Determination of a factor VIII-interactive region within plasmin responsible for plasmin-catalysed activation and inactivation of factor VIII(a)*

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Summary

Plasmin, an active form of plasminogen, activates and inactivates factor VIII (FVIII) by limited proteolysis. We have previously identified lysine-binding site-independent plasmin-interactive sites on the FVIII A2 domain responsible for cleavages at Arg^{336} and Arg^{372}, together with lysine-binding site-dependent plasmin sites on the light chain responsible for cleavage at Lys^{36}. We have now characterised FVIII-interactive regions on plasmin. SDS-PAGE analysis demonstrated that a monoclonal antibody (mAb) against kringle (K)5-catalytic domain (K5-CD) of plasmin significantly blocked plasmin-catalysed cleavages at Arg^{336} and Arg^{372}. K5-CD fragment and this mAb blocked plasmin-catalysed activation and inactivation of FVIII(a). Anti-K1–2–3 and anti-K4 mAbs blocked plasmin-catalysed cleavages at Lys^{36}, and K1–2–3 and K4 fragments inhibited plasmin-catalysed inactivation of $A1^{1-336}$FVIIIa. The K5-CD preferentially bound to the A2 domain (K_d^{app}; 52 nM), whilst the K1–2–3 and K4 bound to the light chain (K_d^{app}; 75 and 106 nM, respectively) in ELISA. Binding was attributed to the A2 484–509 region and A3 1690–1705/1804–1818 region, respectively. 6-aminohexanoic acid, a lysine analogue, significantly inhibited the light chain/K1–2–3 (and K4) binding by ~90%, whilst A2/K5-CD binding was moderated by only ~35%. Furthermore, an anti-CD antibody blocked plasmin-catalysed cleavage by inhibiting the A2/K5-CD interaction. These data demonstrate that the K5-CD of plasmin (and plasminogen) interacts with the A2 domain independent of lysine-binding site, whilst interactions of K1–2–3 and K4 with the light chain are lysine-binding site-dependent. Interactions between the K5-CD and A2 likely constitute the major regulatory mechanism for activation and inactivation of FVIII(a) mediated by cleavage at Arg^{372} and Arg^{336}.

Keywords

Activation/inactivation, binding-site, factor VIII(a), kringle domain, plasmin(ogen)

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Introduction

Normal haemostasis is maintained by a controlled balance between coagulation and fibrinolysis. Blood coagulation is mediated by the generation of thrombin, which induces clot formation by the conversion of soluble fibrinogen to insoluble fibrin. Subsequently, fibrinolysis is governed by the generation of plasmin, which proteolyses insoluble fibrin to produce soluble degradation products. An imbalance of these systems, and the consequent excessive activation or inactivation of coagulation and fibrinolysis, leads to pathological thrombosis and/or haemorrhage, as evidenced, for example, in clinical disseminated intravascular coagulation (1, 2). Thus, components of the coagulation and fibrinolytic processes not only regulate their respective pathways, but also appear to be closely inter-dependent.

The most potent fibrinolytic enzyme, plasmin, is generated by activation of the zymogen, plasminogen, by tissue-type and/or urinary-type plasminogen activators. The enzyme is composed of a heavy chain consisting of five kringle domains and a light chain containing the catalytic domain (CD) (3). The kringle (K) domains contain homologous lysine-binding sites that play an important role in binding to numerous ligands involving fibrin (3). Lysine-binding sites are essentially constructed of three parts; a hydrophobic core, a cationic centre, and an anionic centre. The CD contains the catalytic triad (His^{603}, Asp^{646}, and Ser^{741}) and the streptokinase-binding site (4). It is particularly noteworthy that plasmin has broad specificity and is capable of activating and/or degrading coagulation factors (F)VIII(a), V(a), IXa, and X (5–8).

FVIII is a plasma protein deficient or defective in individuals with the severe congenital bleeding disorder, haemophilia A. It

functions as a cofactor in the tenase complex responsible for phospholipid surface-dependent conversion of FX to FXa by FIXa (9). It is synthesised as a multi-domain, single chain molecule (A1-A2-B-A3-C1-C2) consisting of 2,332 amino acid residues. 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 proteolysed B domain, linked to a light chain consisting of the A3, C1, and C2 domains (10). FVIII circulates as a complex with von Willebrand factor and is converted into its active form, FVIIIa, by limited proteolysis, principally catalysed by either thrombin or FXa (11). 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 FVIIIa cofactor activity (12). Serine proteases including activated protein C (APC), FXa, FIXa, and plasmin inactivate FVIII(a) by cleavage at Arg^{336} within the A1 domain (5, 11, 13). In addition, FXa and plasmin inactivate FVIIIa by cleavage at Lys^{36} in A1 (5, 14).

We have recently identified plasmin-interactive sites on FVIII using anhydro-plasmin (Ah-plasmin), a catalytically inactive derivative of plasmin (15, 16). Plasmin interacts with the A2 domain of FVIII with high affinity (K_d; ~20 nM), in mechanisms largely independent of lysine-binding site. The A2 domain, especially Arg^{484}, contributes to a unique plasmin-interactive site that promotes plasmin-docking during cofactor activation and inactivation by cleavages at Arg^{372} (and Arg^{740}) and Arg^{336} (15). Furthermore, plasmin interacts with the light chain of FVIII with moderate affinity (K_d; ~70 nM), mainly through lysine-binding site-dependent mechanisms. An extended surface, centred on Lys residues and involving the 1690–1705 and 1804–1818 regions in the A3 domain, contributes to a similarly unique plasmin-interactive site that promotes plasmin-docking during cofactor inactivation by cleavage at Lys^{36} (16).

To further clarify the FVIII-plasmin binding mechanism, we attempted to identify FVIII(a)-interactive domains on plasmin using a combination of functional and binding experiments employing isolated FVIII and plasmin subunits and antibodies. Our results indicate that the K5-CD of plasmin interacts with the A2 domain of FVIII through lysine-binding site-independent mechanism, whilst the K1–2–3 and K4 interact with the light chain through lysine-binding site-dependent mechanism. In addition, interactions between the K5-CD and A2 appear to regulate the major activation and inactivation mechanisms of FVIII(a) by cleavage at Arg^{372} and Arg^{336} in the heavy chain of FVIII.

Materials and methods

Reagents

Recombinant FVIII (Kogenate FS®) were generous gifts from Bayer Corp. (Osaka, Japan). FVIIIa, the light chain (^{1649}A3C1C2 and ^{1690}A3C1C2) and heavy chain, A2, A1, and $A1^{1-336}$ subunits were isolated and purified as previously reported (5). Human purified Lys-plasmin and Lys-plasminogen (devoid of other serine proteases), 6-aminohexanoic acid (6-AHA) were purchased from Sigma (St Louis, MO, USA). Plasminogen fragments; K1–2–3 domain, K4 domain, and K5-catalytic domain (K5-CD), were prepared by elastase treatment of plasminogen, and isolated by chromatography on Lys-Sepharose column and size exclusion gel filtration (17). SDS-PAGE of the purified proteins followed by staining with GelCode Blue Reagent (Pierce, Rockford, IL, USA) showed >95% purity. Ah-plasmin, a catalytically inactive derivative of plasmin in which the active-site serine is replaced by dehydroalanine, demonstrated <1% of plasmin activity using a chromogenic assay (15). The K5-CD also demonstrated little plasmin activity, showing little contamination of active K5-CD (data not shown). Protein concentrations were determined by the method of Bradford. Pefabloc (Roche, Basel, Switzerland) and horseradish peroxidase-labeled streptavidin (Chemicon, Boronia, VIC, Australia) were purchased from the indicated vendors. Phospholipid vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine (Sigma) were prepared using *N*-octylglucoside (18). Synthetic peptides corresponding to the A2 residues 484–509, and A3 residues 1690–1705 and 1804–1818 of FVIII were prepared by BioSynthesis (Lewisville, TX, USA).

Antibodies

An anti-FVIII A1 monoclonal antibody (mAb) 58.12, recognizing the N-terminus of the A1 domain, was a generous gift from Bayer Corp. (14). An anti-A2 mAb, JR8, was obtained from JR Scientific Inc. (Woodland, CA, USA). An anti-C2 mAb (NMC-VIII/5), recognising both N- and C-termini of the C2 domain, was prepared as previously described (19). Anti-human plasmin(ogen) K1–2–3, K4, and K5-CD mAbs were purchased from American Diagnostica Inc. (Greenwich, CT, USA) (20). These anti-plasmin mAbs belonged to IgG_1 subclass and directly bound the K1–2–3, K4, and K5-CD, respectively. A goat polyclonal antibody IgG against CD, C-14Ab, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). This recognised the C-terminus of plasmin(ogen). In control experiments using a specific substrate for plasmin (S-2251) none of the anti-plasmin mAbs or the polyclonal anti-CD Ab significantly blocked plasmin activity directly (data not shown). The mAb IgGs were biotinylated using *N*-hydroxysuccinimido-biotin (Pierce). $F(ab')_2$ fragments were prepared using immobilised pepsin-Sepharose (Pierce).

Reconstitution of FVIIIa

The A1/A3C1C2 dimer was reconstituted by mixing 500 nM of $A1^{1-336}$ with an equimolar concentration of ^{1690}A3C1C2 overnight at 4°C in 20 mM HEPES buffer, pH 7.2, 0.3 M NaCl, 25 mM $CaCl_2$,

containing 0.01% Tween 20 (14). The reconstituted dimer (200 nM) was incubated with excess (300 nM) A2 subunit for >15 min at 22°C, to minimise A2 dissociation from FVIIIa (16). Incubation for 15 minutes (min) generated maximum FVIIIa activity.

Cleavage of FVIII by plasmin

Plasmin was added to FVIII as indicated below (mol/mol) in the presence of phospholipid vesicles (10 μM) in buffer containing 20 mM HEPES, pH 7.2, 0.1 M NaCl, 5 mM $CaCl_2$, and 0.01 % Tween 20 (HBS buffer) at 22°C. Samples were obtained at the indicated times and the reactions were immediately terminated and prepared for SDS-PAGE by adding SDS and boiling for 3 min. SDS-PAGE was performed using 8% gels at 150 V for 1 hour (h). For Western blotting, the proteins were transferred to a polyvinylidene difluoride membrane at 50 V for 2 h. Proteins were probed using the indicated anti-FVIII mAbs, followed by anti-mouse peroxidase-conjugated antibody. The signals were detected using the enhanced chemiluminescence system (PerkinElmer Life Science, Boston, MA, USA). Densitometry scans were quantitated using Image J 1.34 (National Institutes of Health, Bethesda, MD, USA).

Clotting assay

Plasmin-catalysed activation and inactivation of FVIII(a) experiments were performed as previously described (5). FVIII(a) activity was measured in a one-stage clotting assay. Aliquots were removed from the mixtures at the indicated times, and plasmin activity immediately quenched by adding 0.5 mM pefabloc and 1,000-fold dilution prior to assay. Control experiments demonstrated that the presence of plasmin and pefabloc in the diluted samples did not affect the FVIII assay.

FXa generation assay

The rate of conversion of FX to FXa was monitored in a purified system (21). Plasmin-catalysed inactivation of FVIIIa was performed in HBS buffer containing 0.1% bovine serum albumin (BSA) and phospholipid vesicles (10 μM). Samples were removed from the mixtures at the indicated times, and plasmin reaction was immediately quenched by pefabloc (0.2 mM) and dilution. All reactions were performed at 37°C. FXa generation was initiated by the addition of FIXa (20 nM) and FX (400 nM) in the presence of phospholipid (10 μM). The reaction was quenched by the addition of EDTA (100 mM). Rates of FXa generation were determined at 405 nm using a microtitre plate reader after the addition of chromogenic substrate, S-2222 (0.46 mM final concentration). The presence of plasmin and pefabloc in the diluted samples did not affect this assay (data not shown). FVIIIa activity was determined as the amount (in nmol) of FXa generated per min and converted into the amount (in nmol) of FVIIIa.

ELISA using immobilised plasminogen fragments

Assays were performed using a minor modification of a previously reported method (15, 16). In detail, plasminogen fragments (100 nM) were immobilised on microtiter wells in 20 mM Tris, and 0.15 M NaCl, pH 7.4, overnight at 4°C. After blocking with 5% BSA, the intact FVIII, A2 domain or ^{1649}A3C1C2 subunit was added and incubated for 2 h at 37°C. The appropriate biotinylated anti-FVIII mAb IgG was added and bound IgG was quantified using horseradish peroxidase-labelled streptavidin. Absorbances were measured at 492 nm in a microplate reader. The amount of non-specific binding of biotinylated IgG in the absence of FVIII was <5% of the total signal. Specific binding was recorded after subtracting the non-specific binding.

Data analyses

All experiments were performed at least three separate times, and average values are shown. The rate constant (k) for FVIIIa inactivation by plasmin was determined using Equation 1.

$$[\text{FVIIIa}]_t = [\text{FVIIIa}]_0 \cdot e^{-kt} \quad \text{(Eq. 1)}$$

where $[\text{FVIIIa}]_t$ is the concentration of FVIIIa at time point (t).

Analyses of interactions between FVIII subunits and plasminogen fragments in ELISA were performed by a single-site binding model using Equation 2,

$$\text{Absorbance} = \frac{A_{max} \cdot [S]}{K_d + [S]} \quad \text{(Eq. 2)}$$

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

Data from studies assessing 6-AHA and FVIII synthetic peptide-dependent inhibition of plasmin interaction with FVIII subunits were analysed by non-linear least squares regression using Equation 3.

$$\%\ \text{binding} = \frac{B_{max} \cdot [\text{FVIII subunit}]}{K_d \cdot \left[1 + \frac{[L]}{K_i}\right] + [\text{FVIII subunit}]} + C \quad \text{(Eq. 3)}$$

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 FVIII subunit and plasminogen fragment; K_i is the (apparent) inhibition constant for L; and C is a constant for the binding of FVIII subunit and plasminogen fragment that was unaffected by L.

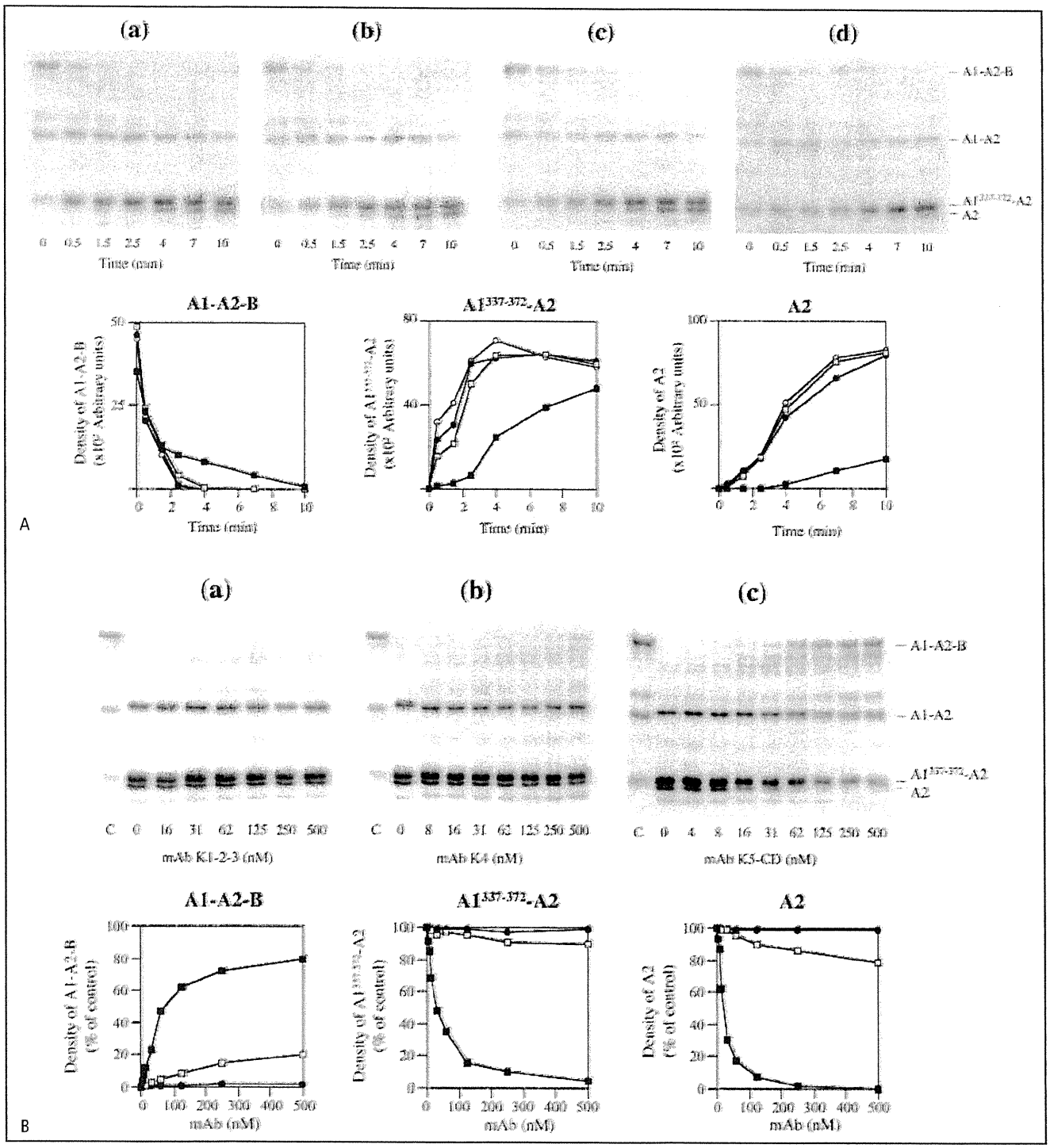

Figure 1: Plasmin-catalysed cleavage of FVIII heavy chain in the presence of anti-plasmin mAbs. A) Upper panels: Plasmin (3 nM) was preincubated with 60 nM control or anti-plasmin mAb F(ab')$_2$ (control IgG (a), anti-K1–2–3 (b), anti-K4 (c), anti-K5-CD (d)) for 1 h, followed by incubation with FVIII (100 nM) and phospholipid (10 μM) for the indicated times. B) Upper panels: Plasmin (3 nM) was preincubated with various concentrations of mAb F(ab')$_2$ [anti-K1–2–3 (a), anti-K4 (b), anti-K5-CD (c)] for 1 h, followed by incubation with FVIII (100 nM) and phospholipid (10 μM) for 3 min. Samples were analysed on 8% gels SDS-PAGE followed by Western blotting using biotinylated anti-A2 mAbJR8. Lower panels in A and B show quantitative densitometry of the A1-A2-B, A1$^{337-372}$-A2, A2 bands from blotting data obtained from A and B, respectively. The symbols used are as follows: ○, control IgG; ●, anti-K1–2–3; □, anti-K4; ■, anti-K5-CD mAb.

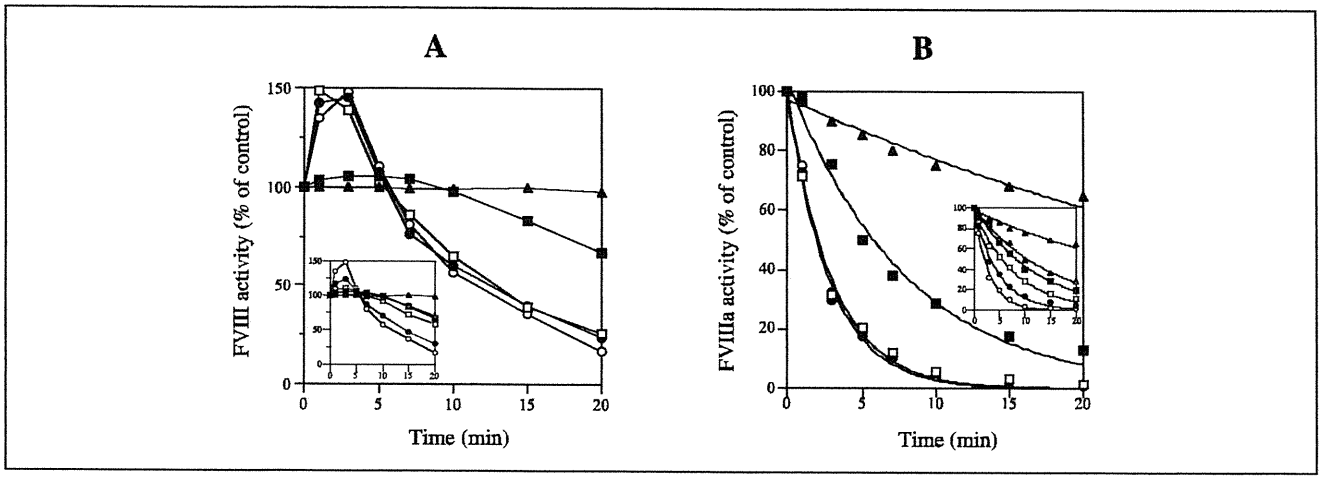

Figure 2: Plasmin-catalysed activation or inactivation of FVIII(a) in the presence of anti-plasmin mAbs. Plasmin (3 nM) was preincubated with 60 nM control or anti-plasmin mAb F(ab')$_2$ (○, control IgG; ●, anti-K1–2–3; □, anti-K4; ■, anti-K5-CD) for 1 h, followed by incubation with FVIII (100 nM, A) or FVIIIa (40 nM, B) in the presence of phospholipid (10 μM). As a control, buffer was used instead of plasmin (▲). FVIII(a) activities were measured at the indicated times in a one-stage clotting assay. At t=0 FVIII and FVIIIa activities were ~50 and ~120 units/ml, respectively (100% level). Insets in A and B show effects of various concentrations of anti-K5-CD (○, 0 nM; ●, 25 nM; □, 50 nM; ■, 100 nM; △, 200 nM; ▲, no plasmin). Data obtained from B was fitted to an equation of single exponential decay. Rate of FVIIIa decay in the absence of plasmin was 0.06 ± 0.01 min^{-1}.

Results

Effects of anti-plasmin mAbs on plasmin-catalysed cleavage of the FVIII heavy chain

We have recently identified plasmin-interactive sites responsible for cleavages at Lys^{36}, Arg^{336}, and Arg^{372} in the heavy chain of FVIII (15, 16). The present studies were designed to further identify plasmin domains containing FVIII(a)-interactive regions responsible for plasmin-catalysed cleavage of the heavy chain. Initially, the effects of anti-plasmin mAbs on these reactions were examined. Three anti-plasmin mAbs were used, against K1–2–3, K4, and K5-CD fragments of plasmin, respectively. Plasmin (3 nM) was preincubated with the anti-plasmin mAbs (60 nM) for 1 h and then added to FVIII (100 nM). ▶Figure 1A (upper panels) shows the results of the cleavage reaction from Western blotting using biotinylated anti-A2 mAbJR8. In addition, A1-A2-B, A1$^{337–372}$-A2, and A2 bands were quantified using scanning densitometry (lower panels). In the presence of control IgG (panel a), intact heavy chain (A1-A2-B) was initially converted into A1-A2 subunits by cleavage at Arg^{740}, followed by the generation of A1$^{337–372}$-A2 and A2 bands by cleavages at Arg^{336} and Arg^{372}, respectively. These data were similar to those previously reported (5). The presence of anti-K5-CD mAb (panel d) delayed both the disappearance of A1-A2-B band and the appearance of A1$^{337–372}$-A2 and A2, demonstrating inhibition of cleavage at Arg^{740}, Arg^{336} and Arg^{372}, respectively. Cleavage at Arg^{336} and Arg^{372} appeared to be particularly inhibited. In the presence of anti-K1–2–3 (panel b) and anti-K4 mAb (panel c), however, cleavage by plasmin was not significantly different from that in the control experiments.

We also evaluated the effects of various concentrations of anti-plasmin mAbs on plasmin-catalysed cleavage (Fig. 1B). An anti-K5-CD mAb dose-dependently blocked cleavage at Arg^{740}, Arg^{336}, and Arg^{372} by ~80%, ~95%, and >95%, respectively, at the maximum concentration employed (500 nM, panel c). The IC$_{50}$ values were ~75, ~25, and ~20 nM, respectively, indicating predominant inhibition of cleavage at Arg^{336} and Arg^{372}. In contrast, ant-K1–2–3 (panel a) and anti-K4 (panel b) mAbs demonstrated little (<5%) and slight (<20%) inhibition of plasmin-catalysed

Table 1: Kinetic parameters characterising plasmin-catalysed FVIIIa inactivation in the presence of anti-plasmin mAb or plasminogen fragment. A) Anti-plasmin mAb (60 nM); B) Anti-K5-CD mAb; C) K5-CD fragment. FVIIIa (40 nM) was incubated with plasmin (3 nM) and phospholipid (10 μM) in the presence of competitors. Rate constants for inactivation were calculated using a single exponential decay from the data shown in Figures 2B and 3C. Data points represent mean values of at least three separate experiments. Rate of FVIIIa decay in the absence of plasmin was 0.06 ± 0.01 min^{-1}.

A				
Control	K1–2–3	K4	K5-CD	
min^{-1}				
0.48 ± 0.04	0.47 ± 0.05	0.47 ± 0.07	0.23 ± 0.03	
B				
0 nM	25 nM	50 nM	100 nM	200 nM
min^{-1}				
0.48 ± 0.04	0.38 ± 0.03	0.27 ± 0.01	0.21 ± 0.02	0.17 ± 0.01
C				
0 nM	7.5 nM	15 nM	30 nM	60 nM
min^{-1}				
0.48 ± 0.04	0.41 ± 0.02	0.31 ± 0.02	0.23 ± 0.02	0.18 ± 0.02

cleavage, respectively. These results supported that the K5-CD of plasmin might contain the region necessary for the docking of FVIII and the subsequent plasmin-catalysed cleavage at Arg^{740}, Arg^{336}, and Arg^{372} in the heavy chain (Arg^{336}/Arg^{372}>Arg^{740}), although steric hindrance could not be excluded. It is noteworthy that the inhibitory pattern of the anti-K5-CD mAb was similar to that of anti-FVIII A2 mAb413 (15). These anti-plasmin mAbs did not significantly affect plasmin-catalysed cleavage of the light chain of FVIII (data not shown).

Effects of anti-plasmin mAbs on plasmin-catalysed activation and inactivation of FVIII(a)

Proteolytic cleavage at the A1-A2 and A2-B junctions (Arg^{372} and Arg^{740}) and within the A1 domain (Arg^{336}) mediates the up- and down-regulation of FVIII(a) activity, respectively (11). We examined, therefore, the effects of anti-plasmin mAbs on plasmin-catalysed activation and subsequent inactivation of FVIII(a). FVIII(a) activity was measured using a one-stage clotting assay. The presence of mAbs, plasmin, and the plasmin-inhibitor, pefabloc, did not affect this assay at the dilutions utilised (data not shown). Control experiments showed that maximum FVIIIa activity was observed within 3 min after adding plasmin (3 nM), followed by a sharp decline to ~20% of the initial activity at 20 min (▶Fig. 2A). The addition of anti-K5-CD mAb (60 nM) inhibited plasmin-catalysed activation and subsequent inactivation of FVIII. This mAb (at 100 nM) completely blocked (by ~90%) the activation phase and partially inhibited (by ~65%) the inactivation phase (inset). The inhibitory effect was dose-dependent. The addition of either anti-K1–2–3 or anti-K4 mAb had little effect on these reactions.

FVIIIa activity at any time point represents contributions from non-activated molecules, activated molecules, and activated molecules that have decayed following FVIIIa subunit dissociation. Therefore, to precisely evaluate the effects of anti-plasmin mAbs on plasmin-catalysed inactivation, the experiments were repeated using FVIIIa as a substrate. In these instances, the addition of anti-K5-CD mAb (60 nM) similarly inhibited FVIIIa inactivation by plasmin by ~60% compared to control (Fig. 2B, ▶Table 1A). This inhibitory effect was dose-dependent (inset). The highest concentration used (200 nM) mediated an ~75% decrease in inactivation rates, as illustrated by the rate constants of plasmin-catalysed inactivation of FVIIIa (Table 1B). Anti-K1–2–3 and anti-K4 mAb had little effect on inactivation. The data were in keeping with the functional (cleavage) findings described above (in Fig. 1).

To exclude the possibility of steric hindrance by the mAb binding, we repeated similar experiments using three plasminogen fragments (isolated K1–2–3, K4, and K5-CD, derived from elastase-proteolysed plasminogen) in place of the anti-plasmin mAbs. The addition of K5-CD (400 nM) to FVIII (100 nM) competitively inhibited plasmin-catalysed activation and subsequent inactivation of FVIII (▶Fig. 3A). In particular, the inhibitory effect for activation was dose-dependent, and the IC_{50} value was ~10 nM (Fig. 3B). The addition of either K1–2–3 or K4 fragments (400 nM) had little inhibitory effect. Furthermore, the addition of K5-CD inhibited plasmin-catalysed FVIIIa inactivation in a dose-dependent manner, as well as FVIII activation (Fig. 3C). Rate constants of plasmin-catalysed inactivation of FVIIIa are summarised in Table 1C. The addition of K5-CD (60 nM) demonstrated ~75% decreases in inactivation rates. Taken together, these results strongly suggested that the K5-CD of plasmin significantly regulated plasmin-catalysed activation and inactivation of FVIII(a).

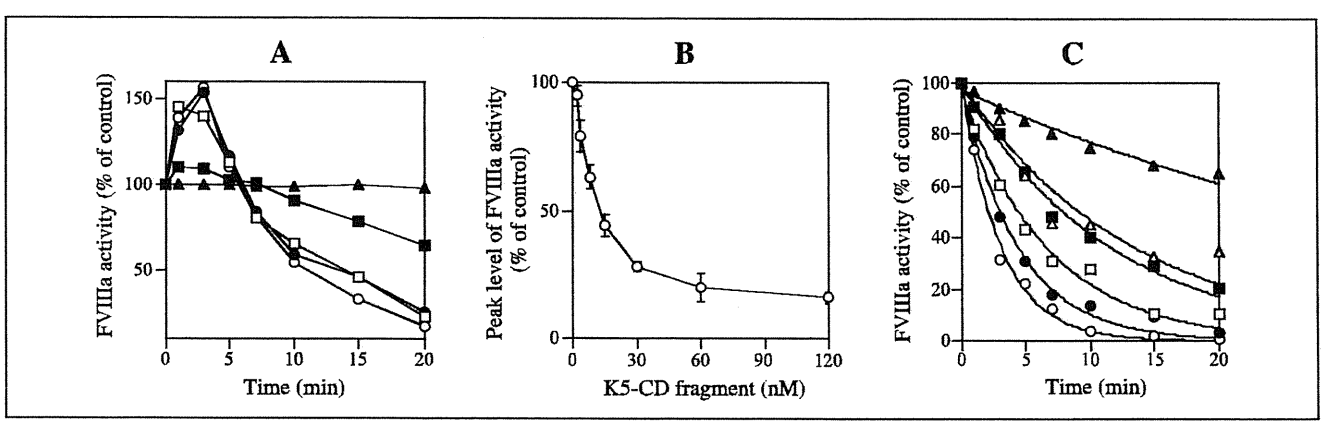

Figure 3: Inhibitory effects of plasminogen fragments on plasmin-catalysed activation or inactivation of FVIII(a). A) FVIII (100 nM) was preincubated with 400 nM plasminogen fragment (○, no fragment; ●, K1–2–3; □ K4; ■, K5-CD) and phospholipid (10 μM) for 15 min, followed by reaction with plasmin (3 nM). As a control, buffer was used instead of plasmin (▲). FVIII activity was measured at the indicated times in a one-stage clotting assay. At *t*=0 FVIII activity was ~40 unit/ml (100% level). B) Peak values of FVIII activity in the presence of various concentrations of K5-CD in A. Peak FVIII activity in the absence of K5-CD was regarded as 100%. C) FVIIIa (40 nM) was preincubated with K5-CD (○, 0 nM; ●, 7.5 nM; □, 15 nM; ■, 30 nM; △, 60 nM; ▲, no plasmin) and phospholipid (10 μM) for 15 min, followed by reaction with plasmin (3 nM). FVIIIa activity was measured at the indicated times in a one-stage clotting assay. At *t*=0 FVIIIa activity was ~120 unit/ml (100% level). Data obtained from C was fitted to an equation of single exponential decay.

Effects of anti-plasmin mAbs on plasmin-catalysed cleavage at Lys^{36} in A1

An extended surface, centred on Lys residues and involving the 1690–1705 and 1804–1818 regions in the A3 domain, is known to contribute to the plasmin-interactive site that promotes plasmin-docking during cofactor inactivation by cleavage at Lys^{36} in A1. To characterise the FVIII-interactive region responsible for Lys^{36} cleavage, the effects of anti-plasmin mAbs on these reactions were examined. As a substrate, A1$^{1-336}$/^{1690}A3C1C2 dimers (200 nM) were reconstituted with the excess A2 (300 nM) as described in *Methods* (16). Plasmin (3 nM) was mixed with anti-plasmin mAbs

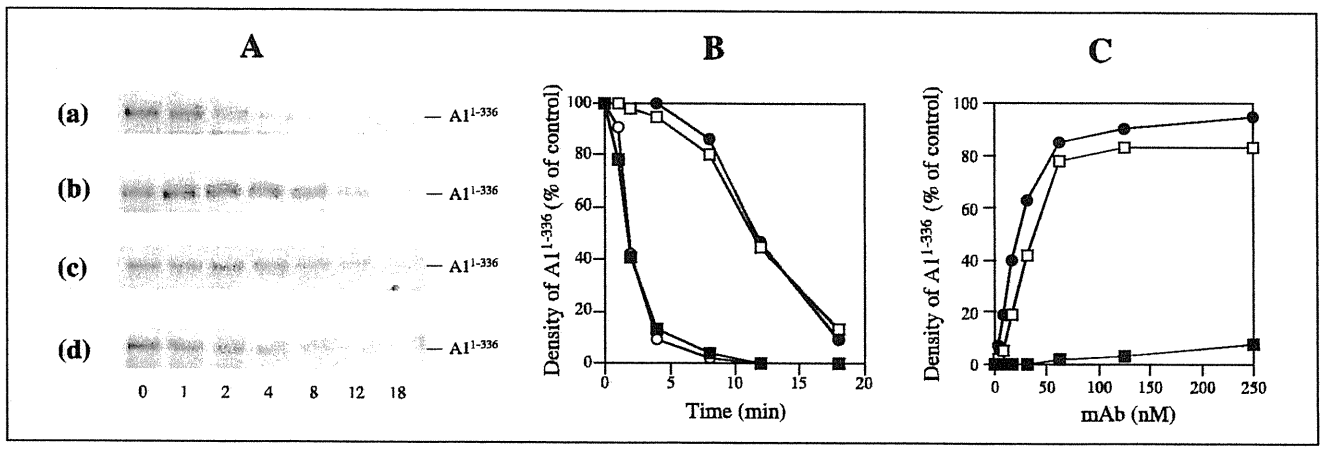

Figure 4: Effects of anti-plasmin mAbs on plasmin-catalysed cleavage at Lys36 in A11–336FVIIIa. A) Plasmin (3 nM) was preincubated with 60 nM control or anti-plasmin mAb F(ab')$_2$ [control IgG (a), anti-K1–2–3 (b), anti-K4 (c), anti-K5-CD (d)] for 1 h. Reconstituted A1$^{1-336}$/^{1690}A3C1C2 (200 nM) incubated with the A2 subunit (300 nM) and phospholipid (10 μM). And then the A1$^{1-336}$FVIIIa was incubated with plasmin and anti-plasmin mAb mixtures for the indicated times. Samples were analysed on 8% gels SDS-PAGE followed by Western blotting using biotinylated anti-A1 mAb58.12 IgG. B) Quantitative densitometry of A1$^{1-336}$ from blotting data obtained from A. Band density of A1$^{1-336}$ at t=0 was regarded as 100%. The symbols used are as follows: ○, control IgG; ●, anti-K1–2–3; □, anti-K4; ■, anti-K5-CD. C) Various concentrations of anti-plasmin mAb F(ab')$_2$ (●, anti-K1–2–3; □, anti-K4; ■, anti-K5-CD) were preincubated with plasmin (3 nM) for 1 h, followed by incubation with A1$^{1-336}$FVIIIa and phospholipid (10 μM) for 8 min, and samples were analysed by Western blotting. Density of A1$^{1-336}$ prior to and post addition of plasmin in the absence of anti-plasmin mAb were regarded as 100% and 0%, respectively.

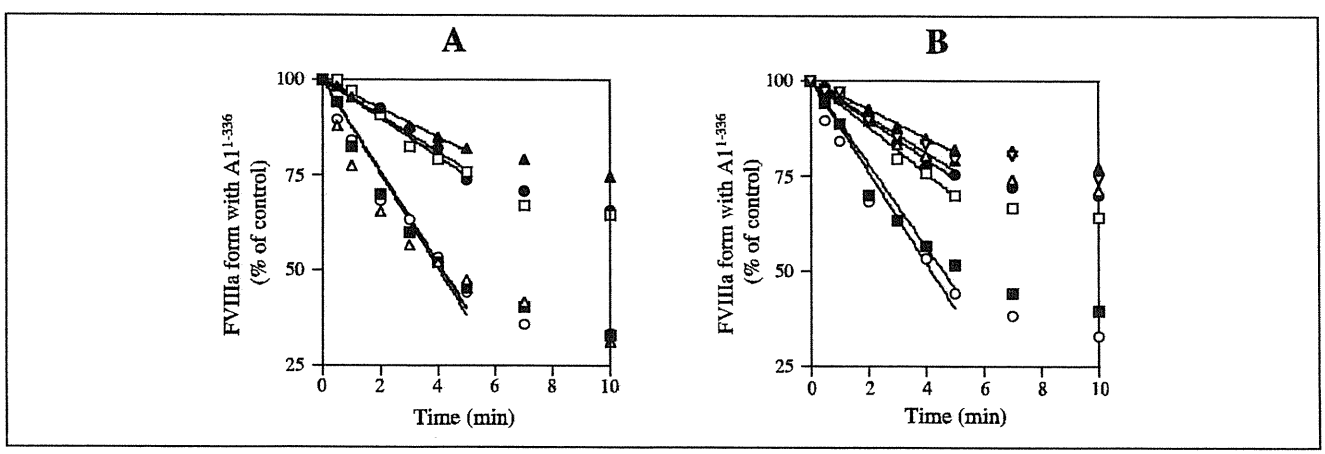

Figure 5: Effects of anti-plasmin mAb or plasminogen fragments on plasmin-catalysed inactivation of A11–336FVIIIa. A) Effects of anti-plasmin mAbs: Reconstituted A1$^{1-336}$/^{1690}A3C1C2 (200 nM) was incubated with A2 subunit (300 nM) and phospholipid (10 μM). Plasmin (3 nM) was preincubated with 60 nM control, anti-plasmin mAb F(ab')$_2$ or 150 nM C-14Ab F(ab')$_2$ (○, control IgG; ●, anti-K1–2–3; □, anti-K4; ■, anti-K5-CD; Δ, C-14Ab; ▲, no plasmin) for 1 h. FVIIIa inactivation was then monitored over time in the presence of plasmin and anti-plasmin mAb using a FXa generation assay as described in *Methods*. B) Effects of plasminogen fragments: A1$^{1-336}$FVIIIa (prepared in A) was mixed with 400 nM plasminogen fragments (○, no fragment; ●, K1–2–3; □, K4; ■, K5-CD; Δ, mixture with K1–2–3 and K4) and PL (10 μM) for 15 min, followed by incubation with plasmin (3 nM). As a control, buffer was used instead of plasmin (◊). In addition, similar experiments were performed using a competitor 6-AHA (100 μM, ▲). FVIIIa inactivation was then monitored over time in the presence of plasmin using a FXa generation assay. Solid lines were drawn from linear regression fitting to evaluate the rate of plasmin-catalysed inactivation of A1$^{1-336}$FVIIIa (16).

Table 2: Rates of plasmin-catalysed inactivation of A11–336FVIIIa in the presence of anti-plasmin mAb (60 nM) (A) or plasminogen fragment (400 nM) (B). Inactivation of $A1^{1-336}$FVIIIa was estimated by the rate obtained using straight line fitting of the first several points (within 5 min) of the data shown in Figure 5. Data points represent mean values of at least three separate experiments. Inactivation rate of FVIIIa decay in the absence of plasmin was 3.0 ± 0.4.

A				
Control	K1–2–3	K4	K5-CD	Anti-CD Ab (C14: 150 nM)
Inactivation rates				
10.8 ± 1.0	4.3 ± 0.6	4.5 ± 0.6	10.6 ± 1.4	9.8 ± 1.0

B					
Control	K1–2–3	K4	K5-CD	Mixture with K1–2–3/K4	6-AHA (100 μM)
Inactivation rates					
10.8 ± 1.0	4.5 ± 0.7	5.0 ± 0.8	10.1 ± 0.9	3.9 ± 0.4	3.4 ± 0.2

(60 nM) prior to incubation with $A1^{1-336}$FVIIIa. Reactions were analysed by Western blotting using anti-A1 mAb58.12 for detection (▶Fig. 4A). This antibody recognises the N-terminal region of A1, and failure to detect $A1^{1-336}$ fragments indicated complete cleavage at Lys^{36} and conversion to $A1^{37-336}$ (14). In addition, the $A1^{1-336}$ product at each time point was assessed by scanning densitometry (Fig. 4B). In control experiments using normal IgG, the $A1^{1-336}$ band disappeared in a time-dependent manner within 8 min (panel a). In the presence of anti-K1–2–3 (panel b) and anti-K4 (panel c) mAbs, $A1^{1-336}$ disappeared very slowly compared to control, indicating that plasmin-catalysed cleavage at Lys^{36} was inhibited. These two antibodies demonstrated inhibitory effects on Lys^{36} cleavage in similar dose-dependent manners (Fig. 4C). In contrast, in the presence of anti-K5-CD mAb (panel d) a similar cleavage pattern to that of control was recorded, even at the highest concentration of mAb (250 nM). These findings suggested that K1–2–3 and K4 contained FVIIIa-interactive site(s) contributing to plasmin-catalysed cleavage at Lys^{36} in the A1 domain.

The effects of plasminogen fragments on plasmin-catalysed inactivation of $A1^{1-336}$FVIIIa

To confirm that the K1–2–3 and K4 domains of plasmin were responsible for plasmin-catalysed inactivation through Lys^{36} cleavage in A1, the inhibitory effects of anti-plasmin mAbs and plasminogen fragments on inactivation of $A1^{1-336}$FVIIIa were further examined. One-stage clotting assays using FVIII-deficient plasma can not be used for measuring $A1^{1-336}$FVIIIa (14), and alternatively, therefore, FXa generation was estimated as previously described (16). Results are illustrated in ▶Figure 5 and ▶Table 2. In control experiments, the presence of anti-plasmin mAbs and plasminogen fragments were shown not to affect this assay (data not shown). Preincubation of plasmin with anti-K1–2–3 or anti-K4 mAb (60 nM) inhibited plasmin-catalysed inactivation of $A1^{1-336}$FVIIIa by 84% and 81%, respectively, on inactivation rate, compared to normal IgG (Fig. 5A, Table 2A). In contrast, anti-K5-CD had little effect on the inactivation rate (<5%). Similarly, isolated K1–2–3 and K4 fragments (400 nM) competitively inhibited the inactivation rate of $A1^{1-336}$FVIIIa, by 81% and 75%, respectively, whilst K5-CD fragments did not significantly (<10%) affect this reaction (Fig. 5B, Table 2B). The equimolar mixture of K1–2–3 and K4 fragments inhibited this reaction more predominantly compared to individual fragment, supportive of additive effect. In addition, the inhibitory effect of this mixture on plasmin-catalysed inactivation of $A1^{1-336}$FVIIIa was similar to that of lysine analogue (6-AHA), again suggestive of the contribution of lysine-binding site(s) on Lys^{36} cleavage by plasmin (16). Taken together, these findings indicated that the K1–2–3 and K4 of plasmin (plas-

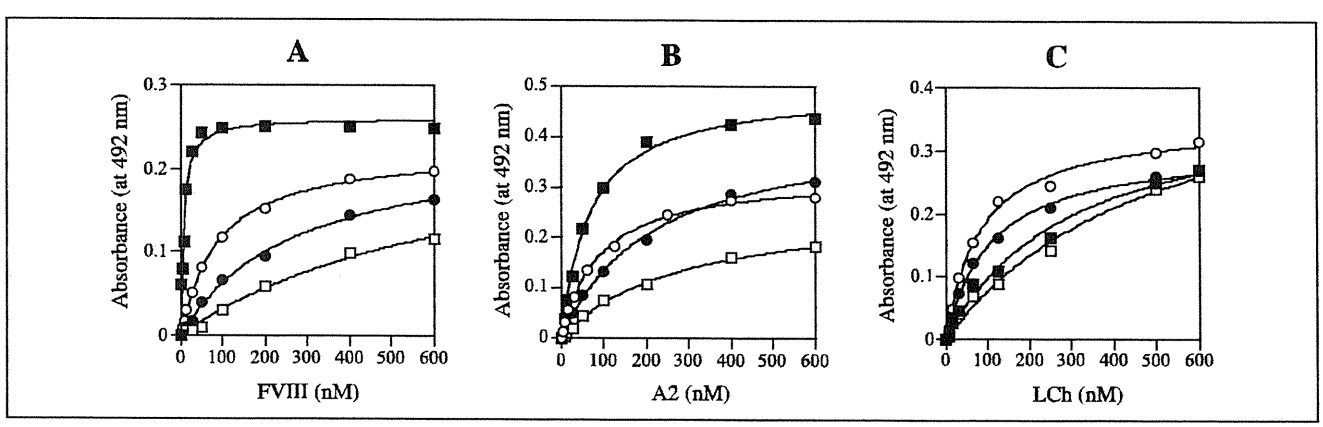

Figure 6: ELISA for direct binding of FVIII to plasminogen fragments. Various concentrations of intact FVIII (A), A2 (B) and ^{1649}A3C1C2 (C) subunits were reacted with 100 nM plasminogen (○), K1–2–3 (●), K4 (□), and K5-CD (■) that had been immobilised onto microtitre wells. Bound FVIII, A2 and ^{1649}A3C1C2 were detected using biotinylated anti-A2 (JR8) or anti-C2 (NMC-VIII/5) IgG. Absorbance values were plotted as a function of FVIII, A2 and ^{1649}A3C1C2 concentration, and data were fitted using a one-site binding model (Equation 2).

minogen) contributed to plasmin-catalysed inactivation of FVIIIa by Lys^{36} cleavage.

Direct binding of FVIII(a) subunits to plasminogen (and plasmin) fragments

As discussed above, the plasmin(ogen) domains, K5-CD and K1–2–3/K4, contain FVIII(a)-interactive region(s) contributing to plasmin-catalysed cleavages of FVIII at Arg^{372}, Arg^{336} and Lys^{36}, respectively. To further characterise the FVIII-plasmin binding model, we examined those regions (domains) of plasmin that could directly bind to FVIII. These interactions were studied using a solid-phase binding assay (ELISA), in which plasminogen fragments were immobilised onto microtiter wells (15). In these experiments, various concentrations of FVIII, A2, and light chain subunits (▶Fig. 6 panels A-C, respectively) were incubated with immobilised plasminogen fragments. Bound FVIII subunits were detected using biotinylated anti-A2 or anti-C2 mAb, which has been shown not to affect the FVIII-plasmin interaction (15). These results are shown in Figure 6 and are summarised in ▶Table 3. Binding reactions yielded saturable binding curves, and were well-fitted using a single-binding site model. This method was not based on true equilibrium-binding assays, however, and the K_d values obtained represented an apparent K_d for the interactions. The K5-CD bound to FVIII with much higher affinity (K_d; 8.7 nM) than K1–2–3 and K4. This result was consistent with the affinity for Ah-plasmin (K_d; 6.7 nM), supporting that the K5-CD had a significant contribution to FVIII binding. Interestingly, the affinity of

Table 3: ELISA for interaction between plasminogen (and Ah-plasmin) fragment and FVIII(a) subunit.

Plasmin(ogen) fragment	ELISA		
	FVIII	A2	Light chain
	K_d^{app}		
	nM		
Lys-plasminogen	117 ± 39	101 ± 25	271 ± 34
K1–2–3	273 ± 47	215 ± 18	75 ± 8
K4	586 ± 121	275 ± 23	106 ± 9
K5-CD	8.7 ± 1.5	52 ± 4	628 ± 62
Ah-plasmin[15]	6.7 ± 2.0	41 ± 9	115 ± 11

Values were calculated by nonlinear regression analysis of the kinetic curves shown in Fig. 6 using a single-site binding model. K_d^{app}: apparent K_d value.

Lys-plasminogen for FVIII (K_d; 117 nM) was much weaker than that of Ah-plasmin, suggesting that plasmin probably reacted with FVIII more effectively than plasminogen. The A2 domain preferentially bound to K5-CD (K_d; 52 nM) than K1–2–3 and K4 (K_d; 215 and 275 nM, respectively), similar to the affinity of A2 for Ah-plasmin (K_d; 41 nM). In contrast, the light chain preferentially bound to K1–2–3 and K4 (K_d; 75 and 106 nM, respectively), compared to K5-CD (K_d; 628 nM), similar to the affinity of light chain for Ah-plasmin (K_d; 115 nM). These results indicated that the K5-CD domain of plasminogen (and plasmin) predominantly interacts with the A2 domain of FVIII, whilst K1–2–3 and K4 predominantly interact with the light chain. The findings strongly support the functional data obtained in activation and cleavage experiments.

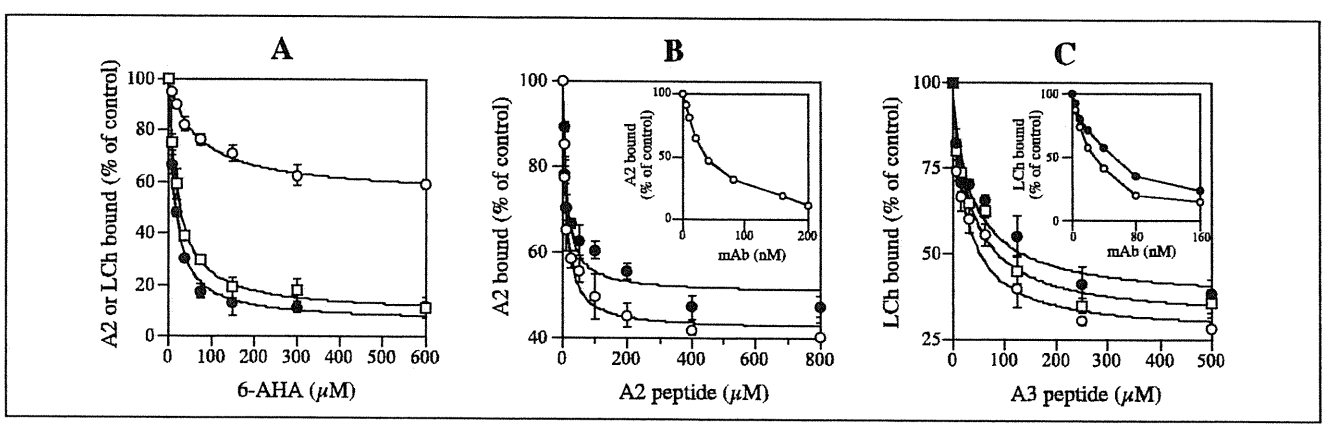

Figure 7: Effect of 6-AHA or FVIII peptide on FVIII subunit binding to plasminogen fragments. A) 6-AHA: The A2 (80 nM) or ^{1649}A3C1C2 (150 nM) subunit in the presence of various concentrations of 6-AHA was incubated with plasminogen fragment (120 nM) immobilised onto microtitre wells. Bound A2 or ^{1649}A3C1C2 was detected using biotinylated anti-A2 (JR8) or anti-C2 (NMC-VIII/5) mAb IgG, respectively. The symbols used are: ○, A2/K5-CD binding; ●, ^{1649}A3C1C2/K1–2–3 binding; □, ^{1649}A3C1C2/K4 binding, respectively. B) A2 peptide: The A2 subunit (80 nM) was mixed with varying amounts of the 484–509 peptide, prior to reaction with the K5-CD (○) or Ah-plasmin (●) immobilised onto microtiter wells. Bound A2 was detected using biotinylated anti-A2 mAb IgG. Inset shows the inhibitory effect of anti-K5-CD mAb on the A2/K5-CD binding. C) A3 peptide: The ^{1649}A3C1C2 (150 nM) was mixed with varying amounts of equimolar mixture of 1690–1705 and 1804–1818 peptide, prior to reaction with K1–2–3 (○), K4 (●), or Ah-plasmin (□) immobilised onto microtitre wells. Bound ^{1649}A3C1C2 was detected using biotinylated anti-C2 mAb IgG. Inset shows the inhibitory effects of anti-K1–2–3 (○) and anti-K4 (●) mAb on the ^{1649}A3C1C2/K1–2–3 and ^{1649}A3C1C2/K4 binding, respectively. The absorbance values corresponding to the A2 or ^{1649}A3C1C2 subunit binding to plasminogen fragment in the absence of competitor were considered to be 100%. The percentage of A2 or ^{1649}A3C1C2 binding was plotted as a function of 6-AHA or FVIII peptide concentration, and the plotted data were fitted by non-linear least squares regression (Equation 3).

Effects of 6-AHA on the interaction between A2 and K5-CD or light chain and K1–2–3 (and K4)

The A2 domain or light chain of FVIII interact with plasmin predominantly through lysine-binding site-independent or -dependent mechanisms, respectively. To investigate whether the interactions between A2 and K5-CD, and light chain and K1–2–3 (and K4) were also regulated by similar mechanisms, the effects of 6-AHA, a competitor of lysine-binding site, were determined in ELISAs. The A2 (80 nM) or light chain (150 nM) was incubated with immobilised plasminogen fragments in the presence of 6-AHA. The known lysine analogue blocked interactions between the light chain and K1–2–3 (and K4) in similar dose-dependent manners by >90% (▶Fig. 7A). The apparent K_i values were 8.0 ± 0.8 and 10.4 ± 1.0 μM, respectively. In contrast, 6-AHA blocked A2 and K5-CD interactions weakly (~35%) with a K_i value of 14.3 ± 2.8 μM. These K_i values (~10 μM) were similar to those for 6-AHA reported earlier (23). In addition, the inhibitory effects were also equivalent to those for the A2 and light chain binding to Ah-plasmin reported previously (15, 16). The data indicated that K5-CD interacted with the A2 domain mainly through lysine-binding site-independent mechanisms, and that K1–2–3 and K4 interacted with the light chain in lysine-binding site-dependent mechanisms.

The inhibitory effects of FVIII A2 or A3 peptides on these interactions were also examined in similar experiments. The molecular structures corresponding to these peptides are known to contain the plasmin-interactive sites on the A2 or A3 domain of FVIII (15, 16). As shown in Figure 7B, the A2 peptide (484–509 residues) inhibited A2 binding to K5-CD by ~60% at the maximum concentration employed (800 μM), with a K_i value of 8.5 ± 1.8 μM. This inhibitory effect was similar to that observed with A2 binding to Ah-plasmin (by ~50%) (15). The mixture with both A3 peptides (1691–1705 and 1804–1818 residues) also inhibited the light chain binding to K1–2–3 and K4 by ~70% (K_i; 16.8 ± 2.8 and 15.9 ± 3.5 μM, respectively) (Fig. 7C). These inhibitory effects were similar to those seen with the light chain binding to Ah-plasmin (by ~60%) (16). In addition, the A2 and K5-CD binding and the light chain and K1–2–3 (and K4) binding were significantly (by ~80%) blocked by anti-K5-CD and anti-K1–2–3 (and K4) mAbs, respectively (Fig. 7B and C, inset). These results suggested that A2 and K5-CD significantly contributed to the A2 and plasmin interactions and that the light chain and K1–2–3 (and K4) were closely involved in light chain and plasmin interactions.

Effect of anti-CD Ab (C-14Ab) on the A2 and K5-CD binding

Our observations indicated that interaction between the A2 of FVIII and the K5-CD of plasmin regulated plasmin-catalysed activation (by cleavages at Arg^{372} and Arg^{740}) and inactivation (by cleavage at Arg^{336}) of FVIII(a). To further study the domain-domain interactions, inhibition experiments were performed using similar ELISA method with polyclonal C-14Ab, recognising the CD of plasmin. Varying amounts of C-14Ab were preincubated with the K5-CD immobilised onto microtiter wells, followed by addition of the A2 subunit (80 nM) (▶Fig. 8A). The C-14Ab inhibited the A2 and K5-CD binding by >80% in a dose-dependent manner (with IC_{50}; ~90 nM). Furthermore, Western blot analysis showed that C-14Ab inhibited plasmin-catalysed cleavage of the heavy chain at Arg^{740}, Arg^{336}, and Arg^{372} in a dose-dependent manner (Fig. 8B). These results demonstrated that plasmin activation and inactivation of FVIII(a) might be regulated by interactions between the A2 domain of FVIII and the CD of plasmin, although the precise contribution of K5 and the effects of possible steric hindrance on these reactions caused by the antibody remain to be fully evaluated.

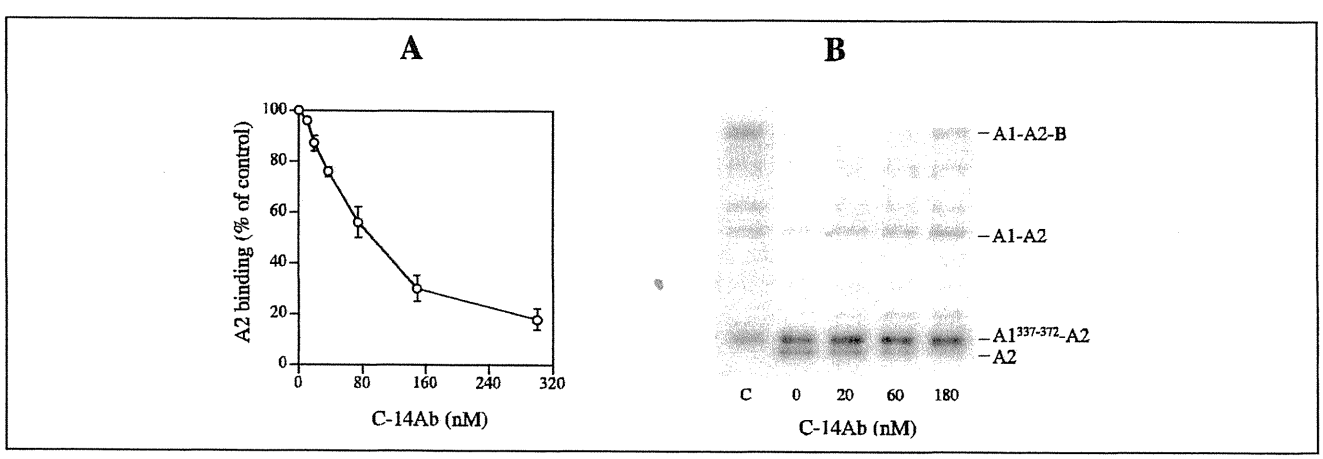

Figure 8: Effect of C-14Ab on the A2 and K5-CD interaction and plasmin-catalysed cleavage of the heavy chain of FVIII. A) A2 and K5-CD interaction: Varying amounts of C-14Ab were preincubated with the K5-CD (100 nM) immobilised onto microtitre wells, followed by addition of the A2 subunit (80 nM) (A). Bound A2 was detected using biotinylated anti-A2 mAb IgG. The absorbance values corresponding to the A2 binding to K5-CD in the absence of competitor were considered to be 100%. B) Plasmin-catalysed cleavage: Plasmin (3 nM) was preincubated with various concentrations of C-14Ab for 1 h prior to incubation with FVIII (100 nM) for 3 min. Samples were run using 8% SDS-PAGE, followed by Western blotting using biotinylated anti-A2 mAbJR8.

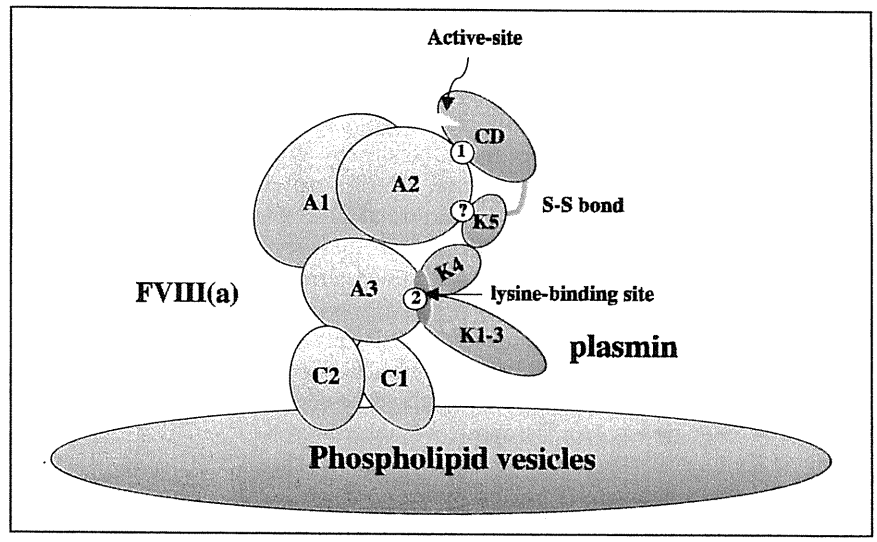

Figure 9: Schematic diagram of putative FVIIIa-plasmin interactions. The putative FVIIIa and plasmin interaction is shown (*1 and 2).

To further confirm that a C-14Ab against CD did not affect the plasmin-catalysed inactivation of FVIIIa through cleavage at Lys^{36}, the similar experiments were repeated. The C-14Ab (150 nM) did not significantly inhibit the plasmin-catalysed inactivation of $A1^{1-336}$FVIIIa by ~10% (Fig. 5A and Table 2). In addition, the presence of C-14Ab did not affect the cleavage at Lys^{36} in $A1^{1-336}$FVIIIa by plasmin (data not shown). These results were similar to those of anti-K5-CD mAb (see Fig. 4 and Fig. 5A). Taken together, this might again indicate the specificity of plasmin-catalysed inactivation of FVIII by cleavage at Arg^{336} by C-14Ab.

Discussion

The opposing mechanisms of blood coagulation and fibrinolysis maintain normal haemostasis by the inter-dependent regulation of several circulating proteases. Thrombin, in particular, plays an integral regulatory role in the formation and dissolution of fibrin, and the effect of plasmin, the principal enzyme of the fibrinolytic system, on a number of proteins that significantly affect thrombin generation (e.g. FV(a), IXa, X) are well described (6–8). In addition, we have recently shown that plasmin is a potent activator and inactivator for FVIII(a), independent of phospholipid surfaces (5). The physiological significance of plasmin-catalysed cleavage of FVIII, resulting in this activation and inactivation of cofactor function, remains to be fully determined, however. Nevertheless, even very low concentrations (~1 nM) of protease, generated from high plasma concentrations of the zymogen, plasminogen (~2.4 μM [3]), appear to promote a catalytic rate greater than that of APC or FXa (15), suggesting that small amounts of plasmin generated in the fibrinolytic response are likely to contribute to the up- and down-regulation of blood coagulation.

Plasmin is composed of five kringle domains and one catalytic domain. To investigate FVIII(a)-plasmin binding domains in the current studies, three major fragments (K1–2–3, K4, and K5-CD) were prepared by digestion of plasminogen with elastase. These isolated fragments have been well characterised in previous experiments to analyse plasmin-binding mechanisms. Each of the five kringles, consisting of ~80 residues and well-conserved between species, has the ability to bind to exposed Lys residues in other proteins (e.g. fibrin, α-antiplasmin, ω-aminocalboxylic acids). Of interest, however, the separate fragments possess different affinities and specificities for fibrin and ω-amino acid ligands. The tightest binding site for fibrin is provided by K5, followed by K1–2–3 (24). However, K4 and CD contribute little to fibrin binding (25). In contrast, the tightest binding site for ω-amino acid ligands is provided by K1–2–3, (K1>>K2>K3) (26), followed by K4, whilst K5-CD binds very weakly (27). Our recent studies (15) showed that 6-AHA had a slight effect on FVIII A2-plasmin interaction, but significantly blocked the light chain-plasmin interaction, indicating that the former interaction is mainly independent of lysine-binding site and the latter is mainly dependent on lysine-binding site. In the present investigations, the A2 domain predominantly bound to K5-CD, whilst the light chain bound more effectively to K1–2–3 and K4 than to K5-CD. These properties and effects of 6-AHA strongly suggest that the light chain interacts with lysine-binding sites within K1–2–3 and K4 and that the A2 domain interacts with K5-CD in lysine-binding site-independent mechanism. Interestingly, the K_d (~8 nM) for the FVIII and K5-CD interaction was similar to that for FVIII and Ah-plasmin (15), and was much lower than that (~120 nM) for FVIII and plasminogen interaction. This discrepancy might be due to steric hindrance of intact plasminogen, or might be due to a change in binding affinity as a result of conformational changes in the isolated of plasminogen fragments, but precise reasons remain to be determined.

Our studies showed that anti-K5-CD mAb and K5-CD fragment competitively blocked plasmin-catalysed FVIII (in)activation and cleavage. These data suggested that interactions between the A2 and K5-CD regulate plasmin-catalysed FVIII(a) activation and inactivation through cleavage at Arg^{372} (and Arg^{740}) and Arg^{336} in the FVIII molecule. Furthermore, an anti-CD polyclonal

Ab blocked the plasmin-catalysed FVIII cleavage and interactions between A2 and K5-CD. These anti-plasmin mAbs and anti-CD Ab did not directly affect plasmin activity (data not shown), indicating that the CD might contain an essential binding region responsible for interaction with the A2 domain, although the contribution of K5 to this specific binding remains to be determined. The sequence 484–509 in A2, particularly Arg^{484}, is known to comprise a pivotal binding site for docking to plasmin for the activation and inactivation of FVIII by cleavage at Arg^{372} and Arg^{336} (15). An A2 peptide with the 484–509 sequence inhibited both A2 and K5-CD binding and A2 and Ah-plasmin binding. It seemed likely, therefore, that residue Arg^{484} in A2 interacted with the K5-CD domain of plasmin.

The extended surface, centred on Lys residues, in the 1690–1705 and 1804–1818 regions of the FVIII light chain, contributes to a unique plasmin-interactive site that facilitates plasmin docking during cofactor inactivation by cleavage at Lys^{36}. Loss of activity resulting from cleavage at Lys^{36} is associated with an altered molecular conformation that markedly affects the affinity of the A1 subunit for A2 (14). The present study demonstrated that the K1–2–3 and K4 domains of plasmin interacted with both regions in the light chain by binding to lysine-binding site in the protease. FXa also catalyses cleavage at Lys^{36} (14, 28). This cleavage appears to be regulated by the association between the heparin-binding exosite of FXa and the 337–372 acidic region in A1 (29). Hence, although plasmin and FXa cleave FVIII at an identical site, the binding mechanisms appear to be distinctive.

What is known about this topic?

- Plasmin activates and inactivates factor (F)VIII by limited proteolysis.
- Plasmin interacts with the A2 domain of FVIII, in mechanisms largely independent of lysine-binding site. The A2 domain, especially Arg^{484}, contributes to plasmin-interactive site(s) that promote(s) plasmin-docking during cofactor activation/inactivation by cleavages at Arg^{372} and Arg^{336}.
- Plasmin interacts with the light chain of FVIII through lysine-binding site-dependent mechanisms. An extended surface, centered on Lys involving the 1690–1705 and 1804–1818 regions in the A3 domain, contributes to plasmin-interactive site(s) that promotes plasmin-docking during cofactor inactivation by cleavage at Lys^{36}.

What does this paper add?

- We characterised FVIII-interactive regions on plasmin using a combination of functional and binding experiments employing isolated FVIII and plasmin subunits and antibodies.
- The kringle (K)5-catalytic domain (CD) of plasmin (and plasminogen) interacts with the A2 domain independent of lysine-binding site, whilst interactions of K1–2–3 and K4 with the light chain are lysine-binding site-dependent.
- Interactions between the K5-CD and A2 constitutes the major regulatory mechanism for activation and inactivation of FVIII(a) mediated by cleavage at Arg^{372} and Arg^{336}.

More recently, the X-ray crystal structure of B domain-deleted human FVIII has been described (30, 31), and interactions between FVIII and serine proteases (FIXa and APC) have been partially clarified (32, 33). On the basis of these reports, a potential structural model of the FVIIIa-plasmin complex on phospholipid membranes is shown schematically in ▶Figure 9. The catalytic and epidermal growth factor (EGF)-like domains in FIXa and APC seem likely to interact with the A2 and A3 domains, respectively, consistent with the contact sites between the FVIIIa-plasmin. Some differences are evident in the FVIII-plasmin binding model, however. For example, FIXa and APC require the presence of a membrane surface to inactivate FVIIIa and consequently increase the catalytic rate, whilst FVIII-plasmin binding appears to be surface-independent. In addition, there are differences in binding affinity between the contact sites in these substrate-enzyme complexes. The binding affinity of the A3 domain of FVIIIa and the EGF domain of FIXa is higher (K_d; ~15 nM [33]) than that of the A2 domain and CD (K_d; ~300 nM [34]). Also, the binding affinity of the A2 domain and K5-CD was significantly greater than that of the light chain and K1–2–3 (and K4). These observations strongly suggest that FVIII-plasmin interaction represents a unique binding model. Further detailed studies, for example to determine the predominant domains (regions) of plasmin involved in A2 or light chain binding, are required to fully characterise FVIII-plasmin binding mechanisms.

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第72回日本血液学会学術集会
教育講演
基本シリーズ S-9 出血性疾患の病態と管理

後天性凝固異常症の病態と治療：後天性血友病を中心に

嶋 緑倫

Key words : Acquired hemophilia, Inhibitor, Factor VIII, Autoantibody

後天性血友病は第VIII因子に対する自己抗体が出現して内因性の第VIII因子が低下するために出血症状を呈する疾患で，第VIII因子遺伝子異常に基づく先天性の第VIII因子欠乏症である血友病Aとは全く異なる疾患である[1,2]。本疾患は従来まれな疾患と考えられてきたが，最近，わが国でも報告例が増加している。後天性血友病はさまざまな基礎疾患を背景に発症し，また，高齢者に多いことからも免疫制御機構の変化が原因と考えられる。迅速な診断と適切な止血および免疫学的治療が必要である。

疫 学

後天性血友病の発生頻度は年間人口100万人あたり1.5人といわれている[3]。したがって，わが国でも年間200人は発症していることになる。平成13年1月から平成14年5月にかけて日本血栓止血学会学術検討部会凝固委員会が中心となって実施された後ろ向き調査によると計75症例の後天性インヒビターの報告があり，内訳は抗第VIII因子インヒビター58例，抗von Willebrand因子インヒビター7例，抗第V因子インヒビターが5例，その他3例であった[4]。その後，平成15年10月から18年9月に日本血栓止血学会学術標準化委員会血友病部会により実施された前向き調査で計57症例の後天性インヒビターの症例が集積されたが，抗第VIII因子インヒビターが55例，第V因子インヒビターが1例，抗第VIII，IX，XI，XII因子複合抗体1例であった[5]。したがって，後天性インヒビターの中では圧倒的に抗第VIII因子インヒビターが多いことがわかる。男女比は男性53%，女性47%と明らかな差はない。後天性血友病の疫学上の特徴として高齢者と分娩後の女性に多いことがあげられる。実際，わが国でも70歳台を頂点とする第一ピークと，20歳から30歳台を頂点とする第2ピークがみられる（図1）。第2ピークはほとんどが女性である。欧米での報告も同様で，中央値は77歳である[6]。年齢別に発症率（/100万人）を比較すると，16～64歳では0.3人であるが65～84歳になると9人，85歳以上になると15人に上昇する[3]。

後天性血友病のもうひとつの特徴として，基礎疾患を背景に発症することが多いことがあげられる。わが国の調査では何らかの基礎疾患が認められたのは75%に上る。多いのが膠原病（17%）と悪性腫瘍（17%）である。内訳は前者では関節リウマチ，SLE，Sjögren症候群，自己免疫性肝炎などで，後者では胃，大腸，腎，肝，胆管，十二指腸，甲状腺，乳癌などである。以下，糖尿病，分娩後，皮膚疾患などが続く（図2）。

欧米の報告例では，基礎疾患の特定されていない特発性のタイプはGreenらの総説によると46%[7]，Morrisonらの報告[8]では55%，Collinsらの報告[3]では63%とわが国の実態調査と比較すると高い。基礎疾患の内訳はわが国と同様に膠原病，悪性疾患，皮膚疾患，薬物などの頻度が高い。分娩関連の症例は1割前後である（表1）。

後天性血友病の予後であるが，前向き調査では調査期間中にインヒビターが消失した例が52%，消失しなかった例が23%，死亡例が25%であった。死亡原因の内訳は出血関連死が50%で頭蓋内出血，腹腔内出血などが多い（図3）。出血以外の死亡原因では肺炎や敗血症などの感染症が多い。したがって，後天性血友病は治癒叶な疾患で予後は必ずしも悪くないが，死亡率が高いことは十分留意すべきである。欧米での報告では後天性血友病の死亡率は9～22%である[3,7]。この原因として，出血症状が重篤であること，基礎疾患を有する症例が多いこと，高齢者に多く抵抗力が弱いこと，免疫抑制療法や基礎疾患に起因する重症感染症の発症の危険性が高いこと

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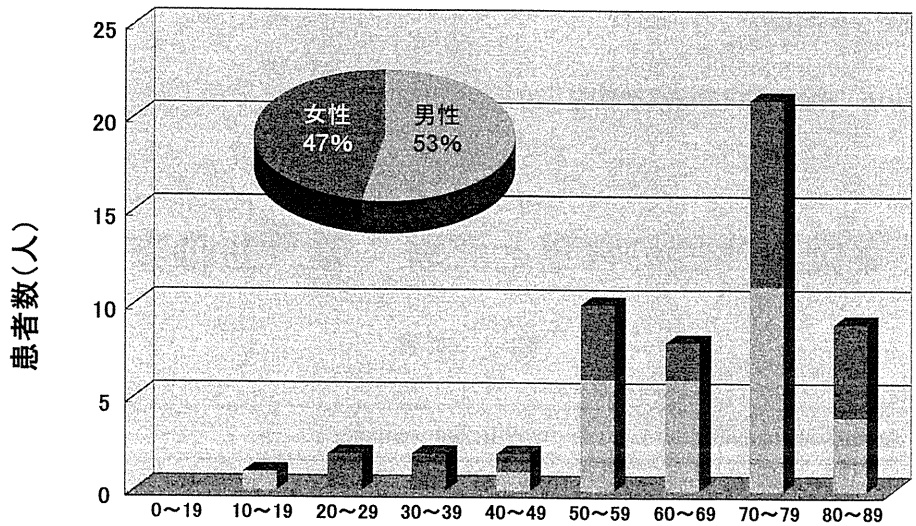

図 1　後天性血友病の性別および年齢分布[5]
男女比 1:0.9，年齢分布 12～85 歳（中央値 70 歳）

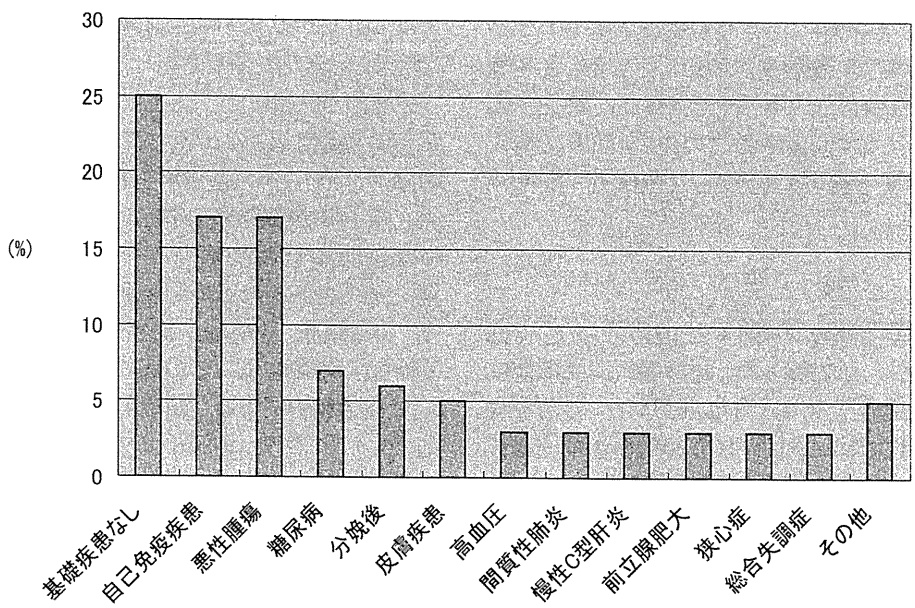

図 2　後天性血友病の基礎疾患および病態[5]

などが考えられる。

臨床症状

後天性血友病は第 VIII 因子因子の欠乏～低下に起因する出血症状であるが，同じ第 VIII 因子異常症の先天性血友病と異なる点もある。一般に，皮下や筋肉内出血が多いが，関節内出血はきわめてまれである。わが国の調査では両者を合わせると 56%である。その他は消化管出血，口腔内出血などの粘膜内出血や血尿などの頻度が高い（図 4）。欧米の報告でも同様である[3,8]。後天性血友病で見られる皮下，筋肉内出血は広範である。したがって，重篤な貧血を合併する場合もまれではない。さらに広範な筋肉内出血による血管や神経の圧迫によるコンパートメント症候群を発症する危険性もある。一方，軽度の出血症状も比較的多くみられ，約 3 割が治療を要さない出血である。なお，後述するが出血症状の重篤度と第 VIII 因子活性レベルやインヒビター力価とは全く相関しない。

診　断

1）臨床症状

高齢者や分娩後の女性で，今までに出血症状がなく，

表 1 後天性血友病の関連疾患

	報告者		
	Green ら[7]	Morrison ら[8]	Collins ら[3]
症例数	215	65	150
特発性	46	55	63
膠原病，血管炎	18	17	17
悪性腫瘍	7	12	15
皮膚疾患	5	2	3
薬剤	6	3	0
妊娠	7	11	2

また，家族歴にも出血者がない成人で，原因不明の出血症状をみたら本疾患を疑う必要がある。過半数の症例で基礎疾患がみられることから，後天性血友病の患者は必ずしも血液内科を受信するとは限らず，むしろ，膠原病/リウマチ科，腫瘍科，皮膚科，産婦人科など様々な科を受診する。本疾患は早期診断早期治療が必須であり，本疾患を啓蒙する必要がある。

2）検査

出血傾向が見られた場合，通常，血小板数，プロトロンビン時間（PT），活性化部分トロンボプラスチン時間（aPTT）を実施する。後天性血友病は第 VIII 因子の低下が本態であり，血小板数や PT は正常であるが aPTT は延長する。さらに延長した aPTT は正常血漿で補正さ

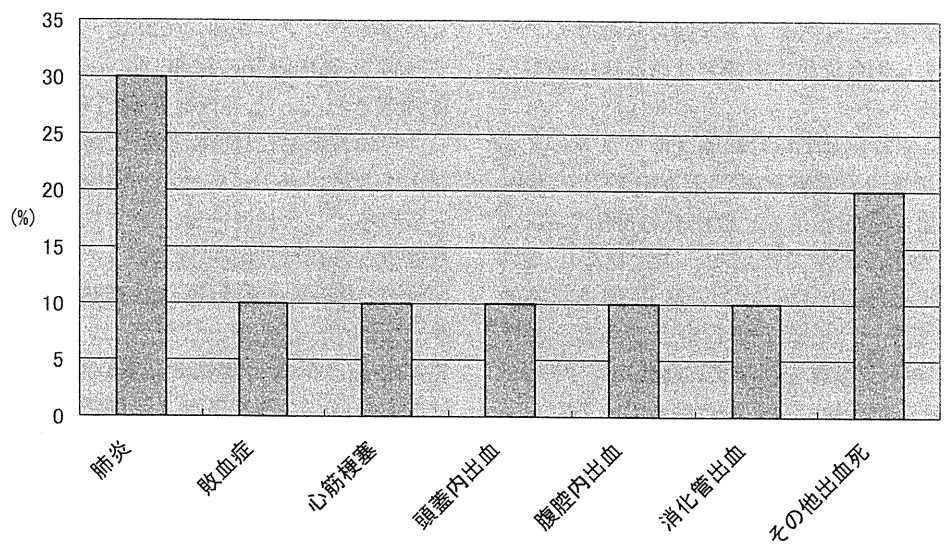

図 3 後天性血友病患者の死因[5]

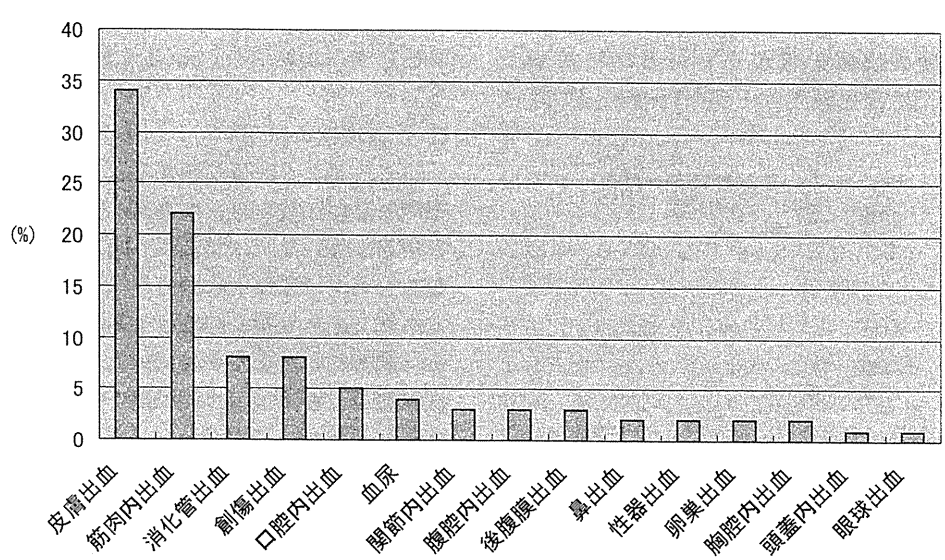

図 4 後天性血友病患者の出血症状[5]

れない。aPTTに基づく補正試験は混合交差試験といわれ，日常診療の中で凝固因子欠乏症とインヒビターとの鑑別に実施される。先天性の血友病であれば患者血漿に正常血漿を混和すると延長したaPTTが短縮するが，後天性血友病では短縮しない（図5）。混合試験でインヒビターパターンをとり，後天性血友病と鑑別すべき疾患にループスアンチコアグラント（LA）がある。LAの場合リン脂質依存性の凝固検査ではいずれも延長する。ただし，後天性血友病では第VIII因子抑制効果は時間温度依存性である。したがって混合試験では反応時間を0と2時間いずれも実施することが望ましい[9,10]。また，aPTTの試薬も混合試験の結果を左右する可能性がある。近年のaPTT試薬は正常コントロール血漿の凝固時間が早くなっており，凝固因子欠乏症に対する感度は帰って悪いことがある。したがって，混合試験でインヒビターパターンをしめさなくても後天性血友病は必ずしも否定できない。

後天性血友病の検査診断で混合試験はあくまでもスクリーニング試験であり，最終診断は第VIII因子の単独低下～欠損と抗第VIII因子インヒビターの検出である。抗体力価が高い場合，測定で使用する欠乏血漿中の第VIII因子を抑制するために見かけ上，第IX因子などの他の内因系凝固因子活性も低下する場合がある。さらに，LAの場合でもリン脂質を抑制するために見かけ上，内因系凝固因子活性が低値になることがある。このような場合，検体をさらに希釈するか，合成発色基質法や第VIII因子抗原で測定することで鑑別できる。後天性血友病の初診時の第VIII因子活性であるが，報告によると<1%が46%，1～5%が39%，>5%が15%と半数以上の症例で有意に第VIII因子が検出される[5]。通常，先天性血友病Aでは第VIII因子の活性測定値と臨床的出血症状の重症度は良く相関する。実際，<1%の重症タイプでは1/週程度の出血症状が見られるのに対して1～5%の中等症では出血頻度は1回/月～2～3回/年と

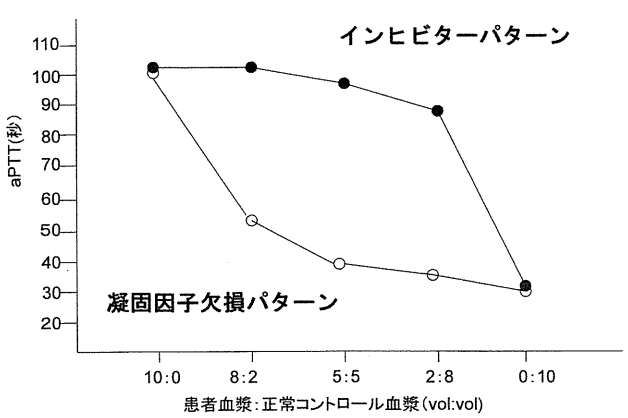

図5　APTT交差混合試験

激減する。軽症タイプでは成人になって外科手術前のスクリーニング試験で初めて診断される場合がある。しかしながら，後天性血友病ではこの原則はあてはまらず，出血症状の重症度と第VIII因子活性レベルは全く相関しない。

後天性インヒビターの確定診断で抗第VIII因子インヒビターの検出は必須である。先天性血友病における第VIII因子や第IX因子製剤による補充療法の反復の結果生じるインヒビター（抗第VIII因子同種抗体）と同様にBethesda法にて測定する[11]。本方法は患者血漿と正常コントロール血漿を等量混合して2時間37℃で加温後残存する第VIII因子活性を測定する。残存活性を50%に低下させる抗体を1 Bethesda単位/m*l*（BU/m*l*）と定義される。高力価のインヒビターの場合，段階希釈した検体サンプルを用いて測定する。一般に，インヒビターは第VIII因子の抑制パターンによりタイプ1と2に分類される。先天性血友病にみられるインヒビターは，検体希釈度と残存活性が比例直線的に相関するタイプ1インヒビターが多い。しかしながら後天性血友病にみられるインヒビターはタイプ2インヒビターが多く希釈倍数と残存活性は相関しない。このような場合は，残存活性が50%に最も近い希釈倍数をもってインヒビター力価を表示する。したがって，インヒビター力価は第VIII因子活性と同様に必ずしも病勢の強さの指標とはならない。さらに予後とも相関しない。わが国でも前向き調査で，生存例と死亡例で年齢，第VIII因子活性，初診時のインヒビター力価およびインヒビター力価のピーク値との相関を調べたところ，いずれも相関しなかった[5]。

治　療

後天性血友病の治療は出血症状に対する止血療法とインヒビターの消失をはかる免疫学的治療法に大別される。

1）止血療法の原則

前述したように後天性血友病の出血症状は広範であり重篤な出血症状もみられるが，30%は軽度な止血治療の対象とはならない。つまり，後天性血友病の治療はあくまでもインヒビターの消失をはかる免疫学的治療が主体であり，止血治療の対象となるのは重度の出血症状である。重度の出血症状の目安として，ヘモグロビンが>3 g/d*l*低下，濃厚赤血球の輸血が必要な場合，頚部出血や消化管出血，コンパートメント症候群，頭蓋内出血，肺出血，術後出血などがあげられている[12]。ただし，初診時に貧血がみられなくてもい後日進行する場合もあり，出血症状の程度や経過の評価にはヘモグロビンやヘマトクリット値の定期的検査が必須である。

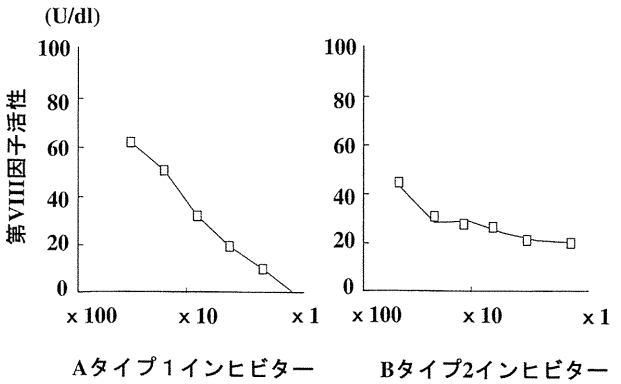

図6 FVIII残存活性によるインヒビターのパターン

前述したように，第VIII因子活性やインヒビター力価は出血症状の重症度や後天性血友病の病勢に相関しない。抗体力価が低値を示しても，タイプ2インヒビターが多く，必ずしも中和できない。したがって，先天性血友病の止血治療と異なり，第VIII因子製剤の有効性は乏しくバイパス止血治療製剤が第一選択となる。

2) バイパス止血治療

バイパス止血療法製剤として使用できる製剤は活性化プロトロンビン複合体製剤（activated prothrombin complex concentrates; APCC）と遺伝子組み換え活性化第VII因子製剤（recombinant activated factor VII, rFVIIa）の2剤である。どちらの製剤も後天性血友病の止血療法製剤として有用で，両者の間の止血効果の差を示すエビデンスはない。しかしながら，それぞれの製剤に長所短所があるのも事実で，症例により，また，出血症状により適応を考慮する必要がある。たとえば，分娩後の症例ではrFVIIaが推奨される[12]。わが国の実態調査では，rFVIIaの使用例が圧倒的に多いが，止血効果はAPCC製剤と同等で有効例はそれぞれ80%，77%である。第VIII因子製剤の有効率は33%と劣る結果であった[5]。

止血療法の開始の判断基準はすでに前述したとおりであるが，文献的にも止血療法をいつまで継続するのかに関する指標がないのが現実である。近年，発表された後天性血友病の国際的推奨によると出血症状の程度や部位など一概に規定することは困難であるが，24～72時間の継続を推奨している。止血効果の評価に関する理想的な検査法は確立していない。しかしながら，血友病インヒビター症例の止血管理の際使用されるトロンボエラストグラフィー（Thrombelastography TEG, Roitating thrombelastopmetry ROTEM），凝固波形解析，トロンビン生成試験も有用である[13]。

(1) 遺伝子組み換え活性型第VII因子製剤（rFVIIa）

rFVIIa製剤の有効性に関するこれまでの成績は，先天性血友病に発症するインヒビター陽性例の止血療法を標的とした報告が多く，後天性血友病に限ったものは少ない[14,15]。rFVIIaの有効率は概ね高く，非外科的出血症状に対しては部分的有効も含めると90%に達する。外傷性の出血に対しても有効率は86%であった。rFVIIaを第一選択の治療製剤した場合とそうでない場合にわけてそれぞれの有効率を多施設に実施された調査によると，第一選択群では止血効果は100%にみられたが，第2選択の場合も75%有効であった[16]。さらに，前向き調査でも有効率は86.6%と高い有効率が報告されている[17]。わが国の調査でも有効率は80%に達しており，後天性血友病の出血症状にrFVIIaは有効であるといえる。

rFVIIaの投与は先天性血友病のインヒビター保有例と同様である。一回の投与量は90～120 μg/kgで2～3時間毎に反復投与する。出血後早期に投与することが望まれる。急性出血時トラネキサム酸を併用することも有用である。

先天性血友病のインヒビター陽性例を対象としたこれまでの報告ではrFVIIaの血栓症発症リスクはきわめて少ないとされているが，基礎疾患の合併例や動脈硬化のリスクが高い高齢者に多いことからも後天性血友病も同様とは言えない。rFVIIaで治療された139例の後天性血友病症例中，血栓症の発症は10例見られている[15]。最近の後天性，先天性インヒビターをあわせた報告では血栓症の発症は<1%と報告されているが[18]，後天性血友病に限った成績ではないので，血栓症の発症リスクが低いと考えられているrFVIIaも後天性血友病の場合は特に動脈硬化の進行例，外傷や敗血症合併例などの組織因子の露出が高い症例では注意を要する。

(2) 活性化プロトロンビン複合体製剤（APCC）

APCC製剤はrFVIIaの市販前から先天性血友病インヒビター保有例の止血療法製剤として使用されてきており，止血効果についても種々の臨床研究により明らかにされている[19]。しかしながら後天性血友病を対象とした止血効果に関する報告は少ない。わが国の調査報告でもrFVIIaを使用した症例数は24例であるが，有効性はかわらないもののAPCC使用例は4例と少なかった。これは世界的にも同様でrFVIIaと比較して後天性血友病におけるAPCCの使用経験はまだまだ少ないのが現状である。使用経験が少ない原因のひとつにAPCC製剤に起因する血栓症の発症リスクに関する懸念が挙げられる。先天性血友病インヒビター保有例での経験では計3.95×10^5回投与において16例の血栓症の発症が報告されている[20]。しかも発症例は過剰投与，肥満や高脂血症などのリスクがみられることも明らかにされている。rFVIIaと同様に後天性血友病に限定した報告例はなく，血栓症やDICの発症については留意すべきであるが，重篤な出血症状に遭遇した場合にはAPCCもrFVIIaと

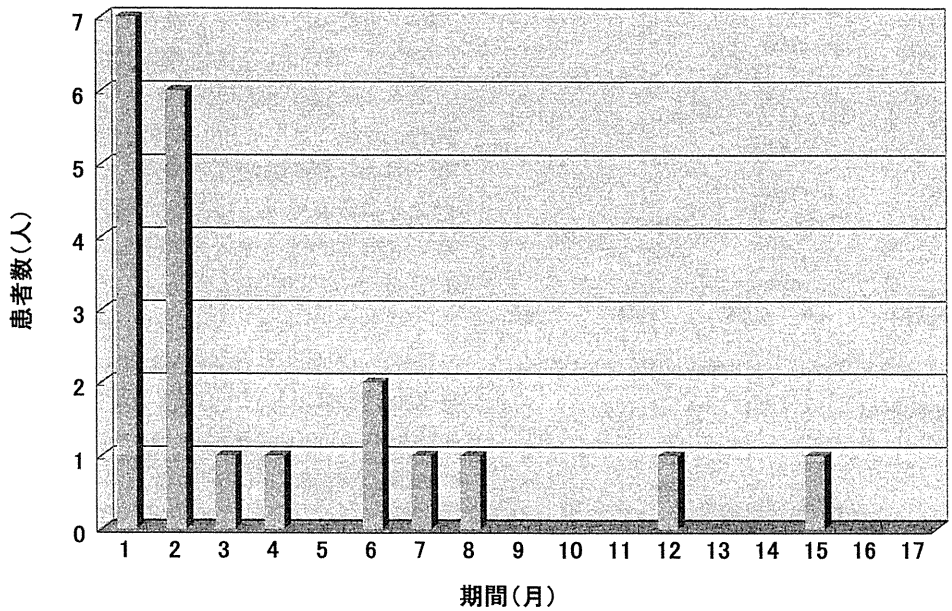

図7　インヒビター消失までの期間
中央値2ヶ月（0.5～15ヶ月）

同様選択できるものと考える。APCCの投与量であるが，血友病インヒビター保有例と同様に50～100単位/kg，8～12時間毎1～3回/日が推奨される[21]。一日最大投与量は200単位/kgでトランサミンとの併用は禁忌である。

(3) 第VIII因子製剤

基本的には後天性血友病の止血療法製剤として第一選択には薦められていない。インヒビター力価が極めて低い症例，出血症状が軽度な場合，バイパス止血療法製剤が使用できない場合などに限定される。第VIII因子が低下するものの有意に存在する中等症～軽症の先天性血友病A患者では酢酸デスモプレシン（DDAVP: 1-deamino-8-D-arginine）が適応になるが，後天性血友病に関するエビデンスはなく，また，第VIII因子製剤と同様薦められていない。

3) インヒビター除去のための免疫学的治療

(1) 免疫学的治療の原則

後天性血友病における最も重要な治療法である。免疫学的治療は，たとえ出血症状が軽度であっても早期に開始することが薦められている[12]。なぜなら，インヒビター力価や第VIII因子活性レベルと出血症状は相関しないからである。つまり，症状が軽く，インヒビターが低力価で第VIII因子が有意に検出されても，生命の危険を伴う重篤な出血症状が出現することは否定できない[9]。249症例を集積した最近の報告によると，治療を実施しなかった症例の死亡率は41%であったが，免疫学的治療を実施した症例の死亡率は20%であった[1]。一方，16症例中5例，36%の患者が治療せず治癒した報告もあるが[22]，自然緩快を期待できるエビデンスは不十分である。

(2) 免疫学的治療の選択

免疫抑制剤がインヒビターの除去に有効であることは以前から知られているが，どの製剤を選択するかに関する指針はなく，また，それぞれの治療製剤の有効性に関する臨床試験はない。しかしながら，文献的考察に基づくエビデンスが近年急速に集積されており，専門医の意見を加えたオピニオンベースの指針も近年発表されている[12]。広範囲の文献的調査では，免疫学的治療法の基本は副腎皮質ホルモン製剤（prednisolone: PSL）単独か，サイクロフォスファマイド（cuclophosphamide: CPA）併用療法である[1]。Huth-KuhneらはPSL単独もしくはPSL+CPA第一選択とし推奨している[12]。両者の優劣に関して実施されたコントロール研究によると42%（13/31症例）でPSL単独で有効であったが，CPA単独の有効率は50%（3/6症例），PSL+CPA併用群（5/10例）であった。さらに，英国の後ろ向き調査によるとPSL単独例のインヒビター消失率は60～70%であったが，経口CPA併用群では70～80%とやや高かったが，生存率や疾患フリーの生存率では両者に差がなかった[3]。これは，CPA併用例の方が抗体除去効果からみると有効率は高いが，免疫抑制効果による死亡例もあり全般的な予後からみるとCPAを併用しても変わらないということになる。Mannucciらは前者を第一選択とすべきとコメントしている[23]。わが国ではPSL単独使用例

が19症例と圧倒的に多く。PSL+CPA併用例は8例である。有効率はPSL単独で抗体消失したのが47%に対し，併用例では75%と高かった。抗体が低下した症例はPSL単独で42%，併用群では25%であった。したがって，わが国の調査でも抗体消失率はPSL+CPA併用群の方が高い。しかしながら，CPA併用に起因する感染症発症リスクなどについても十分考慮する必要もある。

したがって，後天性血友病の免疫学的治療の第一選択はPSL単独投与が望ましいと考えるのが一般的である。ただし，基礎疾患等ですでにPSLを使用した症例ではCPAの併用を考慮する。他の免疫抑制剤では文献的にはアザチオプリン（azathioprin:）[24]，ヴィンクリスチン（vincristine）[25]，シクロスポリン（cyclosporine）[26]の有効性が報告されている。わが国ではステロイドのパルス療法の実施例も報告されているが海外の報告例もなく，有用性については不明である。

PSL投与量は経口で1 mg/kg 4～6週間投与で，CPA併用の場合は1.5～2 mg/kgを最大6週間投与するのが薦められている[12]。

なお，免疫グロブリン製剤の有効性に関するエビデンスはなく，後天性血友病の免疫学的治療性剤としては薦められていない。

(3) 無効例の免疫学的治療

PSL単独あるいはCPA併用療法が無効であった場合，本疾患の治療はきわめて困難になる。従来は，AZPやCyAなどの他の免疫抑制剤が選択されてきたが，近年，抗CD20モノクローナル抗体製剤であるリツキサン（rituximab）が注目されている。残念ながら本剤の有効性を支持するエビデンスはまだ少ないが，375 mg/m^2/周を最大4週までの投与で有効例が報告されている[27~29]。残念ながら保険適応はないが，国際的にはPSLやCPA併用で無効な場合の第2選択治療製剤としてリツキサンがすすめられつつある[30]。さらに，通常の免疫抑制療法が実施できない症例には第一選択としても考慮すべき薦める報告もある[12]。

後天性血友病の経過

わが国の前向き調査では52%の21例でインヒビターが消失し，10例（25%）が死亡，9例（23%）においてインヒビターが残存していた。インヒビターの消失までの期間は0.5～15ヶ月で中央値は2ヶ月である。インヒビターが消失した21例中15例（71%）が6ヶ月以内に消失している[5]。したがって，予後良好な症例は治療開始早期にインヒビターが消失する。後天性血友病の治療経過はaPTT，インヒビター力価と第VIII因子活性によりモニタリングする。再発例は免疫学的治療法の中止後中央値7～9ヶ月（1週～14ヶ月）でインヒビターが出現している[1,3]。したがって，モニタリングはインヒビター消失後，1年間は継続すべきである。インヒビターの低下に伴って，第VIII因子活性は上昇する。しかしながら，第VIII因子活性が上昇してもインヒビターの影響が残っている場合も多く，必ずしも活性に現れた凝固機能を有するとは言えない。さらに，インヒビター消失前の回復期に第VIII因子活性が著増することがある。この場合，反対に血栓症の発症に注意が必要である[31]。

終わりに

後天性血友病の免疫学的機序はいまだに不明な点が多く，治療の標準化も進んでいない。近年，わが国でも本疾患の認知度が徐々に高まりつつあり，今後，さらに症例数が増加するものと思われる。病態の解明と診断・治療法の標準化が急務である。

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