu^{491}, Lys^{493}, Lys^{496}, His^{497}, Lys^{510}, and Lys^{512}. The selected residues were replaced by alanine. The A2 expression cassette was assembled on the basis of MHGX vector and subcloned into pFastBac1 vector. The chimeric gene encoded a polypeptide, six His tag and a factor Xa cleavage site at the N-terminus. The protein was expressed in Sf9 cells and purified by affinity chromatography using CNBr-activated Sepharose 4B with immobilized anti-A2 mAb. To remove the poly-His tag portions, the eluate was mixed with factor Xa and treated with Xarrest agarose (Novagen, Madison, WI). The resulting A2 was >90% pure as judged by SDS-PAGE and Western blotting.

2.4. Clotting assay

Factor VIII(a) activity was measured in a one-stage clotting assay using factor VIII-deficient plasma. Reconstitution of factor VIIIa activity from the recombinant A2 molecule and A1/A3C1C2 dimer was performed as previously described [27]. Maximal factor VIIIa levels were obtained after ~20 min. Plasmin-catalyzed activation and inactivation of factor VIII(a) was performed in 20 mM HEPES buffer, pH 7.2, 0.1 M NaCl, 5 mM $CaCl_2$, 0.01% Tween 20 (HBS-buffer) containing 0.01% BSA, plus phospholipid (10 μM) at 37 °C. Aliquots were removed from the mixtures at the indicated times and plasmin activity immediately quenched by the addition of 0.5 mM pefabloc and 1000-fold dilution prior to assays of factor VIII activity. The presence of plasmin and pefabloc in the diluted samples did not affect the measurements of factor VIII activity (data not shown).

2.5. Cleavage of factor VIII and its subunits by plasmin

Plasmin was added to factor VIII(a) and its subunits in a 1:25 ratio (mol/mol) with phospholipid vesicles (10 μM) in a HBS-buffer at 37 °C. Samples were obtained at the indicated times and the reactions were immediately terminated and prepared for PAGE by adding SDS and 2-mercaptoethanol and boiling for 3 min.

2.6. Electrophoresis and Western blotting

SDS-PAGE was performed with 8% gels under reducing conditions. The proteins were transferred to a PVDF membrane by Western blotting [13]. Proteins were probed using a biotinylated anti-A2 mAb, followed by horseradish peroxidase-labeled streptavidin. The signals were detected using an enhanced chemiluminescence system (PerkinElmer LifeScience, Boston, MA).

2.7. Surface plasmon resonance (SPR)-based binding assay

The kinetics of factor VIII and plasmin interaction were determined by SPR using a BIAcore X instrument (Biacore AB, Uppsala, Sweden) [25]. Ah-plasmin was covalently coupled to the CM5 chip surface at a coupling density of 7 ng/mm^2. Binding (association) of the ligand was monitored in 10 mM HEPES, pH 7.4, 0.15 M NaCl, 0.005% polysorbate 20, at a flow rate of 15 μl/min for 4 min. The dissociation of bound ligand was recorded over a 2 min period by replacing the ligand-containing buffer with buffer alone. The level of non-specific binding corresponding to the ligand binding to the uncoated chip was subtracted from the signal. Reactions were performed at 37 °C. The rate constants for association (k_{asso}) and dissociation (k_{diss}) were determined by nonlinear regression analysis using the evaluation software provided by Biacore AB. Dissociation constants (K_d) were calculated as k_{diss}/k_{asso}.

2.8. ELISA-based assay using immobilized Ah-plasmin

Microtiter wells were coated with Ah-plasmin (50 μl, 100 nM) in 20 mM Tris, and 0.15 M NaCl, pH 7.4, overnight at 4 °C. The wells were washed with

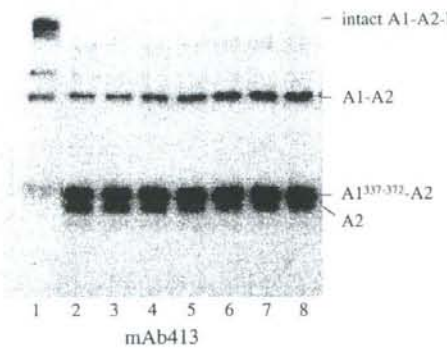


Fig. 2. Effect of anti-A2 mAb413 on plasmin-catalyzed factor VIII cleavage — Factor VIII (100 nM) was preincubated with various concentrations of mAb413 IgG $F(ab')_2$ for 1 h and was reacted with plasmin (4 nM) for 3 min. Samples were run using 8% SDS-PAGE followed by Western blotting using biotinylated anti-A2 mAbJR8. Lane 1 shows the intact heavy chain. Lanes 2–8 show the cleavage of factor VIII heavy chain in the presence of the increasing concentrations of mAb413 (0, 9, 19, 38, 75, 150, and 300 nM, respectively).

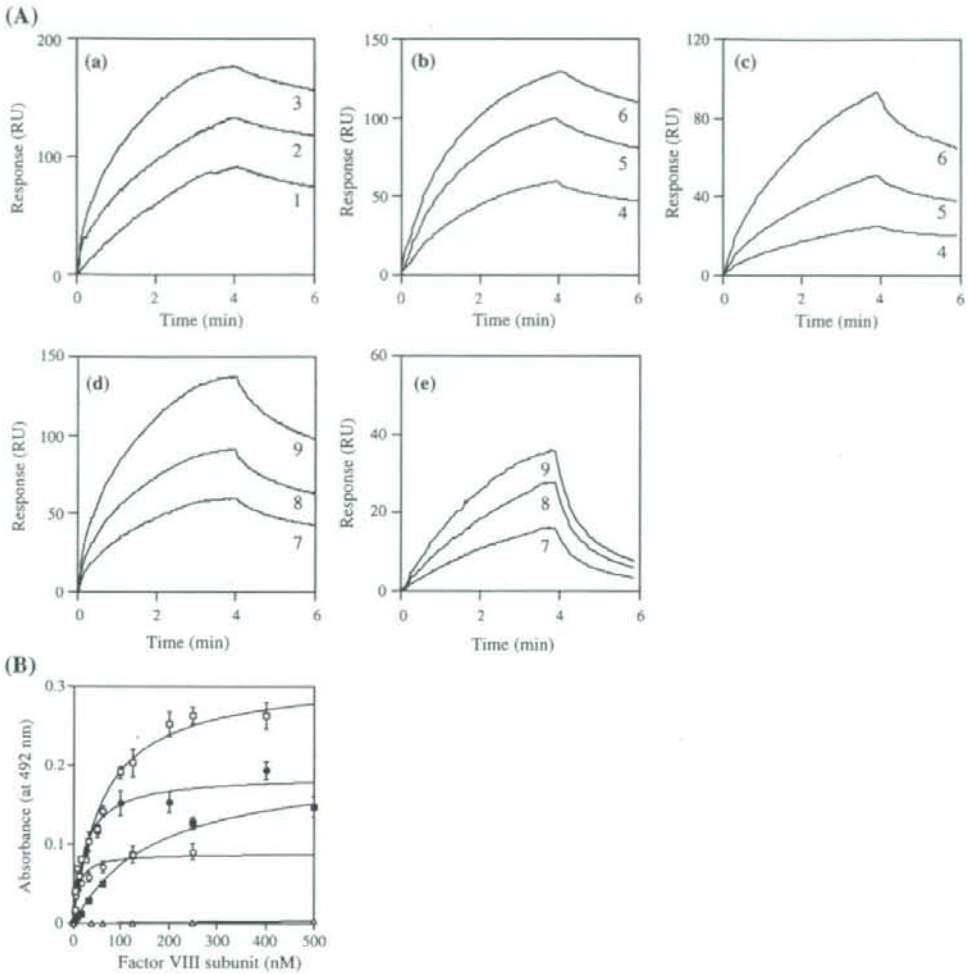


Fig. 3. Direct binding of factor VIII subunits to Ah-plasmin — (A) SPR-based assay for factor VIII subunit binding to Ah-plasmin: Various concentrations of factor VIII (panel a), intact heavy chain (panel b), intact light chain (panel c), the A2 subunit (panel d), and A1 subunit (panel e) were added to Ah-plasmin (~7 ng/mm^2) immobilized onto the sensor chip for 4 min, followed by the change of running buffer for over 2 min as described in Materials and methods. The lines 1–3 show the association/dissociation curves of factor VIII (12.5, 25, and 50 nM, respectively), lines 4–6 show similar curves for intact heavy chain or light chain (20, 40, and 80 nM, respectively), and lines 7–9 show similar curves for the A2 or A1 (40, 80, and 120 nM, respectively). (B) ELISA for factor VIII subunits binding to Ah-plasmin: Various concentrations of the factor VIII (open circles), the heavy chain (closed circles), A2 subunit (open squares), the light chain (closed squares), and A1 subunit (open triangles) were reacted with Ah-plasmin (100 nM) that had been immobilized onto microtiter wells. Bound factor VIII subunits were detected using biotinylated anti-A1 (58.12), anti-A2 (JR8), anti-C2 (NMC-VIII/5) mAb IgG. Absorbance values were plotted as a function of the concentration of factor VIII subunit, and data were fitted using the Eq. (3) according to a single-site binding model described in Materials and methods.

PBS containing 0.02% Tween 20 and were blocked with PBS containing 5% BSA for 2 h at 37 °C. The indicated factor VIII subunit was then added in HBS-buffer containing 1% BSA for 2 h at 37 °C. Biotinylated factor VIII mAb IgG was added and bound IgG was quantified by the addition of horseradish peroxidase-labeled streptavidin and *O*-phenylenediamine dihydrochloride substrate. Reactions were stopped by the addition of 2 M H_2SO_4, and absorbances measured at 492 nm using a Labsystems Multiskan Multisoft microplate reader (Labsystems, Helsinki, Finland). The amount of nonspecific binding of biotinylated IgG in the absence of factor VIII was <5% of the total signal. Specific binding was recorded after subtracting the nonspecific binding.

2.9. Data analyses

Nonlinear least squares regression analyses were performed using Kaleidagrapgh (Synergy Reading, PA). All experiments were performed at

Table 2
Binding parameters for the interaction of factor VIII(a) subunit and Ah-plasmin determined in SPR-based assay and ELISA-based assay

Factor VIII fragment	SPR-based assay			ELISA
	k_{asso}	k_{diss}	K_d^{app}	K_d^{app}
	$\times 10^4\ M^{-1}s^{-1}$	$\times 10^{-3}\ s^{-1}$	nM	nM
Factor VIII	26.3±0.8	0.8±0.1	3.1	6.7±2.0
Heavy chain	40.2±6.9	2.3±0.1	5.6	13.0±3.1
Light chain	2.9±0.4	2.0±0.7	68.2	115±11
A2	14.3±6.2	3.2±1.3	22.6	40.7±8.7
A1	5.2±0.4	10.9±0.9	208	N.d.[b]

Reactions were performed as described under "Materials and methods". Parameter values were calculated by nonlinear regression analysis of the kinetic curves shown in Fig. 3A and B using the evaluation software provided by Biacore AB and using Eq. (3) as described under "Materials and methods". All experiments were performed at least three separate times, and average values and standard deviations are shown.
K_d^{app}: apparent K_d value.
[a] Values were calculated as k_{diss}/k_{asso}.
[b] Not determined.

least three separate times and average values and standard deviations are shown. The rate constant (k) for factor VIIIa inactivation by plasmin was determined by employing the Eq. (1),

$$[\text{factor VIIIa}]_t = [\text{factor VIIIa}]_0 \cdot e^{-kt} \quad (1)$$

where [factor VIIIa]$_t$ is the concentration at time point (t) of factor VIIIa. To evaluate the catalytic efficacy of plasmin for factor VIII, we calculated the activation rate constants based on the values of generated factor VIIIa activity. Assuming that the cleavage event and release of products are rapid, the concentration of free plasmin should be constant. Therefore, the rate constants correlate with the concentration of substrates as follows:

$$\text{factor VIII} \xrightarrow{k_1} \text{factor VIIIa} \xrightarrow{k_2} \text{factor VIIIi} \quad \text{(Scheme 1)}$$

Factor VIIIi represents the inactivated factor VIIIa. These apparent rate constants (k_1 and k_2) in Scheme (1) are based on a series of reactions for factor VIII activation by plasmin and were estimated by nonlinear least squares regression using the Eq. (2) [28],

$$[\text{factor VIIIa}]_t = [\text{factor VIII}]_0 \cdot k_1 \cdot \left(e^{-k_1 t} - e^{-k_2 t}\right)/(k_2 - k_1) \quad (2)$$

where [factor VIIIa]$_t$ is the concentration at time point (t) of factor VIIIa and [factor VIII]$_0$ is the initial concentration of factor VIII.

Analyses of factor VIII subunits and Ah-plasmin interaction in ELISA were performed by a single-site binding model using the Eq. (3),

$$\text{Absorbance} = \frac{A_{max} \cdot [S]}{K_d + [S]} \quad (3)$$

where [S] is the factor VIII subunit; K_d is the dissociation constant; and A_{max} represents maximum absorbance signal when the site is saturated by factor VIII subunit.

Data from studies assessing 6-AHA and A2 synthetic peptide-dependent inhibition of plasmin interaction with isolated factor VIII subunits were analyzed by nonlinear least squares regression using the Eq. (4),

$$\%\text{binding} = \frac{B_{max} \cdot [\text{factor VIII subunit}]}{K_d \cdot \left[1 + \frac{[L]}{K_i}\right] + [\text{factor VIII subunit}]} + C \quad (4)$$

where L represents the concentration of 6-AHA or peptide; B_{max} represents maximum binding; K_d is the dissociation constant for the interaction between factor VIII subunit and Ah-plasmin; K_i is the (apparent) inhibition constant for L; and C is a constant for the binding of factor VIII subunit and plasmin that was unaffected by L.

3. Results

3.1. Effect of the exogenous A2 subunit on plasmin-catalyzed factor VIII(a) activation and inactivation

We have recently suggested that plasmin might interact with the A2 subunit of factor VIII, leading to inactivation of the cofactor by proteolytic cleavage at Arg^{336} [20]. To investigate whether the A2 subunit contains a plasmin-interactive site responsible for plasmin-catalyzed activation and subsequent inactivation of factor VIII, we examined the effect of isolated A2 subunits on these reactions. Factor VIIIa activity was measured using a one-stage clotting assay. Control experiments showed that the presence of exogenous A2 subunit, plasmin, and the plasmin-inhibitor, pefabloc, did not affect this assay at the dilutions utilized. Activation of factor VIII (25 nM) by plasmin (1 nM) was evaluated in the presence of increasing concentrations of isolated A2 subunit (Fig. 1A, panel a). Rates of formation (activation) and decay (inactivation) of factor VIIIa were determined and are summarized in Table 1. Under physiological conditions, factor VIIIa reacts optimally on a membrane surface, and in the present assay phospholipid vesicles (10 μM) were added to the factor VIII solutions prior to reaction with plasmin. We have previously confirmed that the presence of this phospholipid has little effect on the reaction between factor VIII and plasmin [20]. We observed that plasmin-catalyzed activation of factor VIII was significantly inhibited by the addition of isolated A2 subunit in a dose-dependent manner. At the maximum concentration of A2 employed (200 nM), the peak

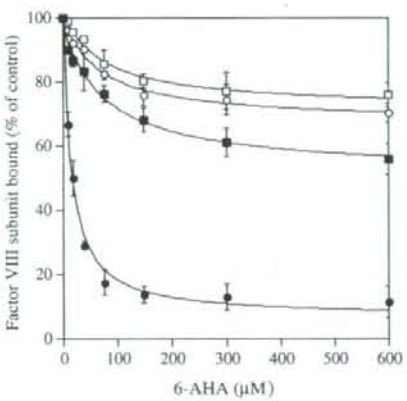


Fig. 4. Effect of 6-AHA on the A2 or light chain subunit binding to Ah-plasmin — The A2 (60 nM, open circles), the light chain (120 nM, closed circles) subunits, the heavy chain (30 nM, open squares), and factor VIII (20 nM, closed squares) in the presence of the indicated levels of 6-AHA were reacted with Ah-plasmin (100 nM) immobilized onto microtiter wells. Bound A2, heavy chain, factor VIII, or light chain was detected using biotinylated anti-A2 (JR8) or anti-C2 (NMC-VIII/5) mAb IgG, respectively. The absorbance values corresponding to the factor VIII subunit binding to Ah-plasmin in the absence of competitor were considered to be 100%. The percentage of factor VIII subunit binding was plotted as a function of 6-AHA concentration, and the plotted data were fitted by nonlinear least squares regression to an Eq. (4).

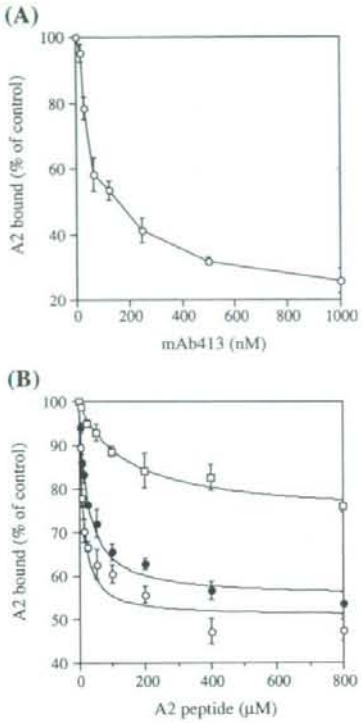


Fig. 5. Effect of anti-A2 mAb413 and A2 peptides on A2 subunit binding to Ah-plasmin — The A2 subunit (60 nM) was preincubated with varying amounts of anti-A2 mAb413 F(ab')$_2$ for 1 h (A) or mixed with varying amounts of the A2 peptides (479–504 peptides; open circles, 484–509 peptides; closed circles, and 489–514 peptides; open squares) (B), prior to reaction with Ah-plasmin (100 nM) immobilized onto microtiter wells. Bound A2 was detected using biotinylated anti-A2 mAb (JR8). The absorbance values for the A2 subunit binding to Ah-plasmin in the absence of competitor were defined as 100%. The percentage of A2 binding was plotted as a function of antibody or A2 peptide, and the plotted data were fitted by nonlinear least squares regression to an Eq. (4) as described in Materials and methods.

factor VIIIa activity was ~50% of that obtained in the absence of A2. The activation rate constant (k_1) for factor VIII in the presence of A2 (200 nM) was ~4-fold lower compared than that observed in the absence of A2. Furthermore, the rate constant (k_2) for inactivation (and/or decay) was ~5-fold less than that observed in the absence of A2.

The activity of factor VIIIa at any time point likely represents contributions from non-activated molecules, activated molecules, and activated molecules that have decayed following factor VIII subunit dissociation. To precisely evaluate the effect of A2 subunit on plasmin-catalyzed inactivation of the cofactor, therefore, the experiments were repeated using factor VIIIa as a substrate. In these instances, the addition of exogenous A2 similarly inhibited factor VIIIa inactivation by plasmin in a dose-dependent manner (Fig. 1A, panel b). The inactivation rate constant (k) with factor VIIIa as substrate was similar to that (k_2) obtained using factor VIII. These results indicated that the A2 domain contributed to plasmin-catalyzed activation/inactivation of factor VIII(a).

3.2. EGR-factor IXa inhibition of factor VIIIa inactivation by plasmin

Factor IXa binds to factor VIIIa with high affinity (K_d: ~5 nM) via the A2 and A3 domains on an anionic membrane surface [29,30]. Since our above observation suggested that the A2 domain associates with plasmin during the activation and/or inactivation, we studied the effect of factor IXa on plasmin-catalyzed inactivation of factor VIIIa. Factor IXa and plasmin are serine proteases that inactivate factor VIIIa by proteolytic cleavage at Arg^{336} [11]. For this reason, an active-site modified EGR-factor IXa was used in place of factor IXa in these experiments. Factor VIIIa (25 nM) was mixed with saturating concentrations of EGR-factor IXa (40 nM) in the presence of phospholipid prior to the addition of plasmin (0.8 nM). In these circumstances, the inactivation rate constant in the presence of EGR-factor IXa was ~2.5-fold lower than that in its absence (0.115 ± 0.010 min^{-1} and 0.264 ± 0.030 min^{-1}, respectively) (Fig. 1B), supporting that factor IXa regulates plasmin-catalyzed factor VIIIa inactivation.

3.3. Effect of anti-A2 antibody on plasmin-catalyzed cleavage of factor VIII heavy chain

Up- and down-regulation of factor VIII activity is mediated by proteolytic cleavages at the A1-A2 domainal junction (Arg^{372}) and at both terminal regions in the A1 domain (Arg^{336} and Lys^{36}) of the heavy chain, respectively. A putative plasmin-interactive site in the A2 domain, which likely overlaps with the factor IXa-interactive sites, may be responsible for the cleavage of the factor VIII heavy chain. To examine this hypothesis, we assessed plasmin-catalyzed cleavage of the factor VIII heavy chain in the presence of an anti-A2 mAb413. This antibody

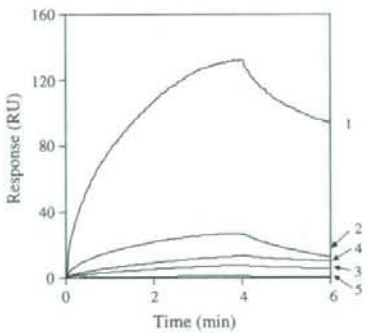


Fig. 6. Binding of recombinant A2 mutants to Ah-plasmin in an SPR-based assay — Recombinant wt-A2 or its mutants (120 nM) were incubated with Ah-plasmin immobilized on a sensor chip (~7 ng/mm^2) for 4 min, followed by a change of running buffer for over 2 min. Curves 1–5 show the association/dissociation curves of wt-A2, and its mutants, K377A, K466A, R471A, and R484A, respectively.

Table 3
Kinetic parameters of the interaction between recombinant A2 mutants and Ah-plasmin in a SPR-based assay

A2 mutant	k_{asso} $\times 10^4\ M^{-1}s^{-1}$	k_{diss} $\times 10^{-4}\ s^{-1}$	K_d^{app} nM
wt-A2	5.14±0.12	11.7±0.19	22.8
K376A	3.90±2.99	4.48±0.23	11.5
K377A	0.32±0.04	10.8±0.32	**338**
H378A	4.83±0.10	7.47±0.17	15.5
K380A	12.7±0.18	7.40±0.33	5.8
K466A	0.057±0.010	0.97±0.20	**170**
R471A	0.079±0.007	3.11±0.30	**394**
R484A	0.025±0.003	14.5±0.60	**5800**
Y487A	5.51±0.13	6.67±0.24	12.1
S488A	3.97±0.09	8.69±0.16	21.9
R489A	8.71±0.14	13.4±0.22	15.4
R490A	3.68±0.13	7.67±0.22	20.8
L491A	7.53±0.12	14.1±0.26	18.7
K493A	5.98±0.09	15.4±0.20	25.8
K496A	8.76±0.15	18.0±0.20	20.5
H497A	0.58±0.02	1.61±0.04	27.8
K510A	7.05±0.13	13.0±0.24	18.4
K512A	1.62±0.02	0.67±0.03	4.14

Reactions were performed as described under "Materials and methods". Parameter values were calculated by nonlinear regression analysis using the evaluation software provided by Biacore AB. The K_d values were calculated as k_{diss}/k_{asso}. A2 mutants, which had the greatest decrease in affinity for Ah-plasmin, and their K_d values are shown in bold. All experiments were performed at least three separate times, and average values and standard deviations are shown. K_d^{app}: apparent K_d value.

recognizes an epitope defined by residues 484–509, overlapping a factor IXa-interactive site, and blocks the interaction between the A2 subunit and factor IXa molecule [29]. Factor VIII (100 nM) was preincubated with the various concentrations of mAb413 IgG F(ab′)$_2$ for 1 h and then treated with plasmin for 3 min. Fig. 2 shows the results from Western blotting of the cleavage reaction using biotinylated anti-A2 mABJR8 as the tracer. In the absence of mAb413, both A1$^{337-372}$–A2 (residues 337–740) and A2 (residues 373–740) fragments derived from the plasmin-cleaved factor VIII were observed (lane 2). In the presence of the antibody (lanes 3–8), the A2 fragment was depressed, whilst the density of the A1-A2 fragment (residues 1–740) was increased in a dose-dependent manner, demonstrating inhibition of cleavage at Arg^{372} and Arg^{336}, respectively. Intact A1–A2-B fragment was not detected in the presence of mAb413, indicating that this antibody did not affect cleavage by plasmin at Arg^{740}. The finding suggested that mAb413 blocked plasmin-catalyzed factor VIII heavy chain cleavage at Arg^{336} and Arg^{372}, but not at Arg^{740}. In contrast, this antibody did not affect plasmin-catalyzed cleavage of the light chain (data not shown). These findings indicated that a region within or close to residues 484–509 in the A2 subunit is critical for plasmin-catalyzed cleavage at Arg^{336} and Arg^{372} during activation and/or inactivation of factor VIII.

3.4. Binding of factor VIII to Ah-plasmin

To obtain direct evidence that the A2 domain contains a major plasmin-interactive site that contributes to enzyme docking and facilitates catalysis of cleavage of factor VIII heavy chain, a series of experiments were performed to assess plasmin binding to factor VIII subunits. In these experiments, an active-site modified plasmin, Ah-plasmin, lacking enzymatic activity, was utilized instead of plasmin. Interactions between the factor VIII subunits and Ah-plasmin were initially evaluated using a SPR-based assay. Several different amounts of factor VIII subunits were added to Ah-plasmin immobilized on a sensor chip. Fig. 3A shows representative curves corresponding to the association and dissociation of immobilized Ah-plasmin with factor VIII (panel a), intact heavy chain (panel b), intact light chain (panel c), A2 (panel d), or A1 subunit (panel e). The factor VIII fragments bound to Ah-plasmin in a dose-dependent manner. The data were assessed by nonlinear regression using a 1:1 Langmuir binding model and were shown to be not significantly different from those calculated using two-site model. Incomplete dissociation curves were obtained, however, because of mass transport issues. The K_d values obtained represent an apparent K_d for interaction, and its derivation may have been subjected to mass transport limitations. Kinetic constants indicated that factor VIII bound with higher affinity than the A2 subunit to Ah-plasmin (K_d: 3.1 nM and 22.6 nM, respectively) (Table 2). The intact heavy chain also bound with ~10-fold higher affinity than the light chain (K_d: 5.6 nM and 68.2 nM, respectively), similar to the calculated K_d value for factor VIII. However, the binding affinity determined for A1 was ~10-fold lower compared with that obtained for A2. The A2 domain readily dissociates from factor VIIIa, and the kinetics for the active form molecule could not be determined. These results indicated a more significant contribution of heavy chain compared with light chain, and a more prominent role for the A2 subunit compared with the A1 subunit.

We further evaluated factor VIII and plasmin interactions by an alternative approach using a solid-phase binding assay in which Ah-plasmin was immobilized onto microtiter wells. Factor VIII bound to Ah-plasmin was detected using biotinylated anti-A1 (58.12), anti-A2 (JR8), or anti-C2 (NMC-VIII/5) mAb IgG. These antibodies did not affect this binding reaction (data not shown). Results are presented in Fig. 3B and Table 2. Reactions of factor VIII subunits with Ah-plasmin yielded saturable binding curves. This method is not based on a true equilibrium binding assay, however, and the K_d values obtained represent an apparent K_d for the interactions. Nevertheless, the K_d value (6.7 nM) obtained for binding of factor VIII to Ah-plasmin in these experiments was similar to that calculated in the SPR-based assay. The binding affinity for the heavy chain was ~9-fold higher than that for the light chain. The A2 subunit also bound to Ah-plasmin with modest affinity (K_d: 40.7 nM), whilst the A1 failed to bind. Overall, the affinity values obtained by the ELISA-based assay were in a good agreement with those obtained in the SPR-based analysis, and were mutually supportive. The reasons for the reduced absorbance signal for factor VIII compared with those observed for the A2 subunits are not known, but may reflect fewer moles of factor VIII bound compared with the isolated subunits as a result of blocking accessible binding sites because of its larger size. Taken together, these results suggest that the A2 domain of heavy chain significantly contributes to factor VIII binding to plasmin.

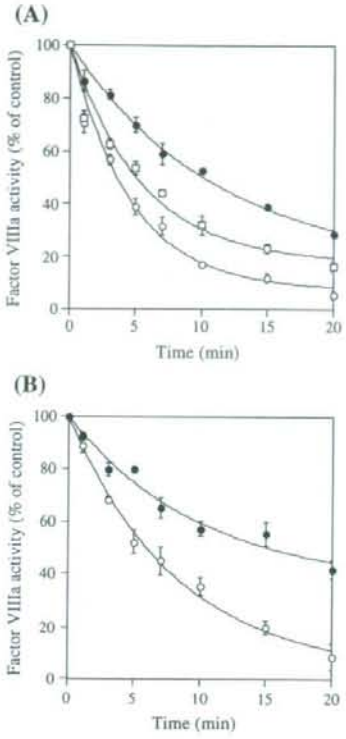


Fig. 7. Inhibitory effect of recombinant A2 mutant on factor VIIIa inactivation by plasmin — (A) Inhibition of R484A mutant on plasmin-catalyzed factor VIIIa inactivation: Isolated factor VIIIa (25 nM) was incubated with plasmin (0.7 nM) and phospholipid (10 μM) in the absence (open circles) or presence of recombinant A2 subunit (100 nM: wt-A2; closed circles, R484A mutant; open squares). Factor VIIIa activity was measured at the indicated times using a one-stage clotting assay. The initial activity of factor VIIIa (100% level) was ~50 U/ml. (B) Plasmin-catalyzed inactivation of factor VIIIa reconstituted with R484A mutant: Factor VIIIa reconstituted with the isolated A1/A3C1C2 dimer (200 nM) and recombinant A2 subunit (200 nM: wt-A2; open circles, R484A mutant; closed circles) was incubated with plasmin (1 nM). Factor VIIIa activity was measured at the indicated times using a one-stage clotting assay. The initial activity of reconstituted factor VIIIa with wt-A2 or R484A was ~35 U/ml (100% level). The value of factor VIIIa activity was plotted as a function of incubation time and the data were fitted to an Eq. (1) as described in Materials and methods.

3.5. Effect of 6-AHA on the binding of A2 subunit to Ah-plasmin

It is well-documented that plasmin interacts with substrates predominantly via exposed lysine-binding sites of the protease [15]. To examine the role of this lysine-binding in the current circumstances, the effects of 6-AHA, a specific competitor of lysine-binding, were examined in the ELISA-based assay. Fixed concentrations of A2 (60 nM) or light chain (120 nM) were incubated with immobilized Ah-plasmin in the presence of varying concentrations of 6-AHA. The 6-AHA blocked the interactions between light chain and plasmin in a dose-dependent manner by >90% (Fig. 4). The apparent K_i value was 6.8± 1.0 μM. In contrast, 6-AHA blocked A2 subunit and plasmin interaction weakly (~30%) with a K_i value of 11.3±2.3 μM. These K_i values (~10 μM) were similar to earlier reports [31] on the effects of 6-AHA on the binding affinity of plasmin, and supported the validity of this competition assay. The observation that 6-AHA markedly inhibited (>90%) light chain-plasmin binding suggests that this interaction is mainly dependent upon lysine-binding sites of the protease. In contrast, the observation that 6-AHA incompletely (~30%) inhibited binding to A2 suggests that plasmin interacts with this subunit predominantly through mechanisms independent of lysine-binding sites. Furthermore, the inhibitory effect of 6-AHA on the interaction between the heavy chain or factor VIII and Ah-plasmin was similar to that for the A2 interaction, indicating a more significant contribution of A2 on factor VIII and plasmin interaction through mechanisms independent of lysine-binding sites.

3.6. Localization of a plasmin-binding site within the A2 domain

Our Western blotting analyses showed that mAb413, recognizing residues 484–509 in the A2 domain, blocked plasmin cleavage of the heavy chain. To confirm that the inhibitory effect of the antibody resulted from blocking of A2 binding to plasmin, we performed a series of competition experiments using an ELISA. The A2 subunit (60 nM) was preincubated with varying amounts of antibody $F(ab')_2$ prior to incubation with immobilized Ah-plasmin. This antibody inhibited the A2 binding to Ah-plasmin by ~80% in a dose-dependent manner, with an IC_{50} of 151±19 nM (Fig. 5A).

To exclude the possibility that this inhibition of A2-plasmin binding resulted from conformational steric hindrance, and to further localize plasmin-binding sites within or around residues 484–509 in the A2 domain, a similar competitive assay was developed using three overlapping synthetic peptides encompassing amino acid sequences within residues 484–509. Control experiments demonstrated that these A2 peptides had little effect on the reaction between A2 subunits and the mAbJR8 used for detection. Both the 479–504 and 484–509 peptides dose-dependently blocked A2 subunit binding to Ah-plasmin by ~50%, and the apparent K_i values were similar (5.7±1.3 μM and 10.3±2.6 μM, respectively) (Fig. 5B). A peptide with a scrambled sequence was used as control and showed little inhibition (data not shown). Peptides 489–514 weakly inhibited binding of the A2 subunit and plasmin with >80% residual binding observed at the highest concentration (800 μM) of peptide. The K_i value for this third peptide (62.8±16.4 μM) was at least 5–10-fold higher than those recorded for the other two peptides. The results suggested that the A2 region comprising residues 479–509, in particular the 479–488 region, represented a plasmin-interactive site.

3.7. Plasmin interaction with the A2 mutants in a SPR-based assay

To further identify the A2 residues responsible for interaction with plasmin, a series of recombinant mutant A2 molecules

were prepared using a baculovirus expression system [26]. We focused on exposed positively charged residues involving lysine, and hydrophobic or hydroxyl group residues within or conformationally close to the 484–509 region. Selected residues were converted to alanine, generating a panel of 17 single-point A2 mutants. The secretion levels and conformational stabilities of the A2 mutants, and specific activities of factor VIIIa obtained from reconstituted mutated A2 and A1/A3C1C2 dimers are described in a recent report [26]. Interactions between our A2 mutants and Ah-plasmin were evaluated using a SPR-based assay (Fig. 6 and Table 3). The data fitted well in a comparative nonlinear regression analysis using a 1:1 Langmuir binding model. The binding affinity of wild-type A2 (wt-A2) for Ah-plasmin was equivalent to that obtained for plasma-derived A2, indicating that the arrangement of the plasmin-interactive site was similar in both preparations. It was especially noteworthy that the binding affinity of the R484A mutant to Ah-plasmin (K_d: 5800 nM) was ~250-fold lower than that of wt-A2, indicating that Arg^{484} plays a major role in plasmin binding. These results were consistent with our earlier competitive binding assays that indicated the presence of a lysine-independent plasmin-binding site within the 479–488 A2 region. In addition, 3 other A2 mutants (K377A, K466A, and R471A) also exhibited reduced binding affinity (by 10–20-fold) compared with wt-A2, suggesting that these residues contributed somewhat to plasmin binding. The remaining A2 mutants bound to plasmin with affinities similar to that of wt-A2. Collectively, these results indicated that residues of Arg^{484}, Lys^{377}, Lys^{466}, and Arg^{471} were involved significantly in A2 binding to plasmin.

3.8. Effect of R484A A2 mutant on plasmin-catalyzed inactivation of factor VIIIa

To investigate the functional role of Arg^{484} residue in the A2 domain that contributed to plasmin binding, we examined the inhibitory effects of R484A mutant on factor VIIIa inactivation by plasmin. Factor VIIIa (25 nM) was mixed with the A2 (100 nM) and incubated with plasmin (0.7 nM). In control experiments, the addition of wt-A2 was associated with an ~3.5-fold decrease in inactivation rate compared with that in the absence of A2 (0.065 ± 0.011 min^{-1} and 0.220 ± 0.029 min^{-1}, respectively) (Fig. 7A). These data again confirmed the functional similarity of recombinant and plasma-derived A2 subunits. For quantitation, the change in the inactivation rate constant obtained by the addition of wt-A2 was regarded as 100% inhibition. As expected, the addition of R484A mutant, possessing ~250-fold reduced affinity for plasmin, weakly inhibited factor VIIIa inactivation by ~20% (0.187 ± 0.026 min^{-1}).

To further elucidate the direct role of Arg^{484} in A2 on plasmin-catalyzed inactivation, we examined the effect of inactivation by plasmin using factor VIIIa reconstituted with the A1/A3C1C2 dimer and R484A A2 mutant as substrate. Reconstituted factor VIIIa with the A1/A3C1C2 dimer (200 nM) and wt-A2 or R484A A2 (200 nM) was reacted with plasmin (1 nM) as described in Materials and methods. Maximum factor VIIIa activity reconstituted with R484A mutant in this condition was similar to that with wt-A2 as previously reported [26]. Reconstituted factor VIIIa with R484A mutant was inactivated by plasmin with an ~2.8-fold decrease in inactivation rate, compared to that with wt-A2 (0.051 ± 0.009 min^{-1} and 0.141 ± 0.011 min^{-1}, respectively) (Fig. 7B). Taken together, these results demonstrated that the A2 domain, in particular Arg^{484}, provides a unique plasmin-interactive site that promotes enzyme docking during plasmin-catalyzed cleavage of the factor VIII heavy chain and cofactor inactivation.

4. Discussion

We have recently suggested that cleavage at Arg^{336} in the factor VIII molecule, a core reaction that regulates factor VIII(a) inactivation by plasmin, may be selectively modulated by interaction of the protease with the A2 subunit [20]. In the current study we present several lines of the direct evidence that the A2 domain does indeed contain a plasmin-interactive site. The presence of this site involved in proteolysis of heavy chain, was demonstrated using two complementary approaches, a SPR-based assay and an ELISA-based assay. Data obtained in conventional factor VIII binding assays using active plasmin are extremely difficult to interpret because plasmin-catalyzed cleavage of the cofactor might deplete the physiological binding response. We developed, therefore, a direct binding assay using Ah-plasmin, a catalytically inactive derivative of plasmin. The advantages of this approach have been confirmed in several previous studies using a range of different serine proteases including Ah-factor Xa [25,32,33]. Direct immobilization of Ah-plasmin may cause conformational changes, however, and crucial regions related to interaction may be buried. We utilized two distinct solid-phase binding assays in this study, therefore, and the results were mutually supportive. The data indicated that plasmin binds to the factor VIII heavy chain with high affinity (K_d: ~5 nM) and to the light chain with modest affinity (K_d: ~70 nM). Our observations further indicated that plasmin binds to the A2 domain with high affinity (K_d: ~20 nM) and does not bind to the A1, highlighting the role of the A2 domain as a plasmin-interactive site.

The A2 domain is known to contain a functionally essential region spanning residues 484–509, a highly basic spacer region exposed on the surface. This region participates in at least three protein interactions; factor IXa [29], alloantibody inhibitors developed in multi-transfused hemophilia A patients [34], and low-density lipoprotein receptor-related protein (LRP) which mediates clearance of factor VIII from the circulation [35]. Plasmin cleaves the factor VIII heavy chain at Arg^{336}, Arg^{372}, and Arg^{740}, resulting in activation and inactivation of the cofactor. In the present study utilizing functional and competitive binding assays with isolated A2 subunits, synthetic peptides and an anti-A2 mAb recognizing residues 484–509, we demonstrated a major contribution of the A2 domain in plasmin docking, mediating proteolytic activity.

Of interest was the observation that EGR-factor IXa inhibited (by ~2-fold) plasmin-catalyzed inactivation of factor VIIIa in the presence of phospholipid. Factor X, however, which binds with lower affinity (K_d: 1–3 μM [14]) to the A1 acidic region consisting of residues 337–372, did not affect plasmin activity

(data not shown). Furthermore, our recent observations indicated that plasmin action against factor VIII was little affected by the presence of either von Willebrand factor or phospholipid [20]. We can speculate, therefore, that factor IXa could protect rapid down-regulation of factor VIIIa by plasmin within the assembled tenase complex in a manner similar to the mechanism whereby factor VIIIa is protected by factor IXa from APC-catalyzed inactivation [36].

To date the best known inactivators of factor VIII(a) are APC and factor Xa. The factor VIIIa interactive sites for APC- or factor Xa-catalyzed inactivation have been already identified. APC interacts with the A3 domain (residues 2009–2018) of light chain and cleaves at Arg^{336} within A1 [37]. Factor Xa interacts with the A1 domain (residues 337–372) of the heavy chain and with the C2 domain of the light chain to cleave at Lys^{36} and Arg^{336} [14,25]. In contrast, based on our present and previous observations [20], plasmin interacts with the A2 domain and light chain for specific cleavage at Arg^{336} and Lys^{36}. Furthermore, the presence of von Willebrand factor and phospholipid modulates inactivation of factor VIIIa by APC and factor Xa [38–40], but has little effect on plasmin-mediated reactions. This evidence suggests that down-regulation of factor VIIIa by these three proteases in the coagulation system is governed by distinct interactive and cleavage mechanisms.

The preparation of a series of single-point A2 mutants allowed us to determine the critical amino acid residues of the plasmin-interactive site within the A2 domain. Recently, Sarafanov et al. [26] identified the binding site for LRP using these A2 mutants. In our study, the mutant R484A markedly reduced the affinity of A2 binding to plasmin (~250-fold) compared with wt-A2. The mutants K377A, K466A, and R471A also mildly reduced this affinity. In addition, the R484A mutant was considerably much less effective than wt-A2 at moderating plasmin-catalyzed inactivation of factor VIIIa. This indicates that Arg^{484} provides a major plasmin-interactive site that promotes enzyme docking during plasmin-catalyzed cleavage of the heavy chain.

Mutations corresponding to R484A and K377A are not listed in the hemophilia A database, but the specific activities of these preparations were similar to that of wt-A2. The specific activities of K466A and R471A correlate with those of the natural mutations, K466T and R471G, seen in mild/moderate hemophilia A [41,42]. Significant conformational disturbances produced by these four mutations are unlikely, therefore, and our findings strongly suggest that residue Arg^{484} is the major functional determinant of the A2 binding site for plasmin. The competitive assays using synthetic peptides and mAb413 with an epitope involving this residue demonstrated that Lys^{377}, Lys^{466}, and Arg^{471} appeared to be principally involved in plasmin docking to A2.

On the basis of the factor VIII homology with ceruloplasmin [43], the currently identified four residues appear to be arranged in a spatially adjacent fashion and exposed on the A2 surface. This provides an extended surface for plasmin binding juxtaposed to the Arg^{336} cleavage site in the A1. Interestingly, comparisons of amino acid sequences among human, porcine, murine, and canine factor VIII molecules (http://europium.csc.mrc.ac.uk) indicate that Arg^{484}, the major contributor for plasmin binding in our study, has less inter-species homology (Arg, Ser, Ser, and Thr, respectively) than the other three residues that are well-conserved. It may be that the mechanism of plasmin action against factor VIII is species-dependent.

The five kringle domains of plasmin, containing homologous lysine-binding sites, play an important role in binding to numerous ligands. A lysine-binding site is essentially constructed in three parts; a hydrophobic core, a cationic center, and an anionic center [44]. The binding mechanism involves hydrogen-bond and/or ion-pair interaction with the cationic or anionic center and van der Waals electronic interaction with the hydrophobic core [45]. In our studies, the lysine analog, 6-AHA, that interacts tightly with lysine-binding sites, inhibited only ~30% A2 binding to Ah-plasmin. Since Arg^{484} in A2 contributed more significantly than the lysine residues to plasmin binding, we suggest that plasmin interacts with Arg^{484} independently of lysine-binding sites. The lysine residues Lys^{377} and Lys^{466} may play a relatively minor role in a lysine-binding sites-dependent mechanism.

In contrast, the binding of factor VIII light chain to Ah-plasmin with mild affinity was completely blocked by 6-AHA, indicating that this interaction is dependent on lysine-binding sites. Plasmin interaction with the light chain, therefore, appears to be quite different from that with the heavy chain (A2 domain). The lysine residues in factor VIII are clustered in both the N-terminal portion (residues 1690–1695) and the middle portion (residues 1804–1818) of the A3 domain of light chain [2,3]. Three-dimensional modeling indicates that these sequences are located over an extended surface in the A3 domain [43], and it is tempting to speculate that the plasmin-interactive site within the light chain may be found in this region. Further studies are required, however, to clarify the lysine-binding sites-dependent mechanisms involved in plasmin interaction with the light chain of factor VIII.

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bjh research paper

Selective factor VIII and V inactivation by iminodiacetate ion exchange resin through metal ion adsorption*

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Summary

The procoagulant activity of factors VIII and V depends on the presence of metal ion(s). We examined the effect of cation-exchange resins with different functional groups on both factors, of which only reaction with iminodiacetate resin resulted in the complete loss of their activity levels in plasma. However, the antigen level of factor VIII was preserved by >95%. This resin reduced divalent cations content present in factor VIII preparations, indicating that it inactivated this factor by direct deprivation of predominant Ca^{2+} ($>Mn^{2+}>>Cu^{2+}$), rather than adsorption of the factor itself. The antigen level of recombinant factor VIII alone was decreased by >95% by reaction with resin, whilst that complexed with von Willebrand factor was preserved by >95%. Iminodiacetate resin-treated plasma was evaluated by measuring factor VIII and V activity in plasma with various levels of either activity. These were significantly correlated to the values obtained using factor VIII- or V-deficient plasma prepared commercially by immunodepletion. We demonstrated that iminodiacetate resin-induced factors VIII and V inactivation is because of direct deprivation of metal ions, predominantly Ca^{2+}, which is more essential for the functional structure of their molecules. Furthermore, iminodiacetate resin-treated plasma would be useful as a substrate for measuring the activity of these factors.

Keywords: factor VIII, factor V, iminodiacetate resin, inactivation, metal ion.

Factors VIII and V function as crucial cofactors in the tenase and prothrombinase complexes that are responsible for factor X activation in an activated factor IX (factor IXa)-dependent reaction and for prothrombin activation in an activated factor X (factor Xa)-dependent reaction on phospholipid surfaces respectively (Mann *et al*, 1988, 1990). Factor VIII deficiency causes a severe congenital bleeding disorder known as haemophilia A. Factors VIII and V are synthesized as multi-domain, single chain molecules (A1-A2-B-A3-C1-C2) with a molecular mass of approximately 300 kDa (Kane & Majerus, 1981; Vehar *et al*, 1984). Both cofactors, which display similar homologies with approximately 40% identical sequences (Vehar *et al*, 1984; Kane & Davie, 1986), are formed by a heavy chain consisting of the A1, A2 and B domains, together with a light chain consisting of the A3, C1 and C2 domains. Factor VIII circulates in plasma as a heterodimer formed by heavy and light chains, whilst factor V circulates as a single chain.

Factor VIII circulates as a non-covalent complex with von Willebrand factor (VWF), which regulates the synthesis and stabilizes the cofactor activity of factor VIII (Weiss *et al*, 1977; Kaufman *et al*, 1989). Quantitative or qualitative defects in VWF result in a decreased level of circulating factor VIII. Critical sites for VWF interaction in factor VIII have been localized to the A3 and C2 domains (Lollar *et al*, 1988; Saenko *et al*, 1994). The association of factor VIII with VWF in this complex results in its increased circulatory half-life (Saenko *et al*, 1999) and enhanced stability of heavy chain–light chain interactions (Fay, 1988). VWF protects factor VIII from proteolysis by several serine proteases including activated protein C (Fay *et al*, 1991; Nogami *et al*, 2002) and factor Xa (Koedam *et al*, 1990; Nogami *et al*, 1999).

Factor VIII possesses a metal-binding motif similar to factor V. Binding reactions of the heavy and light chains of factor VIII(a) and V(a) are non-covalent and require a metal

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ion-dependent linkage with the A1 and A3 domains (Fay, 1988; Adams *et al*, 2004). The high-affinity Ca^{2+} (and Mn^{2+})-binding sites have been identified in the A1 domain of factors VIII and V by site-directed mutagenesis analysis (Sorensen *et al*, 2004; Wakabayashi *et al*, 2004). Cu^{2+}-binding sites are also located in the A1 and/or A3 domains of both molecules (Tagliavacca *et al*, 1997; Adams *et al*, 2004). Factor VIII and V activity can be reconstituted from isolated heavy and light chains in the presence of Ca^{2+}, Cu^{2+} and Mn^{2+} metal ions (Fay, 1988). Occupancy of the high-affinity Ca^{2+} (and Mn^{2+})-binding site promotes the cofactor activity of factor VIIIa by modulating the heterodimer conformations on the anionic membrane surfaces (Fay, 1988; Wakabayashi *et al*, 2005), and is necessary for a stable association between the heavy and light chains of factor V(a) (Sorensen *et al*, 2004). On the other hand, Cu^{2+} enhances the inter-chain affinity by approximately 100-fold rather than contributing to the specific activity of factor VIII (Wakabayashi *et al*, 2001), whilst its functional role for factor V is not known yet. However, these findings indicate that these metal ions are essential in conserving the functional structure of factors VIII and V.

To further elucidate the metal cation(s)-dependent function of both factors VIII and V, we investigated whether cation-exchange resins affected these molecules. We demonstrated that the cation-exchange iminodiacetate resin inactivated factors VIII and V, not by direct adsorption to the resin, but probably by deprivation of metal ions, predominantly Ca^{2+}, from the molecules. Furthermore, the iminodiacetate resin-treated plasma prepared by this new strategy would be very useful as a substrate for measuring the activity of both factors VIII and V.

Materials and methods

Reagents

Purified recombinant factor VIII preparations (Kogenate FS®) and plasma-derived factor VIII/VWF concentrates (Confact F®) were generous gifts from Bayer Corp. Japan (Osaka, Japan) and Chemo-Sero-Therapeutic Research Inc. (Kumamoto, Japan) respectively. VWF was purified from the factor VIII/VWF concentrates using gel filtration on a Sepharose CL-4B column and immune beads coated with immobilized anti-factor VIII monoclonal antibody (Shima *et al*, 1992). Enzyme-linked immunosorbent assay (ELISA) for detection of factor VIII demonstrated >95% purity of VWF. The cation-exchange resins purchased commercially were as follows (Table I): (1) iminodiacetate group: Muromac A-1, Muromac B-1 (both from Muromachi Chemicals, Fukuoka, Japan) and Amberlite IRC748 (Organo Corp., Tokyo, Japan); (2) sulfonate group: Amberlite IR120B Na, Amberlite IR124 Na and Amberlite 200CT Na (Organo Corp.); and (3) carboxylate group: Amberlite IRC50 and Amberlite IRC76 (Organo Corp.) were stored according to the manufacturer's instructions. Coagtrol-N (Sysmex, Kobe, Japan) was used as normal pooled plasma. Factor VIII- or V-deficient plasma prepared by immunodepletion was purchased from Siemens Healthcare Diagnostics (Deerfield, IL, USA). The EVALU-VIII and SYSCOR Sets were purchased from George King Bio-Medical Inc. (Overland Park, KS, USA) and were used as test samples for evaluating the various activity levels of factors VIII and V.

Table I. Functional groups of cation-exchange resins.

Type of functional group	Name®
Sulfonate	Amberlite IR120B Na
	Amberlite IR124 Na
	Amberlite 200CT Na
Carboxylate	Amberlite IRC50
	Amberlite IRC76
Iminodiacetate	Amberlite IRC748
	Muromac A-1
	Muromac B-1

Preparation of resin-treated plasma

The cation-exchange resins were dialysed in 150 mM NaCl buffer with 0·1% ethylenediaminetetraacetic acid (EDTA) for 4 h at 4°C to remove free metal ions from the resins, followed by further dialysis in 150 mM NaCl buffer overnight prior to use. Plasma was mixed with 10% (w/v) resin in polystyrene tubes for 2 h at 22°C with stirring. After centrifuging at 2000 *g*, the supernatants were adjusted by adding 0·5 M HEPES buffer, pH 7·0 in a 50:1 volume ratio.

Measurement of activity and antigen levels of factor VIII and V

The activity levels of factors VIII and V were measured by activated partial thromboplastin time (APTT)-based and prothrombin time (PT)-based one-stage clotting assays respectively (Wolf, 1953; Hardisty & MacPherson, 1962). In some cases, factor VIII activity was also measured in a two-stage clotting assay using thromboplastin generation test (Biggs *et al*, 1955). VWF activity was measured by an assay for ristocetin cofactor of VWF (MacFarlane *et al*, 1975). The antigen levels of factor VIII and VWF were measured by a two-site sandwich ELISA using alloantibodies with C2 epitopes and rabbit anti-human VWF (Dako, Glostrup, Denmark), respectively, as described previously (Shima *et al*, 1995). The levels of factor V antigen were measured by an ELISA using a commercial kit (Matched-Pair Antibody Set for ELISA of human factor V antigen, Affinity Biologicals, Ontario, Canada).

Measurement of metal ions concentrations in factor VIII preparations

Samples were heated at 95°C for 2 min and centrifuged at 3500 *g* for 30 min. Metal ion (Ca^{2+}, Cu^{2+} and Mn^{2+})

