fied from the patient's plasma by Protein A-Sepharose chromatography as previously described (11). Synthetic "acquired" haemophilic blood were prepared by incubating control whole blood with varying concentrations of purified inhibitor IgG, at final inhibitor IgG titres of 5 (#1), 10 (#2), and 20 (#3) Bethesda U/ml (▶Table 1). The remaining FVIII clotting activities and inhibitor titres in corresponding plasma samples, measured by activated partial thromboplastin time (aPTT)-based assay, are also included in the ▶Table 1.

Statistical analysis

Statistical differences between two groups of data were evaluated by Student's t-test. In case of multiple comparisons, two-way factorial ANOVA was employed. P-values < 0.05 were considered to denote statistical significance.

Results

Preparation of VWF-coated glass surfaces containing varying concentrations of FVIII

Various concentrations of recombinant FVIII were incubated with a glass plate which had been coated with FVIII-free VWF. After non-adherent proteins were extensively washed out, amounts of FVIII immobilised onto VWF-coated glass surface (I-FVIII) were determined by the ELISA-based assay. Thus, I-FVIII increased as a function of recombinant FVIII added to a VWF-coated surface, reaching plateau at the FVIII concentrations greater than 5 U/ml (▶Figure 1). As a result, various VWF-coated glass plates with different I-FVIII density (del-FVIII as a control, #A, #B, #C and #D as indicated in the ▶Figure 1) were successfully prepared.

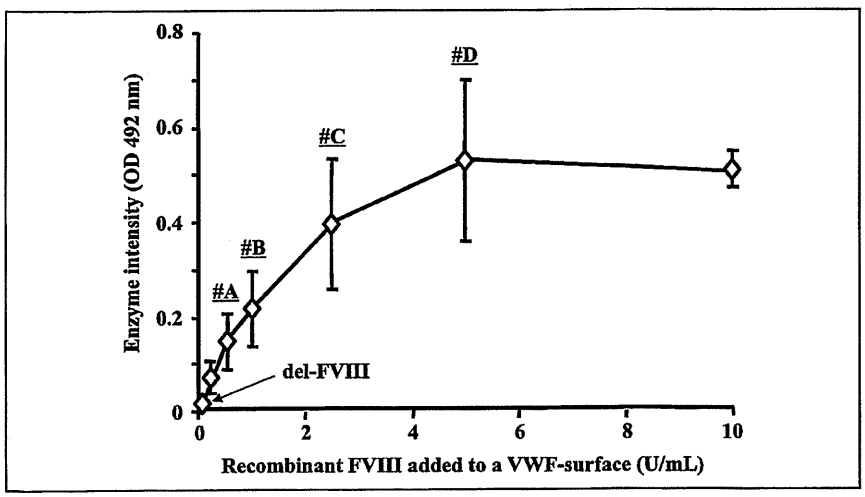

Figure 1: Preparation of VWF-coated glass surfaces containing varying concentrations of FVIII. A glass plate was coated with FVIII-free VWF and recombinant FVIII (0, 0.1, 0.5, 1, 2.5, 5, or 10 U/ml). Each data point represents mean ± standard deviation (SD) of three independent experiments. Note that I-FVIII as determined by the enzyme activity at optical density 492 nm increased as a function of recombinant FVIII added to a VWF-coated surface, reaching plateau at the FVIII concentrations greater than 5 U/ml. Thus, various VWF-coated glass plates with different I-FVIII density (del-FVIII as a control, #A, #B, #C and #D as indicated in the figure) were prepared.

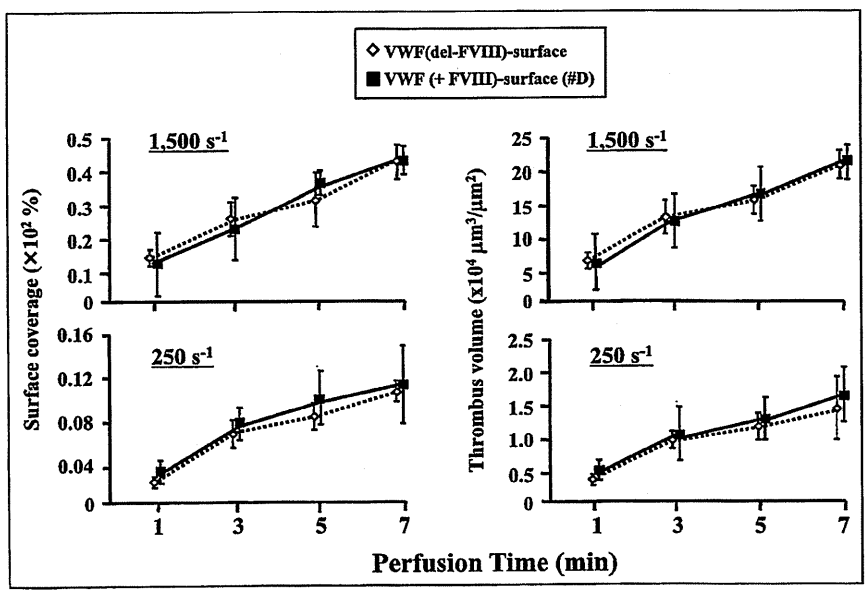

Figure 2: Time course of platelet adhesion and aggregation on a VWF-coated surface in the presence or absence of I-FVIII under high or low shear rate condition. Whole blood from healthy volunteers containing DiOC6 (1 μM)-labelled platelets, mildly anticoagulated with argatroban, was perfused over a VWF-coated glass surface with (#D) or without (del-FVIII) I-FVIII under high (1,500 s^{-1}) or low (250 s^{-1}) shear rate. The process of platelet adhesion and aggregation was evaluated by the surface coverage of thrombi generated at the time points indicated in the figure. Each data point represents mean ± SD of three independent perfusions using blood from three individual donors.

Effects of I-FVIII on platelet adhesion and aggregation on VWF-coated surface under high or low shear rate condition

To evaluate the effects of I-FVIII on basic platelet functions under flow conditions, whole blood was perfused over a VWF-coated glass surface in the presence (#D-plate; ▷Figure 1) or absence (del-FVIII) of I-FVIII under a high (1,500 s^{-1}) or low (250 s^{-1}) shear rate condition. The process of platelet adhesion and aggregation was evaluated by the time-course of surface coverage or volume of platelet thrombi generated on a VWF-coated glass surface. No significant differences in thrombus size were confirmed in those two groups (with or without I-FVIII) under both high and low shear rate conditions (▷Figure 2). Thus, I-FVIII does not seem critically involved in the platelet adhesion and aggregation under flow conditions.

Effects of I-FVIII on fibrin generation within thrombi generated on VWF-coated surface under high or low shear rate condition

Intra-thrombus fibrin generation was evaluated under flow conditions. In contrast to the basic platelet functions, the fluorescent 3D

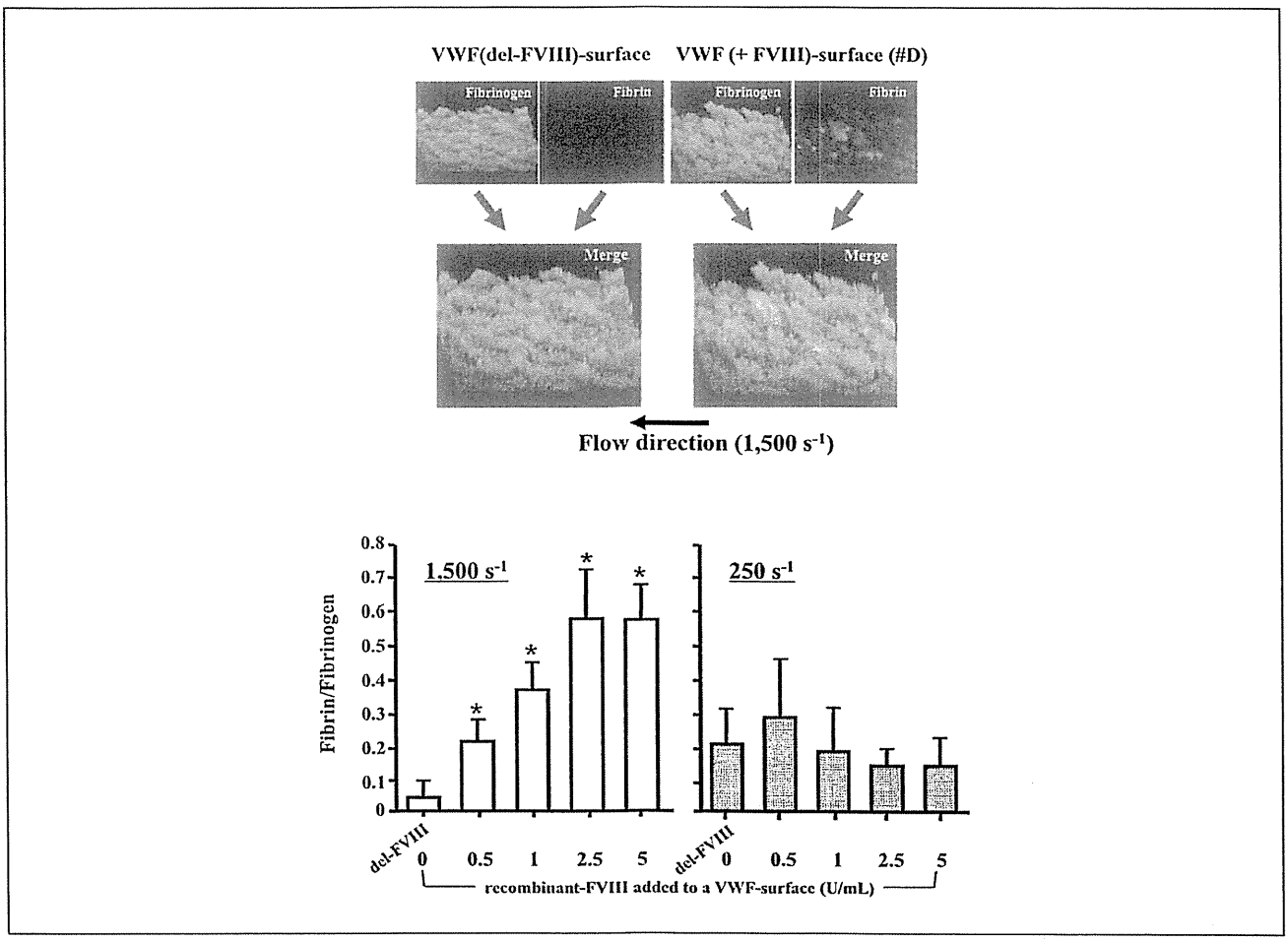

Figure 3: Functional evaluation of FVIII bound to VWF immobilised on a glass surface (I-FVIII). A) Visual evaluation of I-FVIII on fibrin generation within thrombi generated on VWF-coated surface under high shear rate condition. Citrated whole blood from healthy volunteers was perfused over a VWF-coated glass surface with or without I-FVIII under high (1,500 s^{-1}) shear rate. Just prior to perfusion, $CaCl_2$ was added to the sample blood (8 mM) to initiate blood coagulation responses. Thrombi generated on VWF-coated glass surface at 7 min-perfusion in the presence (D) or absence (del-FVIII) of I-FVIII under 1,500 s^{-1} shear were fixed, double-stained (FITC-fibrinogen: green and Cy3-fibrin: red) and viewed by CLSM. The 3D images of thrombi were representative of five independent flow experiments (original magnifications: X 600). Merged 3D images, obtained by superimposing two images of the identical portion, indicate that I-FVIII enhances the intra-thrombus fibrin deposition under high shear rate condition. B) Effects of I-FVIII on fibrin generation within thrombi generated on VWF-coated surface under high or low shear rate condition. Experimental conditions were as described in the legend to panel A. Thrombi generated on various VWF-coated glass surfaces at 7 min after perfusion were fixed, double-stained and viewed by CLSM. Bars represent mean (+ SD) fibrin/fibrinogen ratio in 25 defined areas (each 133 x 100 mm) examined (5 areas randomly selected in 5 independent perfusions of blood from 5 individual donors). Note that the intra-thrombus fibrin generation, as a function of I-FVIII, significantly (*$p < 0.01$) increased as compared to those generated in the absence of I-FVIII (del-FVIII) under high shear rate, while no effects of I-FVIII were observed under low shear rate.

images indicate that I-FVIII enhances the intra-thrombus fibrin deposition under high shear rate condition (▶Figure 3A). Statistical analysis also confirmed that the intra-thrombus fibrin generation, as a function of I-FVIII, significantly increased as compared to those generated in the absence of I-FVIII (del-FVIII) under high shear rate, while no effects of I-FVIII were observed under low shear rate (▶Figure 3B).

Effects of I-FVIII or S-FVIII on intra-thrombus fibrin generation in perfusion of synthetic "acquired" haemophilic blood under high shear rate condition

Synthetic "acquired" haemophilic blood (see ▶Table 1) was perfused over a VWF-surface in the presence or absence of I-FVIII under high shear rate condition (1,500 s^{-1}). In some experiments to evaluate S-FVIII, recombinant FVIII was added in sample synthetic haemophilic blood 30 min prior to perfusion. As shown in the ▶Figure 4, I-FVIII significantly increased fibrin generation within synthetic haemophilic thrombi in the absence of S-FVIII. The fibrin/fibrinogen ratios of haemophilic thrombi in the presence of I-FVIII are nearly equal to that of control thrombi in the absence of I-FVIII (▶Figure 4). Note also that these I-FVIII effects are unvarying regardless of anti-FVIII inhibitor levels in synthetic haemophilic blood, while the effects of S-FVIII was totally abolished at the higher inhibitor levels.

Discussion

The blood coagulation process, essential for thrombosis and haemostasis, is a solid-phase event that occurs on cell surfaces of activated platelets or endothelium (3, 17). Under rapid blood flow *in vivo*, immobilisation of clotting factors on a thrombogenic surface could be crucial for the proper coagulation responses in such solid-phase blood coagulation. In this context, we here proposed a novel concept of "I-FVIII" (FVIII-VWF complex immobilised to a surface), and evaluated the functional relevance of I-FVIII, discriminating from S-FVIII, under experimental whole blood flow conditions.

To evaluate physiologic relevance of I-FVIII, we first compared the overall process of mural thrombus formation on FVIII-free VWF immobilised to a glass surface with that on native FVIII-

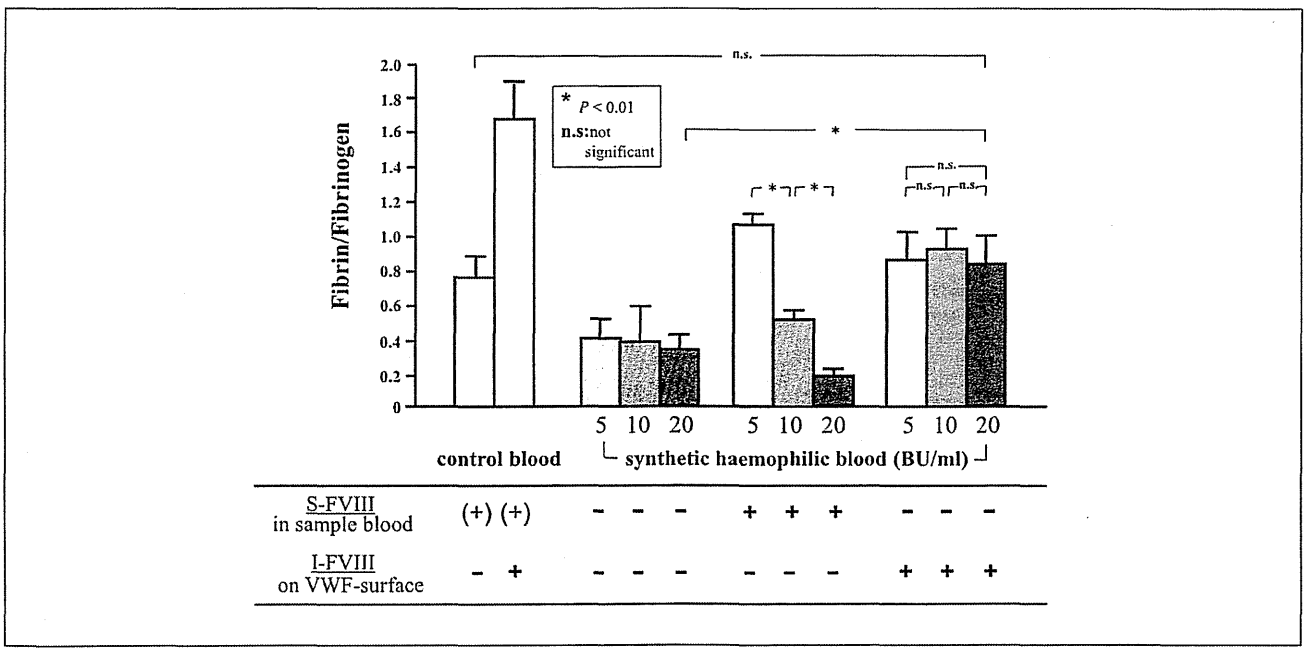

Figure 4: Effects of I-FVIII or S-FVIII on intra-thrombus fibrin generation in perfusion of synthetic "acquired" haemophilic blood under high shear rate condition. Experimental conditions are as described in the legend to Figure 3. Citrated whole blood from healthy donors rendered haemophilic by anti-FVIII human antibody was perfused over a VWF-surface in the presence (#D; indicated as "+ (plus)" in the I-FVIII column) or absence (del-FVIII; indicated as " – (minus) ") of I-FVIII under high shear rate condition (1,500 s^{-1}). Such synthetic "acquired" haemophilic blood was prepared by incubating control whole blood with varying concentrations of purified inhibitor IgG (see Table 1). In some experiments to evaluate S-FVIII, recombinant FVIII (3 U/ml) was added in sample synthetic haemophilic blood 30 min prior to perfusion (indicated as "+" in the S-FVIII column; the "+" symbol in parenthesis represents inherent FVIII present in normal blood). Bars represent mean (+ SD) fibrin/fibrinogen ratio of thrombi generated at 7-min perfusion in 15 defined areas (each 133 x 100 μm) examined (5 areas randomly selected in 3 independent sets of experiment using blood from 3 individual donors). Note that I-FVIII significantly (*p < 0.01) increased fibrin generation within synthetic haemophilic thrombi in the absence of S-FVIII. The fibrin/fibrinogen ratios of haemophilic thrombi in the presence of I-FVIII are nearly equal (n.s., not significant) to that of control thrombi in the absence of I-FVIII (see the right end and left end bars). Note also that these I-FVIII effects are unvarying (n.s., not significant) regardless of anti-FVIII inhibitor levels in synthetic haemophilic blood, while the effects of S-FVIII was totally abolished at the higher inhibitor levels.

VWF complex under flow conditions. In terms of the size of platelet thrombi as well as intra-thrombus fibrin generation, no significant differences were confirmed among those two surfaces under both high and low shear rate conditions (results not shown), suggesting the limited contribution of native I-FVIII in this regard. This consequence is not surprising because only one out of over 50 VWF subunits is presumably occupied by FVIII molecule in native FVIII-VWF complex (6, 18, 19). In order to further explore the functional relevance of I-FVIII, we therefore exploited this FVIII-binding capacity of native VWF immobilised to the surface. We added an excess amount of exogenous FVIII to immobilised VWF and successfully prepared several VWF-surfaces with different I-FVIII density (▶Figure 1).

Despite sufficient levels of S-FVIII inherently present in normal control blood, I-FVIII significantly enhanced blood coagulation in a concentration-dependent manner within platelet thrombi under high shear rate conditions (▶Figure 3), albeit with no appreciable effects on basic platelet functions (▶Figure 2). In general, the increase of fibrin generation is associated with the increase of final thrombus volume under such high shear rate conditions (7). These discrepant results may be due at least in part to the different anticoagulation of sample blood; i.e. a small amount of thrombin inhibitor argatroban was used for the evaluation of platelet adhesion and aggregation, while the intra-thrombus fibrin generation was evaluated with recalcified citrated blood.

Unlike classic coagulation assays such as aPTT that evaluate fibrin clot formation in soluble phase, the intra-thrombus fibrin generation in our experimental approach reflects solid-phase blood coagulation on platelet surfaces and may be more representative of *in vivo* haemostasis (3, 17). Thus, it is assumed that local concentrations of several clotting factors must be sustained for the proper protease-substrate reactions under blood rheological situations *in vivo* (20-22). In this regard, when clotting factors are tightly immobilised on local thrombogenic sites, they may work better under blood flow conditions than those flowing in the bloodstream. Indeed, this scenario may be consistent with our observations that the effects of I-FVIII on solid-phase blood coagulation are very profound under high shear rate conditions (▶Figure 3B), where blood flow is so rapid that soluble blood clotting proteins could be easily washed out from the local thrombogenic sites. In contrast, S-FVIII may be able to contribute more efficiently to flow-dependent fibrin generation in the absence of I-FVIII under low shear rate conditions where blood flow is relatively slow.

In light of recent modelling studies incorporating the coagulation cascade and platelet deposition under flow, thrombus growth is assumed to be limited by the transport of clotting factor zymogens into the interior of thrombus (20, 22). I-FVIII fixed at the central core of generating thrombi could be apparently advantageous for such coagulation responses under flow, as compared to S-FVIII which must bind first to platelet surfaces and penetrate into thrombus against blood flow. Thus, an unusually high density of I-FVIII bound to VWF on the basal layer of a thrombogenic surface can sufficiently compensate for the complete lack of S-FVIII in the bloodstream, as seen in the synthetic "acquired" haemophilic blood (▶Table 1, ▶Figure 4). Interestingly, the effects of I-FVIII on synthetic haemophilic blood, unlike S-FVIII, were unvarying regardless of the anti-FVIII inhibitor titre in the blood under high shear flow conditions (▶Figure 4). Presumably, anti-FVIII IgGs in the bloodstream cannot easily interact with and neutralise I-FVIII when blood flow is quite fast as is the case under high shear rate conditions.

Taken together, these findings may give a clue for a novel therapeutic approach against patients with haemophilia and high titer of anti-FVIII inhibitors. Since I-FVIII bound to VWF at sites of vessel injury is more resistant to inhibitor attack compared to S-FVIII, I-FVIII could effectively enhance the coagulation potentials of blood from such haemophilic patients.

What is known about this topic?

- Coagulation factor VIII (FVIII) plays a pivotal role as a cofactor in factor X activation by activated factor IX, thus drastically amplifying thrombin generation in the coagulation process.
- Physiologic activity of FVIII is so far evaluated mostly by plasma coagulation assays that determine the capability of fibrin clot formation in closed stirring systems *in vitro*.
- However, experimental conditions of such soluble-phase assays differ considerably from *in vivo* haemostatic conditions, in which solid-phase blood coagulation occurs on platelet surfaces under whole blood flow.

What does this paper add?

- We focused on FVIII-von Willebrand factor (VWF) complex immobilised to thrombogenic surfaces as a solid-phase source of FVIII (immobilised FVIII; I-FVIII), and were able to discriminate between I-FVIII and those circulating in the bloodstream (soluble FVIII; S-FVIII).
- Using a perfusion chamber system, we show that I-FVIII, independent of S-FVIII, plays a role in the intra-thrombus fibrin-network formation in mural thrombus generation under high shear rate conditions. In the absence of S-FVIII, I-FVIII normalised in a dose-dependent manner the reduced fibrin deposition in synthetic haemophilic blood regardless of the circulating anti-FVIII inhibitor titre.
- Our results may imply the alternative therapeutic potentials of targeting I-FVIII for patients with haemophilia and high titre anti-FVIII inhibitors.

Conflicts of interest
None declared.

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特 集
臨床血液学　今後の展望（2013年版）—血小板・凝固・線溶系疾患—

血友病

嶋　緑倫

Key words : Hemophilia A, Hemophilia B, Inhibitor

はじめに

定期補充療法の普及に伴い，わが国における血友病患者のQOLは確実に向上している。特に小児期の患者では非血友病者と同等の活動性を維持することが可能になりつつある。しかしながら，頻回の製剤投与には多大な精神的・身体的苦痛を与え血友病患者をささえる家族や支援者にも負担をしいている現状がある。また，抗第VIII因子あるいは抗第IX因子同種抗体（インヒビター）が発生すると血友病補充療法の止血効果は激減～消失するために，患者の止血管理に難渋することになる。したがって，現在の血友病止血治療の課題は，より長時間作用する治療とインヒビター保有例の対策である。

2012年度の血友病に関する文献を総括すると，長時間型製剤の開発と定期補充療法の有用性，医療経済的側面に関する論文が多い。さらにインヒビターの発生要因と製剤に関する問題が再提起されている。また，新たな止血治療のコンセプトや将来の「血友病を治癒する」遺伝子治療関連の研究成果が注目される。

I　長時間型作用補充療法製剤の開発と課題

長時間作用型第VIII因子（FVIII）あるいは第IX因子（FIX）製剤の臨床試験は2012年に入って急速に進んでいる[1]。2013年には各長時間作用型製剤の第3相の臨床試験の成績が明らかにされると思われる。さらに，最近，Fc蛋白融合FIX因子製剤およびFVIII製剤の第3相試験の結果がプレスリリースされた（http://www.biogenidec.com/PRESS_RELEASE_DETAILS.aspx?ID=5981&ReqId=1738359, http://www.biogenidec.com/PRESS_RELEASE_DETAILS.aspx?ID=5981&ReqId=1752097）。Fc融合FIX製剤では115名が治験を終了し，半減期は82時間（対照rFIX製剤は34時間），100単位/kg，10～14日毎の投与で年間出血回数1.38回であったことから2週間に1回の投与でも予防効果があることが発表されている。Fc融合FVIII製剤では153例が治験を終了し半減期の中央値は19時間（対照rFVIII製剤は12.4時間）で，25～65単位/kg 3～5日の投与で年間出血回数1.6回，65単位/kg 1回/週投与で年間出血回数は3.6回であった。したがって，週1回の投与はやや予防効果は劣るが，週2回の投与で十分予防効果を期待できるものと思われる。

奈良県立医科大学　小児科

II　定期補充療法の動向

1. 定期補充療法の目標と医療経済的側面

血友病治療の基本は血漿由来高純度製剤や遺伝子組み換え型製剤の導入や早期定期補充療法が血友病性関節症の発症を抑制するエビデンス[2]の集積により，オンデマンド止血治療から「出血を予防する」定期補充療法に確実にシフトしつつある。この傾向は，20歳までの若年患者で特に顕著で，最新の全国調査[3]によると70%の血友病A患者が定期補充療法を実施している。20歳以降の成人患者の実施率は40%と低いものの，ちょうど，5年前の全国調査では20%であり，この5年間で成人患者の定期補充の実施率は倍増している。血友病B患者では，血友病A患者と比較して定期補充療法の実施率は低いが，この5年間に20歳までの実施率が増加している。長時間作用型製剤の導入後，さらにこの傾向が強くなるものと予想される。長時間作用型製剤の導入により，より高いトラフレベルを長期間維持できる。例えば，通常，定期補充療法の基本的概念はトラフを>1%に維持することであるが，長時間作用型rFIX製剤では投与量によっては週1回投与でも軽症レベル（>5%）や止血レベル（>20%）を維持できる（図1）。したがって，今後，トラフレベルをどこに設定するのかが重要な課題となる。これは，血友病患者のQOLの向上と，医

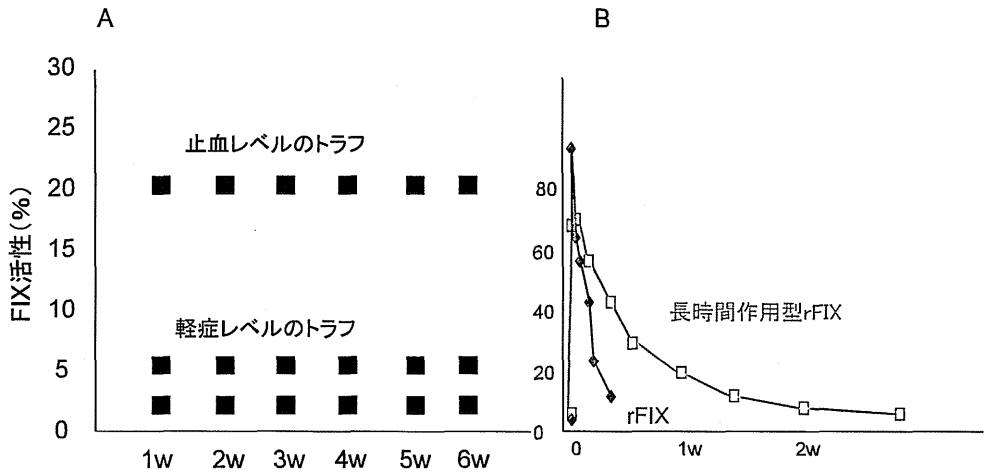

図1 長時間作用型 rFIX 製剤の PK と目標トラフ

A 定期補充療法における現在のトラフ目標は凝固因子活性を＞1%に維持することである。長時間作用型製剤の導入により，トラフを軽症レベル以上＞5%，あるいは止血レベルである＞20%を維持することも可能である。

B 長時間作用型製剤では半減期が伸びるために投与量によりトラフ値を上昇することが可能になる。

療経済のバランスというきわめてむつかしい課題に直面することになる。医療経済的資源は無限ではない。血友病診療における医療コストの 90%以上が高額な治療製剤費である。インヒビターの治療製剤はさらに高額で，わが国でも，毎年の高額レセプトの上位を血友病患者が占めている。 国際的にみると，定期補充療法が実施されている国は欧米諸国を中心で多くの国はいまだにオンデマンド療法が中心である。それぞれの国の財政に応じた治療選択が実施されているのが現状である。

2. 定期補充療法個別化の必要性

血友病医療においても限られた医療資源の中で“cost effectiveness”を考慮することは重要である。定期補充療法の投与量の決定においても，画一的な投与量の設定より，患者毎に様々な因子を総合評価して投与レジメの個別化をはかることはきわめて重要であり，今後，長時間作用型製剤が導入される場合，ますます必要となろう。実際，体重あたり同じ投与量でも，出血予防効果は患者により異なる。また，定期補充療法の基本はトラフを＞1%に維持して，重症を中等症にすることであるが，＞1%では予防効果が不十分な例もあるし，＜1%でも十分な予防効果が得られる場合もある。したがって，トラフや投与量のみではなく，より凝固機能，年齢，活動性，標的関節の有無などを総合的に考慮すべきである[4]。

3. 成人患者の定期補充療法

成人患者の定期補充は出血回数はオンデマンド治療の患者と比較して激減するものの関節症の進行には無効であるというのが定説であり，成人患者の定期補充の有効性に関するエビデンスは少ない。しかしながら，近年，成人患者における定期補充療法の有用性に関する報告が散見されるようになった[5~7]。わが国の全国調査においても定期補充療法を実施している成人の患者が増加している。Noone らは 18～35 歳の重症血友病患者計 124 例を対象に比較検討を行った。対象患者の内訳はいつもオンデマンド群（26 例），生涯の＜50%定期補充群（26 例），生涯の＞50%定期補充群（35 例），いつも定期補充群（15 例）で，出血回数，可動性，重篤な出血，出血に基づく疼痛，就労状況などについて各群別に評価を行った。長期間の定期補充療法群では，概ね，標的関節が少ない（$p<0.001$），重篤な出血が少ない（$p<0.05$），反復出血が少ない（$p<0.01$），外科手術が必要ではない（$p<0.05$）という結果であった。さらに，移動性，日常の活動性，疼痛や不安感などを評価する健康に関する有用性においてはオンデマンド群が明らかに低かった（$p<0.01$）。したがって，成人患者においても定期補充療法は，出血回数を減少させるのみならず，日常生活の QOL も向上することが示唆される[8]。

はたして，成人の重症血友病患者全員に定期補充療法をすすめるべきかについては議論の余地がある。患者の活動性や医療経済などについて考慮する必要がある。また，定期補充を継続することが困難な場合も多い。その理由の上位は，出血回数の減少，症状が消失すること，忘れること，時間が少ない等である[9]。Fischer らは定

期補充を実施していた患者が成人期に達したとき，関節の状況が良好でほとんど出血がみられていない場合は投与量や投与間隔を減少させて中止することもひとつの選択であることを勧めている[10]。ただし，長時間作用型製剤により自己注射の遵守率が高くなる可能性もあり，定期補充の継続を希望する患者が増加することも十分考えられる。

III インヒビター陽性例の動向

インヒビターの発生は血友病の止血治療における重大な問題であり，課題でもある。インヒビターの発生率は重症血友病A患者で20～30%，重症血友病Bで3～5%と血友病Aの方が多い。インヒビター陽性例の治療目標は，インヒビターの発生を防ぐこと，インヒビターの消失をはかること，インヒビター陽性例にも有効な止血治療を確立することである。

1. インヒビター発生要因と予防

インヒビターの発生を防ぐためには，インヒビターの発生要因を明らかにしてインヒビターの発生リスクを評価することがまず必要である。インヒビターの発生リスクは大きく遺伝的因子と非遺伝的因子に分けられる。遺伝的因子としては，第VIII因子あるいは第IX因子の遺伝子異常が重要である。特に，欠失，イントロン22由来逆位，ノンセンス点変異などのいわゆるnull遺伝子異常におけるインヒビター発生率は高くハイリスク遺伝子異常と考えられている。しかしながら，血友病の兄弟を対象とした調査研究によると，必ずしも遺伝子異常とインヒビター発生が一致しない兄弟例もあり，その他の遺伝的なリスクの存在も示唆される。Astermarkらは，IL-10，TNFα，CTLA-4などの遺伝子がインヒビターの発生に関連することを報告している[11]。

早期の定期補充開始によるインヒビターの発生予防効果については，最近の話題のひとつである。この概念は定期補充群がオンデマンド群よりインヒビターの発生率が低かったことがCanal研究により発表されたことと[12]，組織傷害や感染等の炎症のようないわゆるdanger signalを基盤としたインヒビター発生機序の仮説[13]に基づいている。実際，出血症状が出現する10ヶ月ごろから25単位/kg週1回で開始してインヒビターの発生リスクの高い50投与日数までFVIII刺激やdanger signalを防ぎ，以後投与回数を増やして関節出血を防ぐプロトコールでインヒビターの発生率が従来の1次的補充療法群より低下したことが報告された[13]。しかしながらこれは単一施設の報告で，多施設による追試が必要である。

遺伝子組み換え型FVIII製剤（rFVIII）と血漿由来FVIII製剤（pdFVIII）との間でインヒビターの発生率について差があるのかについては未だに結論は出ていない[14~17]。Mancusoらは製剤別，製剤の純度別のインヒビター発生率に関するコホート調査を実施した[18]。対象患者数は721例である。報告によるとインヒビター発生集積率は重症血友病A 565例中27%で，pdFVIII，rFVIIIではそれぞれ22%，49%であった。さらに，high responding例は22%で，製剤別では18%，44%と全インヒビターおよびhigh responding inhibitorともにrFVIII＞pdFVIIIであった。また，製剤の純度別では低中間純度製剤，高純度pdFVIII製剤，rFVIII製剤でのインヒビター発生率はそれぞれ18%，34%，43%と純度によりインヒビター発生率が左右されていることが示唆された。最近発表された2000～2010年に生まれた重症血友病A患者574名を対象としたRODIN studyの調査報告によると，インヒビターの発生率は血漿由来製剤と遺伝子組み換え型製剤との間では有意な差がないこと，von Willebrand因子の含有の程度とも関連しないこと，さらに，第2世代のrFVIII製剤のインヒビター発生率が第3世代のrFVIIIより有意に高いという結果であった[19]。製剤とインヒビターの発生率についてはいまだに議論の多いところであり，結論は出ていない。より明らかにするためには前向き無作為調査が必要である。現在，高純度pdFVIII製剤とrFVIII製剤による前向き調査（SIPPET study; Survey of Inhibitors in Plasama Product Exposed Toddlers）が実施されている。わが国でも厚生労働省エイズ対策研究事業血友病の治療とその合併症の克服に関する研究班においてインヒビター発生患者の実態調査が実施された。41施設から116例のインヒビター症例が登録された。インヒビター発生郡と非発生群で有意差のある背景因子は単編量解析では血友病の重症度（p 0.0243），血友病Aインヒビターの家族歴（p 0.0001），FVIII製剤初回投与時の年齢で，ロジスティック回帰解析ではインヒビターの家族歴のみ有意であった。pdFVIII製剤のみを使用した群とrFVIII製剤のみを使用した群との間でインヒビターの発生率に差は見られなかった[20]。

2. 今までに治療歴のある患者（PTPs）におけるインヒビター発生

製剤変更後のインヒビター発生に関して，これまで十分なエビデンスは報告されていない。製剤変更後のインヒビター発生リスクを評価するためには，今までに治療歴のある患者（PTPs: Previously Treated Patients）でのインヒビター発生率に関するデータが必要である。Alfonsoらの総説[21]によるとPTPsにおけるインヒビター発生率は1995年のColvinらの報告[22]では1.5%，Darbyら（2004年）の報告[23]では15歳以上で2.0%，5～14歳で2.9%，Kempton（2006年）らの報告[24]では

2.1%，最近の Hay らの報告（2011 年）[25] では 10～49 歳で 5.3%，50～59 歳で 5.2%である。したがって，2～5%が概ね PTPs におけるインヒビター発生率といえる。興味深いことには年々，PTPs のインヒビター発生率が上昇していることである。実際，1995 年と 2011 年では 3 倍の差がある。PTPs におけるインヒビターの発生に関する認識が高まってきたこと，インヒビターの測定がより頻回になっていることが，インヒビター発生率の増加の原因と考えられるが，一方で定期補充療法の普及により製剤の投与量が増加していることや製剤の変更がより多くなってきたことも否定はできない。

Aledort らは全長型の rFVIII 製剤（FL-rFVIII）と B ドメイン除去 rFVIII（BDD-rFVIII）の投与を受けた PTPs におけるインヒビター発生についてメタ解析を行った[26]。解析対象となった調査研究は 29 で計 3,012 例の PTPs が含まれている。そのうち，インヒビターの発生例は 35 例で発生率は 1.25%であった。BDD-rFVIII に関連したインヒビター発生率は FL-rFVIII より高値であった。したがって，著者らは BDD-rFVIII の免疫原性が FL-rFVIII より高いことを述べている。しかしながら，本研究については BDD-rFVIII に変更しなかったコントロール群の欠如による統計的弱点が指摘されている[27, 28]。

いずれにしても，現時点では製剤変更とインヒビター発生との関連性に関する明らかなエビデンスはない。PTP におけるインヒビター発生率に関する基礎的データと十分なコントロールを考慮した調査研究が必要である。現在 2 つの調査研究プロジェクト（EUHASS: European Hemophilia Surveillance Scheme[29]），（The National Institutes of Health inhibitor study[30]）が実施されている。

3. 免疫寛容導入療法の動向と展望

インヒビターの消失をはかる免疫寛容療法（ITI: Immune tolerance induction）の有用性は国際的に認知され，ITI は我が国でもインヒビター陽性例の重要な治療法になっている。しかしながら，開始のタイミング，投与量，製剤の選択などはいまだに標準化されていない。ITI の有効性と製剤の投与量や種類との関連性について，2002 年から国際共同研究が開始された。低用量群の出血症状が多かったことが原因で本研究は中止となったが，最近，これまでの研究結果が発表された[31]。

1. 免疫寛容導入誘導療法国際研究の結果

本研究は 17 か国，計 70 施設が参加した前向き無作為調査でエビデンスレベルは高い。我が国も参加している。患者の基準は，重症血友病 A，インヒビター力価の過去最高値が 5～200 BU/m*l*，登録時のインヒビター力価が 10 BU/m*l* 未満，インヒビター力価が 12 か月以内に 10 BU/m*l* 未満になること，登録時の年齢が 8 歳未満である。ITI のプロトコールは，高用量群（200 単位/kg 連日投与），低用量群（50 単位/kg 3 回/週）で，計 115 症例が無作為別に両治療群に振り分けられた。終了した症例は 78 例で寛容成功例は 37 例，部分成功例 3 例であった。

1）ITI 成功因子

単変量解析によると有意な ITI 成功因子は，今までのインヒビター最高値（p 0.026）と ITI 中の最高値（p 0.002）であった。しかしながら，多変量解析では経過中のインヒビター力価最高値のみが有意であった。従来，ITI 開始時のインヒビター力価が有意な成功因子と考えられているが（ITTR: International Immune Tolerance Registry[32], NAITR: North American Immune Tolerance Registry[33]），本研究ではすでに ITI 開始時のインヒビター力価が＜10 BU/m*l* の患者の基準があるため，成功因子としての有意性は明らかでなかった。しかしながら，この結果は，ITI 開始時のインヒビターは ＜10 BU/m*l* であればその範囲内でのインヒビター力価は成功率に関係しないことを示している。

2）各治療段階における成功までの期間（表 1）

インヒビター消失およびインヒビター消失から正常回収率までの期間では高用量（HD）群が統計学的有意にと低用量（LD）群で比較して短縮していたが，正常回収率までの全 ITI 期間において有意差はみられなかった。

3）ITI 治療期間中の出血回数（表 2）

ITI 期間中の出血回数は LD 群のほうが有意に多かった（p 0.019）。各治療段階別に評価すると，出血回数の有意差はインヒビター消失までの期間で明らかであったが，消失後，回収率が正常になるまでの期間において有意差はみられなかった。

4）製剤と ITI 成功率

VWF 含有製剤の成功率が 91%であるのに対して，高純度 FVIII 製剤の成功率は 29%と，vWF 含有 FVIII 製剤がより有効であることを後ろ向き調査で報告されている[34]。しかしながら，ITI 成功率と製剤との関連性についてはいまだに議論の多いところである[35]。今回の国際研究では，90%が rFVIII 製剤を選択しており，この問題に関して明らかなエビデンスをもたらすことはできなかった。最近，ITI 未実施の不良リスク群（過去のインヒビター力価最高値＞200 BU/m*l*，ITI 開始時のインヒビター力価＞10 BU/m*l*，インヒビター診断から＞5 年経過）を対象に vWF 含有製剤あるいは rFVIII 製剤 200 単位/kg 投与による ITI の前向き調査（RESIST 研究）が実施されている[36, 37]。

表1 両治療群における ITI 各段階までの期間

	中央値		P value
	低用量群	高用量群	
BU 陰性化までの期間（n＝60）	n＝29 9.2（4.9～17.0）	n＝31 4.6（2.8～13.7）	0.017
回収率正常化までの期間（n＝50）	n＝27 13.6（9.7～18.9）	n＝23 6.9（3.5～11.9）	0.001
半減期正常化まで（n＝47）	n＝25 15.6（10.8～22.0）	n＝22 10.6（5.9～20.5）	0.096

表2 各治療群と研究段階における出血率（全出血数/月）

ITI の各段階	治療群	平均	中央値	IQ Range	P value
BU 陰性化まで	低用量群	0.63	0.56	0.09～0.89	0.0001
	高用量群	0.28	0.00	0.00～0.44	
回収率正常化まで	低用量群	0.157	0.00	0.00～0.07	0.283
	高用量群	0.087	0.00	0.00～0.00	
半減期正常化まで	低用量群	0.150	0.00	0.00～0.15	0.552
	高用量群	0.033	0.00	0.00～0.00	
定期補充投与期間	低用量群	0.175	0.151	0.00～0.22	0.112
	高用量群	0.102	0.000	0.00～0.23	

2. 血友病 B インヒビターの ITI

血友病 B インヒビターの ITI については症例数が少なく，明らかな推奨プロトコールが存在しない。NAITR では成功率は 5/16 例（31%）であった。また，血友病 B インヒビターの ITI 実施においてはアレルギー症状やネフローゼ症候群の発生リスクを十分に念頭をおく必要がある[34]。特に，アレルギー歴のある患者についてはあらかじめ脱感作を検討すべきである[38]。

3. 今後の ITI の課題

国際研究の結果から投与量については最終的な成功率には差がないことが明らかになったが，インヒビター消失までの期間が HD 群では有意に短く，また，出血回数も少ないことが判明した。実際，これらの結果をわが国の ITI 治療にどのように反映するかがきわめて重要である。年齢，投与の実行性，医療経済的側面など様々な要因を考慮して決定する必要がある。幼少期の患者では比較的投与量が少なく HD の ITI も考慮してもよいかもしれない。ただ，ITI の成功率を現行の治療法でさらに向上させるには限界があり，新たな免疫的アプローチの確立が必要と思われる。

IV 新規血友病止血治療製剤の開発

理想的な血友病の止血療法治療製剤の条件として，長時間作用すること，投与が平易であること，インヒビター保有例にも有効であることなどが挙げられる。現状では，これらの条件を満たす製剤はないが，最近，まったく新たな概念の止血治療製剤が開発されている。

1. 抗 TFPI 療法

現在の凝固反応は Monroe らの提唱した細胞基盤型凝固モデルで理解されている。本モデルによると，出血がおこると，組織因子（TF: Tissue factor）が活性型第 VII 因子（FVIIa）と結合して第 X 因子（FX）が活性型第 X 因子（FXa）に変換される（*Initiation*）。FXa は微量のトロンビンを産生させるが，この段階ではフィブリンを形成するには不十分であるが血小板，FVIII，第 V 因子（FV）を活性化することにより凝固反応は増幅し（*Propagation*），結果的にトロンビンが爆発的に産生され（*Thrombin burst*）安定したフィブリンが形成される。Initiation 相の FVIIa/TF の機能は，組織因子経路インヒビター（TFPI: Tissue factor pathway inhibitor）により制

御されている。したがって，TFPIをブロックすることにより，止血効果を期待することができる。このコンセプトで，抗TFPI物質，アプタマー，抗体，ペプチドなどが開発されているが，最近，ヒト型抗TFPI抗体の第1相臨床試験が開始された。血友病Aの家兎モデルに本抗体を投与するとAPTTは正常化し，出血症状を抑制，予防できることが報告された[39]。

2. FVIII代替バイスペシフィック抗体

FVIIIはFIXaによるFX活性化反応の補因子として機能していると理解されてきたが，その補因子機能の発現機序はわかっていなかった。FVIIIはFXa生成反応におけるKmを低下させ，Vmaxを約2万倍増加させる。したがって，FVIIIは凝固反応系の律速段階であるFXa生成反応のアクセルの役目を有する。FVIIIがFIXaとFXとの間に介在することにより両分子が作用しやすい立体的位置に維持することがFVIII補因子作用であるとの仮説のもとに，一方の手がFIXa，もう一方がFXと結合する2重特異性のバイスペシフィック抗体が創製された（図2）本抗体はヒト型遺伝子組み換え型抗体として改変され血友病A患者血漿のAPTTやトロンビン生成を改善すること，抗FVIII抗体を投与して作製されたサル後天性血友病Aモデルに投与すると出血症状が抑制することが発表された（図3）[38]。

本製剤は抗体製剤であり，皮下投与が可能である。さらに，半減期も長く，1～2週毎の投与で出血予防レベルを維持できる可能性がある。さらに，抗TFPI抗体と同様，本抗体の作用機序にFVIII自体は関与しておらず，インヒビターの存在には全く影響されない。すなわちインヒビター保有患者においても非保有患者と全く同等の作用を期待することができるメリットがある。

V　血友病遺伝子治療に関する動向

遺伝子治療は将来の血友病治療として最も期待されている。基礎研究に関しては20年前から現在までに1,000編もの論文が発表されている[40]。FIX遺伝子サイズが小さいことから血友病Bの遺伝子治療に関する研究が血友病Aより進んでいる。Adeno-associated Virus（AAV）が病原性はなく染色体に取り込まれないためにもっとも有用なベクターとして考えられてきた。当初は筋肉内注射による投与法が主体であったが，発現は一過性であり，また，ベクターに対する免疫反応のために投与8週後にFIX遺伝子がトランスフェクトされた肝細胞は消失することが判明し，臨床応用は困難と考えられた。St Jude Children Hospital と University College London の

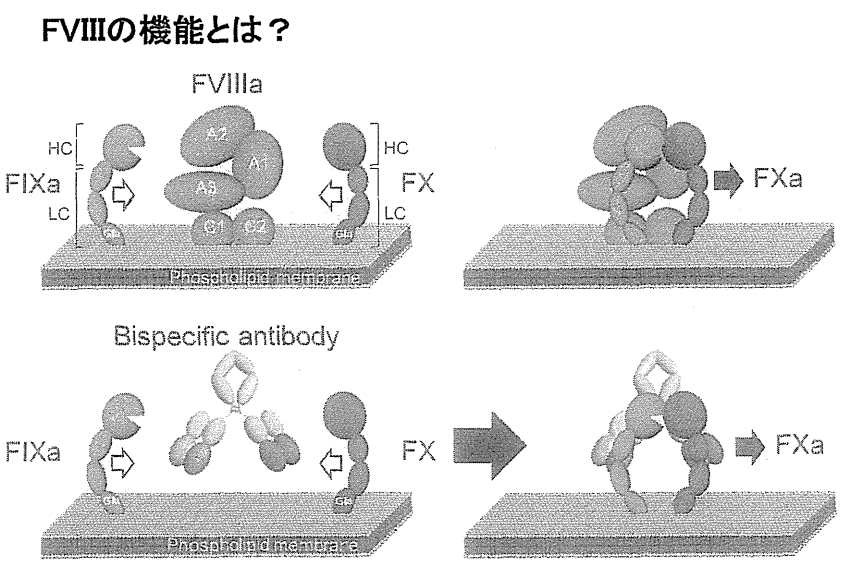

図2　バイスペシフィック抗体のFVIII代替作用機序
活性化された第VIII因子（FVIIIa）は活性型第IX因子（FIXa）と第X因子（FX）の間に介在して両者がいいい位置関係になるようにサポートする。そのために，セリンプロテアーゼであるFIXaが基質のFXに結合・開裂させ活性型第X因子（FXa）が生成される。バイスペシフィック抗体は，一方の手がFIXa，もう一方手がFXを認識するために本抗体はFVIIIaと同様にFIXaがFXに反応しやすい位置関係をもたらすことによりFVIIIaと同様の作用を発揮する。

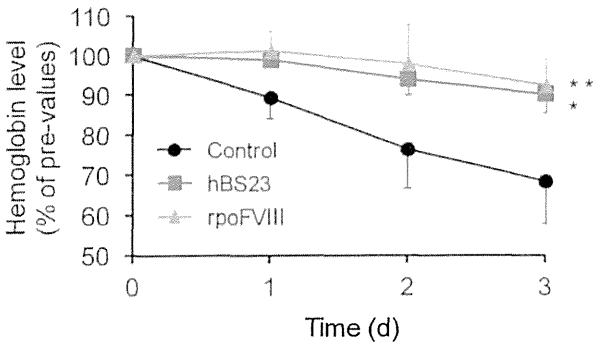

図3 バイスペシフィック抗体の出血惹起血友病Aサルモデルにおける止血効果
抗FVIII抗体を投して確立したサル血友病Aモデルに出血刺激を行うとヘモグロビンレベルは低下するが，あらかじめバイスペシフィック抗体（hBS23）を投与するとヘモグロビンの低下は抑制された。また，その効果はブタFVIII（rpoFVIII）と同等であった。

研究チームは生理的な凝固因子の産生部位である肝臓に特異的な遺伝子発現が可能となるAAV8ベクターを新たに開発した。本ベクターは，安全性が高く，マウスやサルでの実験では従来のベクターより100倍発現効果が高く，系静脈投与が可能である[41,42]。最近，6名の血友病B患者を対象に米国と英国で本ベクターによる臨床試験が実施された[43]。6名はベクターの投与量別に，低用量（LD: 2×10^{11} vg/kg），中等量（ID: 6×10^{11} vg/kg），高用量（HD: 2×10^{12}）の3群に分けられた。第一例（LD）はベクター投与後2年間にわたり2%のFIX活性を維持し定期補充療法を中止することができた。第2例（LD）は第一例と同様2%のFIX活性を維持できたが，重症の関節症のために定期補充療法の投与の継続が必要であった。第3例（ID）も2%を維持したが，定期補充の続行が必要であった。症例4（ID）は4%を15か月にわたり維持し，定期補充の必要はなくなった。症例5（HD）は5〜7%まで上昇したが，肝酵素ALTの上昇とともに発現レベルは3%まで低下した。酵素レベルはステロイド投与で低下し，オンデマンドで追加投与を実施している。症例6（HD）ではFIXレベルは8〜10%に増加したが，投与8週後にALTの上昇がみられFIXの発現レベルは一過性に低下した。本症例もステロイド投与でALTは低下し，FIXも5%で維持し，現在出血もなく予防投与も不要な状態で維持している。ALTの上昇がみられたのはいずれもHD群で，ベクターによる肝機能異常はベクターの投与量に関係することが示唆される。

今回の対象患者はHIV陰性，HCV RNA陰性，抗AAV8抗体陰性とかなり限定されている。今後，広範囲の患者に適応できるか，血友病Aの遺伝子治療についても応用が可能であるかなどが課題であるが，血友病Bの遺伝子治療の臨床応用は着実に近づいている[44]。

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The mild phenotype in severe hemophilia A with Arg1781His mutation is associated with enhanced binding affinity of factor VIII for factor X

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Summary

The clinical severity in some patients with haemophilia A appears to be unrelated to the levels of factor (F)VIII activity (FVIII:C), but mechanisms are poorly understood. We have investigated a patient with a FVIII gene mutation at Arg^{1781} to His (R1781H) presenting with a mild phenotype despite FVIII:C of 0.9 IU/dl. Rotational thromboelastometry using the patient's whole blood demonstrated that the clot time and clot firmness were comparable to those usually observed at FVIII:C 5–10 IU/dl. Thrombin and FXa assays using plasma samples also showed that the peak levels of thrombin formation and the initial rate of FXa generation were comparable to those observed at FVIII:C 5–10 IU/dl. The results suggested a significantly greater haemostatic potential in this individual than in those with severe phenotype. The addition of incremental amounts of FX to control plasma with FVIII:C 0.9 IU/dl in clot waveform analyses suggested that the enhanced functional tenase assembly might have been related to changes in association between FVIII and FX. To further investigate this mechanism, we prepared a stably expressed, recombinant, B-domainless FVIII R1781H mutant. Thrombin generation assays using mixtures of control plasma and FVIII revealed that the coagulation function observed with the R1781H mutant (0.9 IU/dl) was comparable to that seen with wild-type FVIII:C at ~5 IU/dl. In addition, the R1781H mutant demonstrated an ~1.9-fold decrease in K_m for FX compared to wild type. These results indicated that relatively enhanced binding affinity of FVIII R1781H for FX appeared to moderate the severity of the haemophilia A phenotype.

Keywords

Haemophilia A, FVIII, clinical phenotype, FX, association

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Introduction

Haemophilia A results from a deficiency or defect of the coagulant protein, factor (F)VIII, and is the most common of the severe, inherited bleeding disorders. Clinical phenotype in haemophilia generally correlates well with the level of FVIII activity (FVIII:C), and on this basis, patients are classified into three categories: severe (FVIII:C <1 IU/dl), moderate (1-5 IU/dl), and mild type (>5 IU/dl). Patients with severe haemophilia A typically develop spontaneous haemorrhage into joints or muscles that require frequent FVIII replacement (1, 2). The severity and frequency of bleeding may be different, however, in haemophiliacs with similar plasma levels of FVIII, and a mild bleeding phenotype has been reported in 10-15% of individuals with severe plasma FVIII deficiency (1, 2). The reasons for this heterogeneity of clinical expression in severe haemophilia A remain to be fully clarified. A recent study by Santagostino et al. (3) using multivariable logistic regression analysis suggested that non-null mutations (for example missense mutations) of FVIII genes (*F8*) might represent the main determinant for bleeding tendency. Other coagulation factors, including natural anticoagulants, platelets, and fibrinolytic proteins appear to have negligible roles as modulators of disease severity (4, 5).

Accurate measurements of blood coagulation *in vitro* are essential for complete clinical assessment of clotting function. Conventional one-stage clotting assays (prothrombin time; PT and activated partial thromboplastin time; APTT) are useful for routine laboratory examination, but they only partially reflect coagulation in a non-physiological environment and are based on the classical concepts of intrinsic and extrinsic cascade mechanisms. It might be, therefore, that the use of this basic methodology might contribute to the discrepancy between the coagulant activity and clinical phenotype. More recently, interest has focused on global coagulation assays, developed from a better understanding of the coagulation reaction involving tissue factor (TF)-triggered, cell-based mechanisms generating thrombin on activated platelets. Tests of this nature such as thromboelastography, specific throm-

bin generation tests, and clot waveform analysis have been established (6-8).

FVIII, which circulates in plasma in complex with von Willebrand factor, functions as a cofactor in the tenase complex, and is responsible for phospholipid (PL)-dependent conversion of FX to FXa by FIXa (9). FVIII is synthesised as a single chain molecule consisting of 2,332 amino acid residues with a molecular mass of ~300 kDa arranged into three domains, A1-A2-B-A3-C1-C2, based on amino acid homology. FVIII 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 together with heterogeneous fragments of proteolysed B domain linked to a light chain consisting of the A3, C1, and C2 domains (10). Assembly of the tenase complex is markedly enhanced by conversion of FVIII to FVIIIa following limited proteolysis by thrombin (or FXa) (11). The coagulant activity of the tenase complex is dependent on direct interaction of FVIIIa with both FIXa and FX on the PL vesicles (12).

We have identified a patient with severe haemophilia A (FVIII:C 0.9 IU/dl) with a mild bleeding tendency and with an *F8* point mutation at Arg^{1781} to His (R1781H). The haemophilia A database (HAMSTERs [13]) indicates that haemophiliacs with this mutation are clinically heterogeneous and reflect phenotypes ranging from severe to mild/moderate. We have investigated mechanisms of FVIII deficiency in this individual using several global coagulation assays and a recombinant FVIII mutant. The assays demonstrated that coagulation function of the patient's native plasma (0.9 IU/dl) with the R1781H mutant was comparable to that usually obtained at levels of 5-10 IU/dl FVIII:C. This represented a 5-10-fold increased haemostatic potential. In addition, the R1781H mutant showed an ~1.9-fold decrease in K_m for FX compared to wild type. The results suggested that the mild bleeding tendency in this patient with the R1781H genetic defect appeared to be related to the relatively enhanced binding affinity of FVIII for FX.

Material and methods

Reagents

Recombinant FVIII was a generous gift from Bayer Corp. Japan (Osaka, Japan). Recombinant lipidated TF (Innovin®; Dade Behring, Marburg, Germany), ellagic acid (Sysmex, Kobe, Japan), thrombin-specific fluorogenic substrate (Bachem, Bubendorf, Switzerland), and thrombin calibrator (Thrombinoscope, Maastricht, Netherlands) were obtained from the indicated vendors. Human thrombin, FIXa, FX, FXa (Hematologic Technologies Inc. Essex, VT, USA), recombinant hirudin (Calbiochem, San Diego, CA, USA), FXa substrate S-2222 (Chromogenix, Milano, Italy) and plasma-derived FVIII-deficient plasma (George King Biomedical, Overland Park, KS, USA) were commercially purchased. PL vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, 30% phosphatidylethanolamine (Sigma-Aldrich, St Louis, MO, USA) were prepared as previously described (14). The B-domainless FVIII expression construct RENeo FVIII and baby hamster kidney cells were gifts kindly provided by Dr. Pete Lollar.

Patients' plasma

Whole blood was obtained by venipuncture into tubes containing 1:9 volume of 3.8% (w/v) trisodium citrate. After centrifugation for 15 minutes (min) at 1,500 x g, the plasma samples were stored at -80°C, and thawed at 37°C immediately prior to the assays. The levels of FVIII:C and FVIII:Ag were measured as reported previously (15). The blood samples were obtained from patients diagnosed by our research group and enrolled in the Nara Medical University Haemophilia Program. All samples were obtained after informed consent following local ethical guidelines.

DNA extraction and genotyping of F8

Genomic DNA was extracted from white blood cells with fully informed patient's consent. Intron 22 inversion and intron 1 inversion analysis was performed by long distance PCR and by multiplex PCR, and 26 exons and their flanking regions of *F8* were amplified by PCR using specific primers with a simple modification as previously described (16). The PCR products were purified and sequenced with a BigDye® terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). ABI PRISM SeqScape® (Applied Biosystems) software was used for mutation detection. The nomenclature was based on the current recommendations from HGVS (Human Genome Variation Society). The nucleotide number was assigned according to the FVIII cDNA sequence from A of the initiator ATG site as +1. The amino acid sequence numbering assigned the first residue of mature FVIII as +1, and thus the initiator methionine was at the -19 position, as used in the *F8* HAMSTeRS mutation database.

Mutagenesis, expression, and purification of wild-type and variant FVIII

Recombinant wild-type FVIII and FVIII variant Arg1781His (R1781H) were constructed, expressed, and purified as described previously (17). Resultant FVIII forms were typically >90% pure as judged by SDS-PAGE with albumin representing the major contaminant. FVIII concentrations were measured using an enzyme-linked immunosorbent assay, and FVIII:C levels were determined by one-stage clotting assay. FVIII samples were quick-frozen and stored at -80°C

Rotational thromboelastometry (ROTEM)

ROTEM was performed using the Whole Blood Haemostasis Analyzer® (Pentapharm, Munich, Germany) (6). After drawing, citrated whole blood was kept at rest for 30 min at room temperature and was used within 2 hours. At the start of measurement, 20 µl of $CaCl_2$ (final concentration, f.c., 12.5 mM) was added to the whole blood (280 µl). Clot formation was assessed using clotting

time (CT; the time from the start of measurement until detection of clot firmness of 2 mm amplitude) and clot formation time (CFT; the time from the initiation of clotting until detection of clot firmness of 20 mm amplitude).

FXa generation assays

(i) plasma-based chromogenic assay

Coatest®SP FVIII kit (Chromogenix) was used according to the manufacturer's instructions. Plasma samples were mixed with excess amounts of FIXa/FX/PL in the supplied buffer, and the mixtures were incubated at 37°C as described previously (18). Hirudin (2.5 IU/ml) was added to the mixtures to completely exclude feedback activation induced by the generated thrombin. FXa generation was initiated by the addition of $CaCl_2$ and S-2765. The initial velocity rates were determined at 405 nm using Labsystems Multiskan Multisoft microplate reader (Labsystems, Helsinki, Finland).

(ii) purified FXa generation assay

The rate of conversion of FX to FXa was monitored in a purified system at 37°C (19). FVIII (5 nM) was activated by thrombin (10 nM) in the presence of PL vesicles (20 μM). Thrombin activity was inhibited after 1 min by the addition of hirudin (2.5 IU/ml), and FXa generation was initiated by the addition of FIXa and FX at the indicated concentrations. Aliquots were removed at appropriate times to assess the initial rates of product formation and were mixed with EDTA (f.c. 50 mM) to quench the reactions. Rates of FXa generation were measured by the addition of specific chromogenic substrate, S-2222 (f.c. 0.46 mM). Reactions were read at 405 nm using microplate reader.

Clot waveform analysis

APTT measurements were performed using the MDA-II®Haemostasis System (Trinity Biotech, Dublin, Ireland). The clot waveforms obtained were computer-processed using the commercial kinetic algorithm (7). The minimum value of the first derivative (min1) was calculated as an indicator of the maximum velocity of coagulation achieved. The second derivative of the transmittance data reflects the acceleration of the reaction at any given time point. The minimum value of the second derivative (min2) was calculated as an index of the maximum acceleration of the reaction achieved. Since the minimum of min1 and min2 are derived from negative changes, the data were expressed as |min1| and |min2|, respectively. The clot time was defined as the time until the start of coagulation.

Thrombin generation assay

The calibrated automated thrombin generation assay (Thrombinoscope) was performed as previously described (8). Although a small amount of TF is generally used as a trigger reagent, the sensitivity of this original assay was relatively low and differences in coagulation function at low levels of FVIII:C (<2-3 IU/dl) were not seen (8). The addition of a small amount of ellagic acid to the mixtures containing TF little affects the lag-time (representing FVIIa/TF-induced activation of the extrinsic pathway), but mediates higher levels of peak thrombin and ETP (representing the subsequent activation of intrinsic pathway). This modified thrombin generation assay, therefore, sensitively reflects global coagulation in intrinsic as well as extrinsic, cell-based pathways, and enables the evaluation of coagulation function at very low levels of FVIII:C (lower limit <0.4 IU/dl [8]). Briefly, plasma samples (80 μl) were preincubated for 10 min with 20 μl of trigger reagent containing TF, PL, and ellagic acid (final concentration (f.c.) 0.5 pM, 4 μM, and 0.3 μM, respectively). Measurements were then recorded after the addition of 20 μl reagent containing $CaCl_2$ and fluorogenic substrate (f.c. 16.7 mM and 2.5 mM, respectively). The development of fluorescent signals was monitored using a Fluoroskan Ascent microplate reader (Thermo Fisher Scientific, Boston, MA, USA). Data analyses were performed using the manufacture's software, and the standard parameters; peak thrombin, time to peak, and endogenous thrombin potential, were derived.

Data analyses

All experiments were performed on at least four separate occasions; the mean values and standard deviations are shown. Nonlinear least squares regression analysis was performed using Kaleidagraph (Synergy, Reading, PA, USA). The K_m and V_{max} values for FVIIIa/FIXa-catalysed activation of FX were calculated from the Michaelis-Menten equation.

Results

Patient's profile

A 27-year-old male had been diagnosed at 2 years of age following uncontrolled bleeding from a post-traumatic frontal cutaneous incision, and was characterised as severe haemophilia A with FVIII:C level <1.0 U/dl in a one-stage clotting assay. There was no family history or past history of bleeding. The current levels of FVIII:C and FVIII:Ag were 0.9 IU/dl and 1.8 IU/dl, respectively, showing negative cross-reactive material. The case showed FVIII:C level 4-5 IU/dl in FXa generation chromogenic assay, however. The levels of all other plasma procoagulant, anti-coagulant, and fibrinolytic proteins were within normal range (data not shown). To date, bleeding episodes have been rare, regular prophylaxis has not been necessary and consequently he has required extremely small total amounts of FVIII concentrate products as compared to typical patients with severe haemophilia A. He has no chronic complications (e.g. arthropathy) commonly associated with the severe phenotype.

The *F8* genotype of this patient demonstrated a missense mutation comprising a single nucleotide conversion of G5399 to A in exon 16, resulting in an amino acid substitution from arginine to histidine at codon 1781. This mutation has been already enrolled in the haemophilia A database (HAMSTERs [13]), and some clinical and laboratory profiles of these patients had been described,

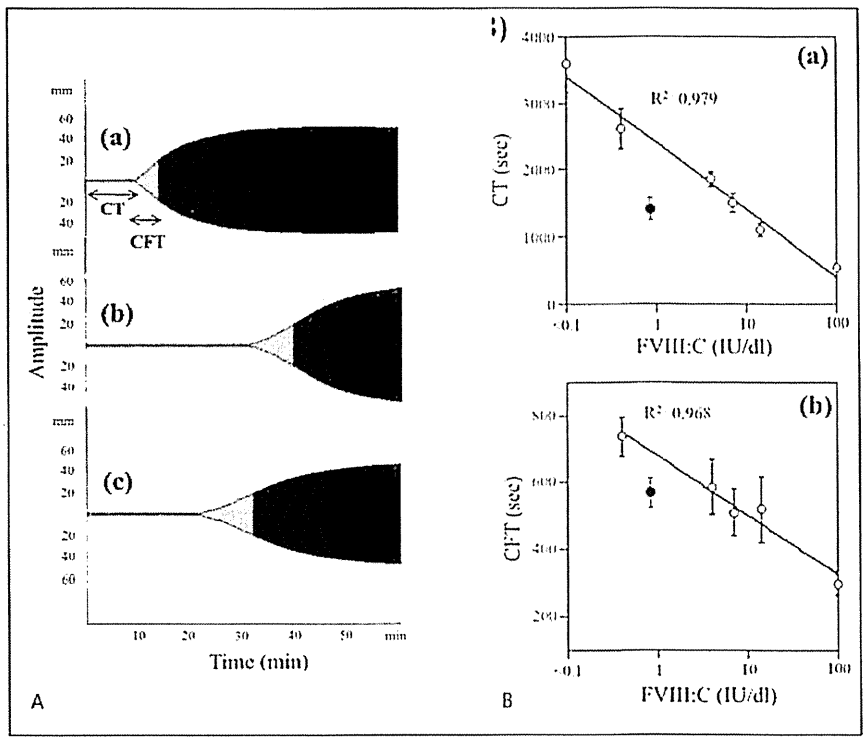

Figure 1: ROTEM in the haemophilia A patient with R1781H. A) $CaCl_2$ was added to FVIII-deficient whole blood (<0.2 IU/dl) mixed with the indicated concentrations of FVIII (a: 100 IU/dl, b: 1.0 IU/dl) and to patient's whole blood with R1781H (c) at the start of the assay. The thromboelastograms were recorded using ROTEM as described in *Methods*. Representative data are illustrated. B) Parameters (CT (a) and CFT (b)) obtained at various concentrations of FVIII (open circles) and those obtained from patient's sample (closed circles). The solid line illustrates the linear regression fitting the data. The dashed lines show the FVIII:C levels corresponding to the parameters obtained from the patient sample. All experiments were performed at least four separate times, and the average values and standard deviations were calculated.

but there has been any little functional and expression studies on FVIII with R1781H mutation.

ROTEM analysis of whole blood from the patient with the R1781H mutation

It was evident that the plasma FVIII:C level (0.9 IU/dl) in this patient with the R1781H mutation did not correlate with his clinical phenotype. To investigate this discrepancy, we first evaluated whole blood coagulation function using ROTEM. A representative thromboelastogram and the derived parameters (CT and CFT) are shown in ▶Figure 1A-c and B, respectively. The values obtained were 1,493 ± 106 seconds (sec) and 580 ± 12 sec, respectively. To assess the level of FVIII:C that could be expected to reflect these parameters, various amounts of FVIII were added to whole blood obtained from a volunteer patient with severe haemophilia A (<0.2 IU/dl), and assayed using same method. Representative data in these control plasmas with added FVIII (100 IU/dl and 1.0 IU/dl) are illustrated in ▶Figure 1A-a,b, and the parameters obtained are shown in ▶Figure 1B. The coagulation function observed in patient's sample with FVIII:C (0.9 IU/dl) was significantly greater than that of the control sample at FVIII:C 1.0 IU/dl, and both parameters obtained were comparable to those observed at FVIII:C 5-10 IU/dl.

Thrombin generation in the presence of FVIII R1781H

Specific thrombin generation assays have been developed to examine global coagulation function based on the principles of cell-based clotting. Representative thrombograms in the patient's plasma or control samples (prepared from FVIII-deficient plasma mixed with various amounts of FVIII) are illustrated in ▶Figure 2A, and levels of peak thrombin derived from these curves are shown in ▶Figure 2B. The levels of peak thrombin in patient's plasma and control plasma with FVIII:C 1.0 IU/dl were 300 ± 15 nM and 169 ± 14 nM, respectively, indicating that thrombin generation in the patient's plasma (0.9 IU/dl) was much greater than that in control with similar FVIII:C level. This result was comparable to that obtained with plasma with a FVIII:C of ~10 IU/dl. The other parameters (time to peak and ETP) showed similar tendencies (data not shown). The results suggested a significantly greater coagulation potential in this case with R1781H mutation than in the equivalent control.

To examine whether the properties of this patient's sample were unique, we utilized the same method to examine coagulation function in four cases of mild/moderate haemophilia A with similar FVIII:C levels. These patients had been diagnosed with point mutations at R372H, T295I, G2325R, and R2307Q (FVIII:C 1.0, 3.4, 4.9, and 5.8 IU/dl, respectively). The levels of peak thrombin obtained in all cases were comparable to those in the equivalent control samples with similar levels of FVIII:C measured in a one-stage clotting assay (▶Figure 2B). The findings appeared to reflect the clinical phenotypes in these patients (data not shown), and indicated that the enhanced haemostatic potential was unique to the R1781H mutation.

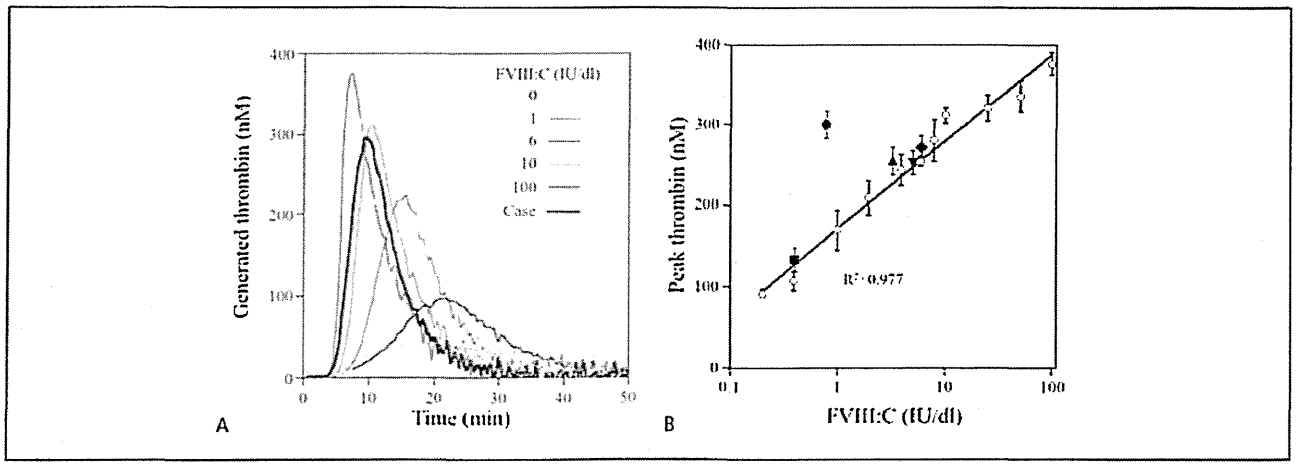

Figure 2: Thrombin generation in the patient with R1781H. A) FVIII-deficient plasma, mixed with various concentrations of FVIII (0-100 IU/dl) was incubated with TF (0.5 pM), ellagic acid (0.3 M) and PL vesicles (4 M), followed by the addition of fluorogenic substrate and CaCl2 at the start of the assay as described in *Methods*. Representative selected curves are illustrated. The bold black line shows the thrombin generation curve obtained from the patient's sample. B) Peak thrombin parameter obtained from the thrombin generation assays. The symbols used are samples with various amounts (0-100 IU/dl) of FVIII (open circles), R1781H (closed circle), R372H (closed square), T295I (closed triangle), G2325R (closed inverted-triangle), and R2307Q (closed diamond). The solid line illustrates the linear regression fitting the data. The dashed line shows the FVIII:C level corresponding to the peak thrombin parameter obtained with the patient's sample. All experiments were performed at least four separate times, and the average values and standard deviations were calculated.

Enhancement of intrinsic FXa generation in the presence of FVIII R1781H

Intrinsic FXa generation, corresponding to the upstream process of thrombin generation, was measured using the chromogenic assay to further investigate the mechanism(s) of enhanced thrombin generation associated with the FVIII R1781H mutation. The initial rate of FXa generation in the presence of R1781H was ~3-fold greater than with control plasma at FVIII:C 1.0 IU/dl ($0.240x10^{-3}$ and $0.082x10^{-3}$, respectively), and was comparable to plasma at FVIII:C ~5 IU/dl (▶ Figure 3). The findings were similar to those obtained by ROTEM and thrombin generation assays. The results suggested that the mutation R1781H, located in the FVIII A3 domain, governed the formation of the tenase complex consisting of FVIIIa, FIXa, and FX on the PL surface, and accelerated thrombin and FXa generation. Overall, therefore, the coagulation potential estimated by global coagulation assays in this case (corresponding to FVIII:C ~10 IU/dl, rather than 0.9 IU/dl) correlated with the clinical phenotype.

Clot waveform analysis in patient's plasma with R1781H

In clot waveform analyses, the data obtained from the waveforms using patient's plasma and control plasmas prepared as described above are illustrated in ▶ Figure 4A. The clot time with the patient's plasma was significantly shorter than that with the equivalent control plasma (55.2 ± 0.4 sec and 78.8 ± 1.7 sec, respectively), and was comparable to that of the control with FVIII:C <15 IU/dl (estimated ~10 IU/dl). In contrast, |min2| obtained with the patient's plasma was lower than that with the control at FVIII:C 1.0

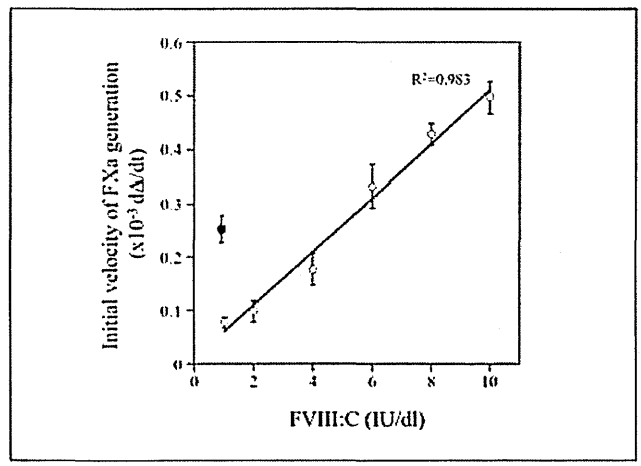

Figure 3: Endogenous intrinsic FXa generation in the patient with R1781H. FVIII-deficient plasma, mixed with small amounts of FVIII (1-10 IU/dl, open circles) was incubated with a mixture of FIXa/FX/PL in the presence of hirudin, followed by the addition of CaCl2 as described in *Methods*. The rates of initial velocity of endogenous FXa generation were determined at 405 nm after the addition of S-2765. All experiments were performed at least four separate times, and the average values and standard deviations were calculated. The solid line illustrates the linear regression fitting the data. The dashed line shows the FVIII:C level corresponding to FXa generation using the patient's sample (closed circles).

IU/dl (0.21 ± 0.01 and 0.26 ± 0.01, respectively). These results were in keeping with the earlier characteristics of the R1781H mutation. As described above in the FXa generation assays (see ▶ Figure 3), the moderated haemostatic potential appeared likely to be associ-

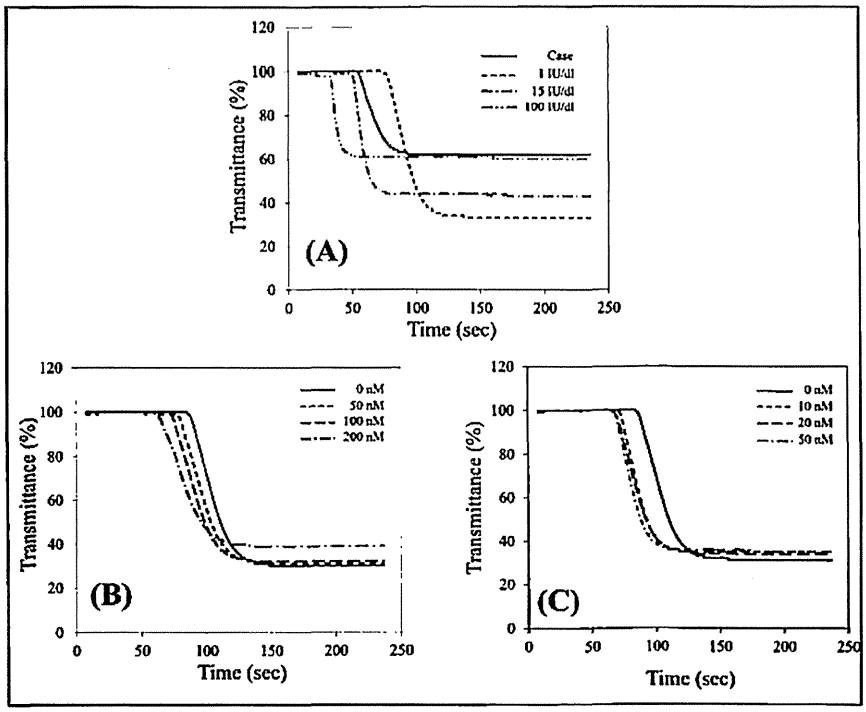

Figure 4: Clot waveform analysis in the patient with R1781H, and in control plasma (FVIII:C, 0.9 IU/dl) with added FVIII or in the presence of exogenous FX or FIXa. $CaCl_2$ was added to FVIII-deficient plasma mixed with various amounts of FVIII (1, 15, and 100 IU/dl) and to patient's plasma (A). Alternatively, various amounts of FX (0-200 nM) (B) or FIXa (0-50 nM) (C) were mixed with FVIII-deficient plasma (FVIII:C, 0.9 IU/dl), followed by the addition of $CaCl_2$. Clot waveforms were visualised in an APTT-based assay as described in *Methods*. Representative data are illustrated in A-C.

ated with the limited reactions of FVIII(a), FIXa, and FX. Furthermore, the clot time was shortened and |min2| was modestly reduced in a dose-dependent manner in the presence of incremental amounts of FX added to control plasma (with FVIII:C 0.9 IU/dl) (▶Figure 4B), and were consistent with the waveform pattern in the presence of the mutant R1781H sample. In contrast, no effects such as FX were evident in the presence of varying amounts of FIXa (▶Figure 4C). It may be speculated that these findings represented an alteration in the association between R1781H FVIII(a) and FX in patient's plasma.

Thrombin generation in the presence of FVIII R1781H mutant

Differences between the coagulation potential of patient's plasma and that of control plasmas might have been related to the concentrations of procoagulant, anticoagulant and/or fibrinolytic components other than FVIII in the test samples. Experiments were repeated, therefore, using a constructed FVIII mutant where Arg^{1781} was converted to His by site-directed mutagenesis, and stably expressed in BHK cells as a B-domainless FVIII form. This R1781H mutant exhibited a modest reduction of specific activity (~60% level of wild type) measured in a one-stage clotting assay.

Thrombin generation assays were repeated as described above using FVIII-deficient plasma mixed with FVIII wild type or R1781H mutant. The levels of peak thrombin in the presence of wild type and R1781H mutant at the same level of FVIII:C (1.0 IU/dl) were 233 ± 2 nM and 338 ± 5 nM, respectively, supporting that thrombin generation in the presence of the R1781H mutant was much greater than that in wild-type FVIII at equivalent levels of FVIII:C, and that 1.0 IU/dl R1781H was comparable to 5-6 IU/dl wild-type FVIII. In addition, the other parameters (time to peak and ETP) also showed similar tendencies (data not shown). The findings were consistent with the earlier data and were in keeping with the suggestion that the mutation alone contributed to the significantly greater coagulation potential in our case with FVIII R1781H.

Effect of FVIII R1781H mutant on the K_m for FX activation

The results illustrated above (see ▶Figure 4) indicated a possible significant alteration in the association between FVIII(a) and FX in the R1781H patient. Purified FXa generation assays were performed, therefore, using a tenase complex containing FVIIIa wild type or R1781H mutant. The results are shown in ▶Figure 5. The K_m value obtained from the fitted curve for FX in the presence of R1781H mutant was 32.5 ± 3.8 nM, and was ~1.9-fold lower than to that obtained using wild-type FVIII (58.5 ± 6.1 nM). In contrast, the V_{max} obtained for the two forms of FVIIIa were similar (203 ± 71 min^{-1} and 225 ± 13 min^{-1}, respectively). These results suggested a significant increase (~1.6-fold) in catalytic efficiency (V_{max}/K_m) for the tenase in the presence of R1781H relative to native tenase. The K_m and V_{max} values for FIXa or PL vesicles obtained using the two FVIIIa forms were both similar (data not shown). These results supported the concept that the R1781H mutant affinity for FX, was greater than that of the wild type, and were in keeping with the conclusion that the increase in tenase activity in the presence of FVIII R1781H resulted from a relative enhancement in the association between FVIIIa and FX.