表2　プロテインCおよびプロテインS活性に影響する要因

要因	プロテインC	プロテインS
生理的		
妊娠	妊娠22週まで著明に上昇	減少
経口避妊薬・ホルモン療法	—	低値
年齢	年齢とともに上昇（PCはPSとATより成人レベルへの到達が遅れる）	
性別	—	女性は低値
後天的欠乏		
抗ビタミンK療法	低値	低値
ビタミンK欠乏	低値	低値
未熟児	低値	低値
肝疾患	低値	低値
消費（DIC，重症感染症，敗血症，VTE）	低値	低値
抗体出現（SLE，水痘，悪性新生物）	低値	低値

＊サラセミア，鎌状貧血，もやもや病などでもProtein CおよびProtein Sがいずれも低下することが報告されている．
＊Lupus anticoagulantの存在下では，いずれもClotting assayの結果に影響が出る．（文献23，24より改変）

抗炎症作用を発揮する（図3右）[3]．TMもHMGB-1を介した抗炎症作用が明らかとなり，臨床における有用性が期待されている．可溶性TMは髄液に移行しないが，APCはEPCR依存的に正常なBBBも通過し中枢神経に作用する．APCにはPAR-1, EPCR及びPAR-3を介した急性神経障害に対する保護作用があると考えられている．

成人までのVTEの発症率は，新生児から乳児早期に最も高く，学童期に低くなり，思春期に再び上昇する．この新生児と思春期の血栓傾向には，凝固制御因子の成熟と内因性線溶活性の低下が関与する．止血機構の成熟に従って，3大抗凝固因子濃度も年齢とともに上昇する[24]．AT濃度は学童期まで，PCおよびheparin cofactor（HC）-II濃度は成人までに上昇する．PSはC4BPと結合しないfree PSが活性を示すが，新生児期はC4BP値が低くfree-PS濃度が相対的に高い．PC，PS及びATのうち最も成人域への到達が遅れるのがPCである．生後2週間までのPC活性値には，とくに個人差が大きいのかも知れない．本邦では高橋ら[25]の基準値が主に使用されているが，測定法の統一（標準血漿，amidolytic/clotting法など），ビタミンK欠乏の確認，さらに3大因子の同時測定での検討が必要であろう．新生児期にPCとPS活性を乖離させる後天的要因は明らかではない（表2）[23]．本邦成人では，PS多型が多いため，PS/AT比が遺伝性PS欠損症のスクリーニングに利用される．小児ではPC/PS比による単独PC低下の同定が遺伝性PC欠損症の診断に有用かもしれない．

小児PC欠乏症に対する治療と予防

血栓症の治療は，抗凝固療法と血栓溶解療法からなる[26]．初回の場合は素因を明らかにし，これに応じて抗凝固療法による再発予防を行う．遺伝性血栓症（栓友病）でなければ新生児血栓症の進行と再発の危険性は低い．PC, PS及びAT欠損症の血栓症リスクは高いため，FVLやPTG20210Aと異なり，この家族歴を有する小児にはこの3因子のスクリーニングが海外でも推奨されている[27]．

小児PC欠乏症による血栓性疾患の治療管理を表3に示す[28]．濃厚血小板と新鮮凍結血漿による補充療法の必要性はとくに新生児・乳児では高い[29]．小児のPC欠乏に対する補充療法は，成人より強調されるべきであろう．生理的な止血効果を期待する凝固因子の最少血中活性値は20～30％程度である．新生児の血漿PC活性もこれ以上に保ちたい．TM製剤（リコモジュリン®，旭化成ファーマ）が有効性を発揮するには，十分なPC濃度が望ましいと考えられる[30]．

現在国内で唯一のPC製剤は血漿由来APCで，先天性PC欠損症による血栓症のみが保険適応となっている．海外における重症敗血症に対する組み換えAPC製剤（rAPC）による大規模な無作為化臨床試験（PROWESS試験，レベル1b）では，APC群の有意な生存率の改善が報告された．しかし，最近成人の重症敗血症と敗血症性ショックに対するrAPCの効果は否定された[31]．小児では頭蓋内出血が問題となるため[32]，新生児PC欠損症例への使用には注意が必要である．APCやTM製剤は，小児（とくに新生児）には病態を考慮して，製剤の特性を生かした投与量と方法

表3　遺伝性プロテインC欠乏症による血栓症（DIC，電撃性紫斑病，急性静脈血栓塞栓症など）の治療管理

疾患	治療製剤	投与量	投与の指標
DIC，PF，急性VTE	PC製剤[a]	100 U/kgを初回ボーラス後に50 U/kgを6-12 h毎（急性期は6 h毎）	PC活性 50 IU/dL；D-dimerの減少と正常化まで
	FFP	10-15 mL/kg，8-12 h毎；但し，PC製剤の使用まで	PC活性 >10 IU/dL；PC製剤の使用まで，D-dimerの低下と正常化まで
	非分画ヘパリン[b]	15-20 U/kg/hをPC補充とともに	抗Xa活性 0.3-0.7 U/mL
	低分子ヘパリン[b]	1.0-1.5 mg/kg 12 h毎をPC補充とともに	抗Xa活性 0.5-1.0 U/mL
侵襲的処置の際の予防	PC製剤[a]	100 U/kg初回ボーラス後に30-50 U/kgを12-24 h毎	PC活性 20-50 IU/dL；術後にD-dimerが陰性化または急速に低下するまで
維持	PC製剤[a]	30-50 IU/kgを1-3日毎	D-dimer陰性化
	通常はWarfarin療法と併用	0.1-0.2 mg/kg経口を毎日（PC製剤の補充なしの場合） 0.05-0.1 mg/kg経口を毎日（PC製剤の補充ありの場合）	INR　2.5-3.5，思春期は一般により高いINRが要求される PC製剤補充の場合は，INR 1.5-2.5

[a] 日本では活性化PCのみが適応製剤である．小児・新生児では年齢と病態に応じて，注意深くモニターしながら，過剰投与に注意して使用する．
[b] ヘパリンの使用は血小板減少を伴うDICの場合に出血のリスクをあげるため注意する．DIC：disseminated intravascular coagulation，INR：international normalized ratio，PF：purpura fulminans，VTE：venous thromboembolism．（文献28より改変）

を確立することが必要であろう．

小児血栓症のうち特発性の再発例及び遺伝性栓友病患者にはワーファリンの長期投与が必要となる．PC欠乏の場合はとくに奇異性血栓症に注意して少量から開始し，細やかにINRをモニターする．Sick dayの管理のみならず，長期的な骨，成長に対する晩期効果にも注意する．新生児乳児期発症のPC欠損症は，発症時に重症型であっても年齢とともに活性もある程度上昇し，比較的血栓症を起こすことが少なくなるようである．Warfarin過剰のカウンターに用いられるPPSB-HT®（日本製薬）のPC濃度は極めて高い．海外でも重症PC欠損患者の長期管理に関する情報は少ない．PC欠損症に対する補充療法の確立が必要である．PC欠損症には血友病と同様に肝移植後の治癒例が報告されている[33]．今後新たな細胞移植療法や遺伝子修復療法の開発が期待される．

おわりに

小児血栓症は稀であるが，新生児の*PROC*変異保有者はハイリスク集団として注意すべきであろう．非遺伝性PC欠乏症の鑑別は困難である．胎児水頭症や感染性電撃性紫斑病と診断された小児にPC欠乏症が隠れている可能性がある．遺伝性PC欠損症による小児血栓症は生命を脅かし重篤な障害を残す．妊産褥婦ではPS欠損症とAT欠損症とが管理上問題となることが多い．新生児血栓症の予後を改善するには，周産期における産科との細やかな連携が必須である．周産期死亡率の低い日本でこそ，新生児血栓症に対する適切な抗凝固療法を確立し，家族を守る包括的医療を実践する意義は大きい．

日本小児科学会の定める利益相反に関する開示事項はありません．

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Journal of Thrombosis and Haemostasis, **11**: 1417–1420 DOI: 10.1111/jth.12287

OFFICIAL COMMUNICATION OF THE SSC

Towards standardization of clot waveform analysis and recommendations for its clinical applications

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To cite this article: Shima M, Thachil J, Nair SC, Srivastava A. Towards standardization of clot waveform analysis and recommendations for its clinical applications. *J Thromb Haemost* 2013; **11**: 1417–20.

Introduction

Automated coagulation analyzers can provide a wealth of information in addition to that provided by conventional clotting tests such as the prothrombin time (PT) and the activated partial thromboplastin time (APTT), which are often considered of limited use for clinical purposes [1]. One particular type of analysis, the clot waveform, which was originally described using the Multichannel Discrete Analyzer (MDA series; Organon, Technika, Durham, NC, USA), defines changes in light transmittance that occur during the process of clot formation. A number of recent reports have described the use of this type of automated clotting instrument for clot waveform analysis (CWA), and there appears to be significant advantages in using this assay for the assessment of global coagulation function. In this communication, we propose standardization of methods for the CWA using currently available clotting analyzers and overview the potential clinical applications.

Principles of clot waveform analysis

Visualization of clot waveforms

Changes in light transmittance or absorbance are determined by continuous measurements during the APTT and are designated the clot waveform (CW) (Fig. 1). This clotting process is categorized into three parts: the pre-coagulation, coagulation and post-coagulation phases. Pre-coagulation is described as the first segment of the trace, from the beginning of the signal to the onset of coagulation. After the onset of coagulation, light transmittance is decreased or absorbance is increased by the formation of fibrin, and this is defined by a slope in the waveform. At the end of coagulation, light transmittance or absorbance tends to stabilize and is characterized again by a linear segment. If fibrinolysis is enhanced due to acquired or congenital abnormalities of hemostasis, light transmittance may increase or absorbance may decrease again in the post-coagulation phase.

Coagulation analyzer

There are two types of clotting machines for CWA. One utilizes a system to detect transmittance during the APTT clotting reaction, and is represented by the MDA-II or CS series. In this type, transmittance is decreased after initiation of clotting (Fig. 1A). The other type monitors the absorbance, and is represented by the ACL series. In this type, 0% absorbance defines the pre-coagulation phase, and the absorbance increases after the initiation of clotting (Fig. 1B). Other analyzers having similar features should also be able to provide CWA data easily, particularly if manufacturers incorporate the relevant software. Even if an automated CWA is not available, there are several analyzers that are able to provide adequate raw data (Table S1). CWA is possible using such analyzers by statistical evaluation of this raw data of transmittance or absorbance (Fig. S1).

Recommended method for standardization of CWA

APTT assay

Plasma should be prepared from fresh citrated whole blood as for the standard APTT assay. Pooled plasma from nor-

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Report on clot waveform analysis from the working group of the FVIII and IX Subcommittee of the SSC

Received 30 October 2012
Manuscript handled by: S. Eichinger
Final decision: F. Rosendaal, 26 April 2013

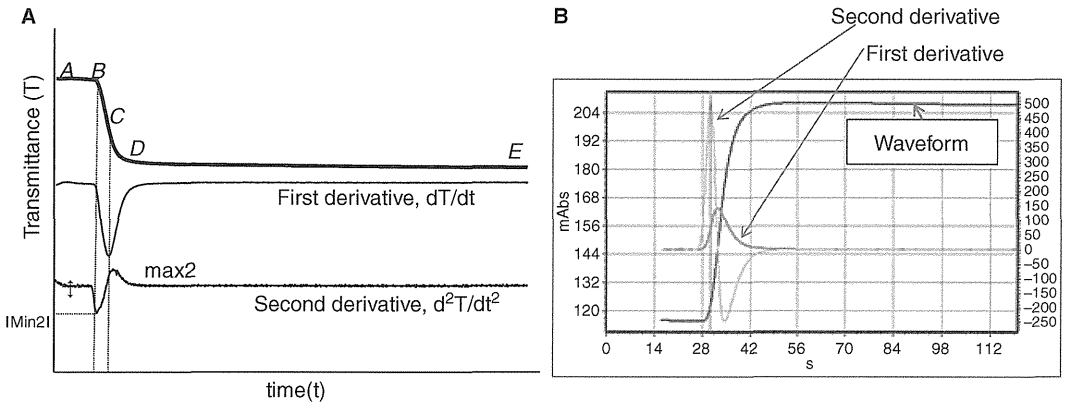

Fig. 1. APTT clot waveforms (A) Clot waveform of normal plasma by monitoring of transmittance (CS2000i). The upper trace shows the changes in light transmittance observed during the performance of APTT with normal reference plasma by CS200i (Sysmex Kobe, Japan). Point 'A' marks the beginning of the recording by the instrument, which occurs 8 s after the addition of CaCl2. Point 'B' indicates the initiation of coagulation, namely fibrin formation. The clot waveform is separated into the pre-coagulation phase (A–B), the coagulation phase (B–D) and the post-coagulation phase (D–E). (B) Clot waveform of normal plasma by monitoring of absorbance (ACL-Top). The upper blue colored trace shows the changes in absorbance observed during the performance of APTT with normal reference plasma by ACL-Top (Instrumentation Laboratory). The red colored curve is the first derivative of the absorbance corresponding to the coagulation velocity. The light blue colored biphasic curve is the second derivative of the absorbance corresponding to the coagulation acceleration.

mal individuals or commercial normal plasma is available as reference plasma. Colorless APTT reagents, without opacity, are recommended to detect sensitive changes in transmittance or absorbance, and for subsequent precise measurements of the various parameters. Although any APTT reagent that fulfills this criteria should be usable for CWA, among the reagents tested so far (Table S1), Thrombocheck APTT-SLA/0.02M $CaCl_2$ is suitable for MDA-II and CS series and HemosIL SynthASil for ACL-Top series. Other instrument and reagent combinations should also be possible to use but these need to be tested. APTT reagents used for the detection of anti-phospholipid antibodies are not recommended for the CWA because their sensitivity for assessing low clotting function is not sufficiently high. The APTT for CWA is performed in a similar manner to that for the standard APTT assay.

CWA parameters

The first derivative of the transmittance reflects coagulation velocity, and the second derivative reflects coagulation acceleration. Clotting time (CT), maximum coagulation velocity (Min1), maximum coagulation acceleration (Min2) and maximum coagulation deceleration (Max2) are common basic parameters. Among these measurements, Min2 has been reported to be correlated with clotting function in hemophilia [2].

Clinical applications of CWA

Clotting function of various bleeding disorders

Initial evaluation of clotting function by CWA is undertaken by qualitative assessment of the CW pattern. In particular, two characteristic CW patterns are observed in various coagulation abnormalities compared with normal reference plasma (Fig. S2). In normal plasma, the pre-coagulation phase is short and the slope, reflecting the coagulation phase, is steep. In factor (F) XII, X, IX, VIII, V and II deficiencies, the pre-coagulation phase is prolonged but the changes in slope are different [1]. Changes in slope are more evident in FVIII and FIX deficiencies than in other deficiencies. Thus, qualitative analysis of CW may have diagnostic value in various clinical settings of impaired clotting function.

Evaluation of clotting function in hemophilia A and B

While assays of FVIII:C and FIX:C are most important for the clinical management of hemostasis in patients with hemophilia, CWA provides a potentially widely available platform for assessment of global hemostasis in these patients [3] (Figs S3 and S4). This assay could then also provide a novel method not only for diagnosis and correlations with the bleeding phenotype but also for monitoring of hemostasis in cases of replacement therapy for serious hemorrhage or surgery.

Furthermore, the aPTT CWA is also useful for assessing very low levels of FVIII or FIX, for example less than 1 IU dL^{-1}. Studies in a number of patients with severe HA diagnosed by conventional clotting assays, demonstrated that CWA patterns differed from patient to patient. The APTT clotting time was prolonged in all patients with severe HA, but there was variation in the slope [2]. Using mixtures of severe HA plasma and exogenous FVIII ranging from zero to 1.0 IU dL^{-1}, the slope and the APTT clot time and the min2 appeared to change in a dose-dependent manner. Similarly, in further studies

of 36 patients with severe HA, significant correlations between min2 and very low levels of FVIII:C were confirmed [4]. These results indicated that in some patients, the presence of trace amounts of FVIII mediated higher coagulation acceleration, characterized by the steeper slope, although it was possible that factors other than FVIII:C alone may have influenced clotting kinetics reflected in the waveform profile. Nevertheless, the data suggested that CWA could discriminate between different levels of FVIII:C in this critical category of severe HA, defined as having < 1.0 IU dL^{-1} FVIII:C by conventional assays (Figs S3 and S4). The evidence suggests that CWA can provide more specific data on global hemostasis in such patients, which could correlate better with the clinical phenotype.

Correlation between clinical severity and CWA parameters

Some HA patients, classified as severe on the basis of standard coagulation assays, exhibit milder clinical symptoms. It appeared possible, therefore, that CWA might provide valuable data for evaluating *in vivo* clotting function in various types of hemophilia A. To investigate this possibility, severe hemophilia A patients based on < 1 IU dL^{-1} of FVIII:C were divided into clinically severe and non- severe groups [4]. Clinically severe patients were characterized by the presence of spontaneous bleeding episodes at the age of < 1 year, the onset of joint or muscular bleeding before the age of 3 years old, or the presence of severe bleeding such as intracranial bleeding or refractory oral bleeding. The differences between the severe and the non-severe phenotype were significant for four CW parameters: clot time, maximal coagulation velocity (Min1), maximal coagulation acceleration (Min2) and maximal coagulation deceleration (Max2), These results strongly suggested, therefore, that CW parameters reflect clinical severity (Fig. S5).

Monitoring hemostatic therapy in the patients with inhibitors

The hemostatic benefits of various agents used for bypassing therapy, including activated prothrombin complex concentrates (APCC) and recombinant factor VIIa (rFVIIa), can be monitored by CWA [4,5]. In addition, CWA was also utilized effectively in a recent clinical phase 1 study for the assessment of a new bypassing agent based on mixtures of plasma-derived FVIIa and X [6]. In two hemophilia A patients with high responding inhibitors, CWA demonstrated improved hemostasis. Moreover, CWA was shown to reflect the prophylactic effect of regular infusions of FVIII during immune tolerance induction therapy (ITI) [7]. The findings confirmed that CWA is very sensitive to low levels of clotting factors, and suggested that the technique could also be useful for monitoring therapy using FVIII or FIX concentrates in patients with inhibitor.

Clotting function of acquired hemophilia

FVIII:C levels do not reflect clinical severity in many cases of acquired hemophilia A, and it may be difficult to determine clotting function precisely in these patients. CWA illustrates severely impaired patterns in these cases, however, characterized by a remarkably prolonged pre-coagulation phase and low values for maximum coagulation velocity and acceleration [8]. Assessment of clotting function by aPTT CWA, in addition to the measurement of FVIII activity, can be useful, therefore, to confirm decisions on hemostatic treatment and the monitoring of bypass therapy in these complicated clinical circumstances.

Advantages and limitations

There are several advantages to the use of CWA. The method has broad utility as a simple global test of hemostasis and is capable of providing sensitive, quantitative parameters as well as qualitative waveform patterns. Furthermore, CWA can be usefully applied in various difficult clinical settings. Not all current coagulation analyzers can be used for CWA, however, although the number of appropriate analyzers is increasing. Finally, the CWA is based on APTT-based coagulation mechanisms using an 'intrinsic' trigger. A modified CWA using trace amounts of tissue factor may extend the application of this technique.

Among the global hemostasis tests, CWA is perhaps the simplest to establish and standardize. It therefore needs to be tested more widely using standardized methods in different clinical situations to decide its place in the assessment of hemostasis and its disorders.

Addendum

M. Shima chaired the working party, performed the research, analyzed the data and wrote the manuscript. J. Thachil and S.C. Nair performed research and collected data. A. Srivastava supervised the study.

Disclosure of Conflict of Interests

M. Shima is supported for APTT reagents from Sysmex.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Data sheet of the transmittance and presentation by waveform.

Figure S2. APTT clot waveforms of various clotting factor deficiencies.
Figure S3. Dose-dependent waveform changes in plasma containing various concentrations of FVIII.
Figure S4. Waveform changes in hemophilia A with various levels of FVIII.
Figure S5. CWA parameters and clinical severity of severe hemophilia A.
Table S1. Coagulation analyzers and APTT reagents for clot waveform analysis.

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Simultaneous measurement of thrombin and plasmin generation to assess the interplay between coagulation and fibrinolysis

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Summary
Normal haemostasis is maintained by a controlled balance between coagulation and fibrinolysis, involving thrombin and plasmin the respective key enzymes. Simultaneous evaluation of both enzymes facilitates, therefore, an overall understanding of normal and pathological haemostasis. Combined thrombin and plasmin generation (T/P-G) assays have been recently described, and we have adapted the technique to investigate the interplay between coagulation and fibrinolysis in patients with various haemostatic disorders. Our modified T/P-G was initiated by the addition of a mixture of optimised lower concentrations of tissue factor and tissue-type plasminogen activator. Thrombin generation (TG) and plasmin generation (PG) were monitored simultaneously using individual fluorescent substrates in separate microtitre wells. The relationship between coagulation and fibrinolysis was demonstrated by analysing the effects of thrombin inhibitors, activated protein C and thrombomodulin. The most evident impairments in TG were observed with plasma samples deficient of coagulation factors participating in the prothrombinase complex. Defects in PG were observed with deficiencies of factor (F)V, FX, fibrinogen, and plasminogen. TG appeared to be a prerequisite for the initiation of PG, and overall PG was governed by fibrinogen concentration. TG in patients with haemophilia A correlated with levels of FVIII activity, but there was no significant relationship between PG and FVIII:C, confirming that the abnormal haemostasis in haemophilia A results in a severe imbalance between coagulation and fibrinolysis. The findings demonstrate that global haemostasis depends on a sensitive balance between coagulation and fibrinolysis, and that the modified T/P-G assay could provide an enhanced understanding of haemorrhage and thrombosis in clinical practice.

Keywords
Thrombin generation, plasmin generation, coagulation, fibrinolysis, global haemostatic assay

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Financial support:
This work was partly supported by grants for MEXT KAKENHI 24591558 and Baxter Coagulation Research Fund 2012.

Received: April 27, 2013
Accepted after minor revision: July 11, 2013
Prepublished online: August 29, 2013
doi:10.1160/TH13-04-0345
Thromb Haemost 2013; 110: 761–768

Introduction

Normal haemostasis is maintained by a controlled balance of pro- or anti-coagulation and fibrinolysis. Abnormalities of these mechanisms lead to various clinical symptoms of haemorrhage, thrombosis, or both. Many laboratory techniques have been devised to evaluate haemostasis, using separate assays for the measurement of the activities of individual coagulation factors. Thrombin and plasmin are the key enzymes involved in these processes, however, and in order to understand overall haemostatic function, simultaneous assessment of the kinetics of thrombin and plasmin generation could offer significant advantages.

Other assays of global haemostasis, such as thromboelastography (TEG) (1) and the thrombin generation assay (TGA) (2) are known to be useful methods that reflect the individual process of coagulation and/or fibrinolysis. TEG evaluates both blood coagulation and subsequent clot lysis in whole blood by measuring viscoelastic changes in fibrin polymerisation. With this method, however, it is difficult to clearly identify the individual phases of coagulation and fibrinolysis. In addition, samples of whole blood are required within a very restricted time after venepuncture. TGAs can be performed using platelet-rich plasma (3), whole blood (4) or platelet-poor plasma, and the thrombogram parameters reflect global coagulation capacity. Enhanced thrombin generation suggests a tendency towards thrombosis (5, 6), whilst depressed thrombin generation is evident in patients with a bleeding tendency such as haemophilia A and B (7, 8). The TGA, however, is not significantly affected by fibrinolytic activity, and appears unlikely to reflect overall haemostasis *in vivo*.

In recent years, global assays of coagulation and/or fibrinolysis that evaluate coagulation capacity (thrombin generation; TG), fibrinolytic ability (plasmin generation; PG), or both, have been developed and utilised (4, 9, 10). More recently, simultaneous assays of thrombin and plasmin generation, using trigger reagents con-

taining mixtures of tissue factor (TF) and tissue-type plasminogen activator (tPA), have been reported by two groups (11, 12). The assay described by Simpson et al. (11), termed the STP-assay (simultaneous thrombin and plasmin generation), measures TG and PG in separate microtitre wells. Van Geffen et al. (12) utilised single fluorometer wells in their novel haemostasis assay (NHA). The concentrations of TF and tPA used as trigger in the NHA were less than those in the STP-assay, and both assays provided simultaneous assessment of TG and PG kinetics. The NHA, however, appeared to demonstrate the interplay between coagulation and fibrinolysis more clearly than the STP-assay (12). In the present study, we have established a simultaneous thrombin and plasmin generation (T/P-G) assay in separate wells similar to the STP-assay, using optimised, low concentrations of TF and tPA in order to retain sensitivity to the haemostatic interactions.

Materials and methods

Reagents

Recombinant human TF (TF; Innovin®, Dade, Marburg, Germany), recombinant tissue-type plasminogen activator (tPA; American Diagnostica Inc., Stamford, CT, USA), thrombin-specific fluorogenic substrate (Z-Gly-Gly-Arg-AMC, Bachem, Bubendorf, Switzerland), plasmin-specific fluorogenic substrate (BOC-Glu-Lys-Lys-MAC, Peptide Institute Inc., Osaka, Japan), carboxypeptidase inhibitor (CPI; Calbiochem, San Diego, CA, USA), recombinant thrombomodulin (TM, Recomodulin®, Asahi-Kasei Phama Corp., Tokyo, Japan), argatroban (Slonnon®, Daiichi Sankyo Co. Ltd., Tokyo, Japan) were obtained from the indicated vendors. Plasma samples deficient of factor (F)II, FV, FVII, FVIII, FIX, FX, FXII, and FXIII were obtained from George King Inc. (Overland Park, KS, USA). Plasma samples deficient of fibrinogen, plasminogen, and α2-plasmin inhibitor (α2-PI) were purchased from Affinity Biologicals Inc. (Ancaster, ON, Canada). Fibrinogen, FV, FX, plasminogen and plasmin (Hematologic Technologies, Burlington, VT, USA) and α-thrombin (Sigma-Aldrich, St. Louis, MO, USA) were obtained from the indicated vendors. Phospholipid (PL) vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, 30% phosphatidylethanolamine (Sigma-Aldrich) were prepared as previously described (13).

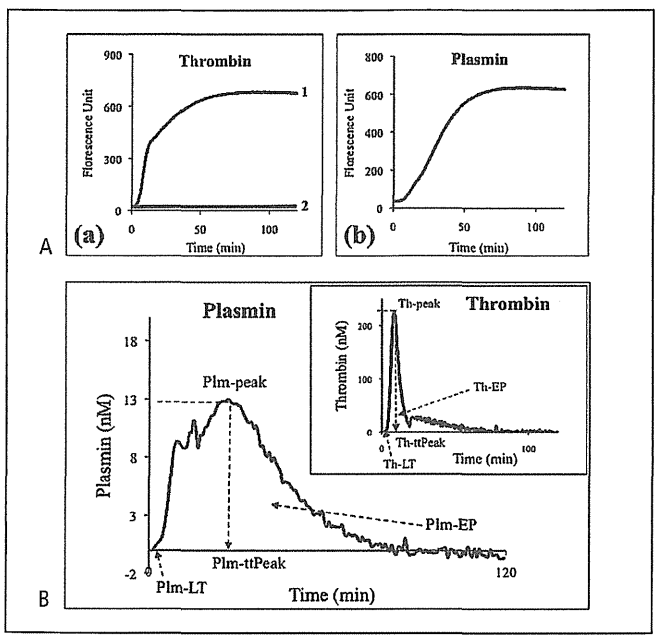

Figure 1: Simultaneous T/P-G assay. A) Mixtures of TF, tPA, and PL (f.c. 1 pM, 3.3 nM, and 4 □M) were added to normal plasma prior to evaluating TG and PG as described in *Methods*. Representative curves of TG (a) and PG (b) are illustrated. Each curve of TG obtained by trigger reagent or by EDTA alone shows line 1 or 2 in panel (a), respectively. B) First derivative (velocity) of TG (inset) and PG obtained in (A). LT: lag time, Peak: peak thrombin or peak plasmin, ttPeak: time to peak, EP: endogenous potential of thrombin or plasmin.

Fresh plasma samples

Normal plasma was prepared from 20 normal healthy individuals. Blood was drawn into evacuated anticoagulant tubes (blood: 3.8% (w/v) trisodium citrate; 9:1). After centrifugation for 15 minutes (min) at 1,500 g, the plasmas were stored at -80°C, and thawed at 37°C immediately prior to the assays. Patients' plasmas were obtained from 20 haemophilia A patients. FVIII:C levels in 12 patients were <0.2 IU/dl (severe type), and in eight patients were 1.0-5.0 IU/dl (moderate type). FVIII:C levels were measured by conventional one-stage clotting assay. All blood samples were obtained after informed consent following local ethical guidelines during routine follow-up of patients in the Nara Medical University Haemophilia Program.

Simultaneous thrombin and plasmin generation (T/P-G) assay

The T/P-G assay was established using a modification of the method reported by Simpson et al. (11). Briefly, trigger reagents (TF, PL, and tPA) were mixed in 20 mM HEPES, pH 7.2, 150 mM NaCl and 0.01% Tween 20, at concentrations of 6.5 pM TF, 26 μM PL, and 21 nM tPA. Final concentrations of reactants in plasma samples were 1 pM TF, 4 μM PL, and 3.3 nM tPA. The assay blank contained 77 mM EDTA, to evaluate the non-specific effects of thrombin that did not play a role in coagulation reactions in this assay (e.g. complexed with α2-macroglobulin). Two fluorometric specific substrates, Z-Gly-Gly-Arg-AMC and Boc-Glu-Lys-Lys-MCA, were used for the detection of the key enzymes, thrombin and plasmin, respectively. The final concentration of each substrate was 100 μM. Plasma samples (80 μl) were added to wells in flat-bottom, black polystyrene (Nunc; Thermo Scientific, Waltham, MA, USA), 96-well plates, and six wells were dedicated to each sample. Twenty μl of the trigger reagent (TF/PL/tPA) were added to four plasma wells, and equivalent volume of EDTA, instead of TF/PL/tPA, was added to the other two wells. And then two duplicated plasma wells were used to measure thrombin generation (TG) using thrombin substrate and plasmin generation

Table 1: Parameters of simultaneous T/P-G assay in normal plasmas (n=20).

Parameters	TG				PG			
	25%	Median	75%	Min-max	25%	Median	75%	Min-max
LT (min)	2.1	3.0	4.1	1.7–5.2	3.0	3.7	4.1	2.3–6.7
Peak (nM)	149	193	245	84–284	12.2	14.1	15.2	9.5–16.6
ttPeak (min)	7.5	8.2	9.0	6.8–11.2	21.0	22.5	23.2	12.8–29.2
EP (nM*min)	3,071	3,207	3,420	2,705–3,560	568	590	608	515–688

(PG) using plasmin substrate. The other two wells were used as EDTA blank wells. The thrombin and plasmin assay wells were kept separate to avoid concerns of potential interference and interactions with signal detection. The microtitre plates were placed in the fluorometer and allowed to warm to 37°C for 10 min. Following the addition of reactant solution, 10 µl of appropriate fluorometric substrate was added to each well, and thrombin substrate (10 µl) was added to EDTA blank wells. The dispenser of the fluorometer was flushed with 20 µl warmed 100 mM $CaCl_2$, and then 20 µl of $CaCl_2$ was added to all assay wells. The development of the fluorescent signal was monitored at 45-second intervals over a period of 2 hours using a Fluoroskan Ascent microplate reader (Thermo Electron Co., Waltham, MA) with a 390 nm (excitation) and 460 nm (emission) filter set. Standard curves were prepared using serially diluted purified α-thrombin and plasmin as previously described by Hemker et al. (14). Since the curve of TG obtained from EDTA blank wells (*line* 2) was regarded as non-specific reaction of thrombin, these data was subtracted from those of TG obtained from trigger-induced coagulation (*line* 1 in ▶Figure 1A-a). Data analyses were performed using excel software. From the first derivative (velocity) of thrombin and plasmin generation obtained above, the parameters; lagtime (LT), endogenous potential (EP), peak levels (Peak), and time to peak (ttPeak), were recorded.

Data analyses

Measurements were obtained in several separate assays as indicated, and the mean ± standard deviation are shown.

Results

The modified T/P-G assay with normal plasmas

The aim of the present study was to establish a simultaneous T/P-G assay in separate microtitre wells that would permit assessment of the interplay between coagulation and fibrinolytic mechanisms. For this purpose, we modified the STP methodology that

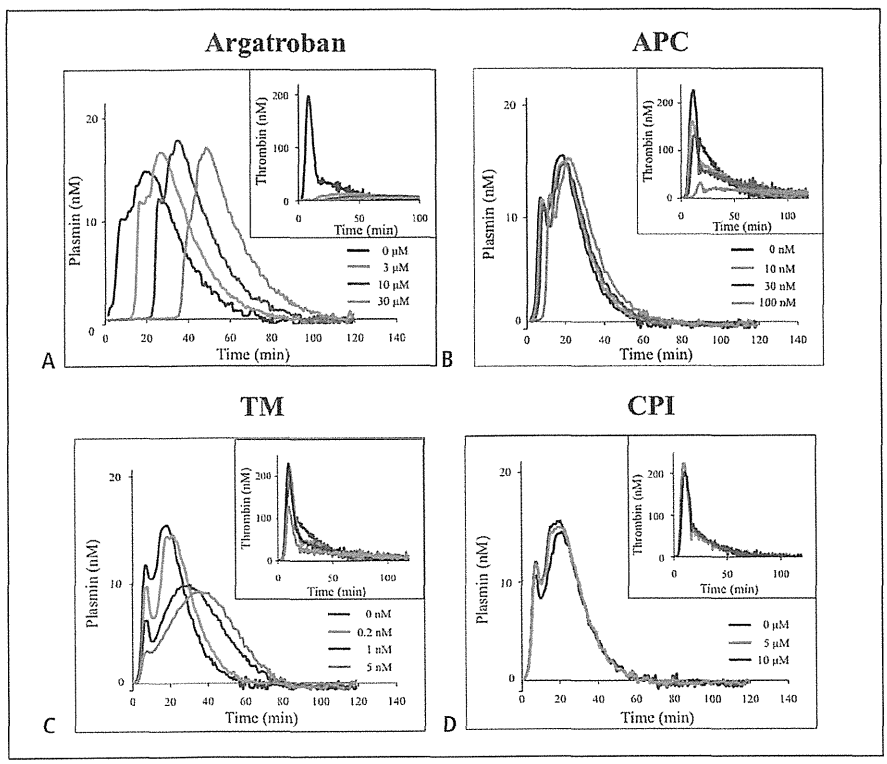

Figure 2: Effects of various coagulant inhibitors on simultaneous T/P-G. *Normal* plasma was incubated with various amounts of argatroban (panel A; 0–30 µM), APC (panel B; 0–100 nM), TM (panel C; 0–5 nM), and CPI (panel D; 0–10 µM), prior to measurements of TG (inset) and PG as described in *Methods*. Representative curves of TG and PG are illustrated.

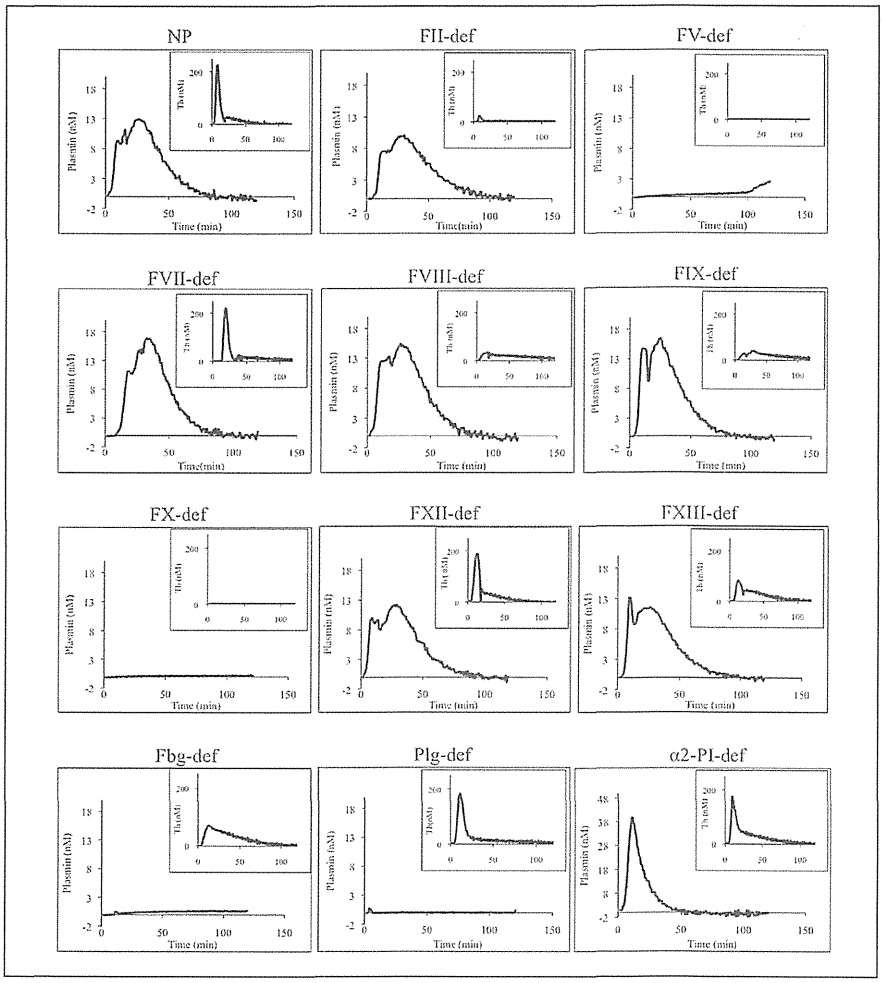

Figure 3: T/P-G with individual factor-deficient plasmas. Individual coagulant factor-deficient plasmas were incubated with trigger reagents, prior to measurements of TG (inset) and PG as described in *Methods*. Normal plasma (NP) was used as control. Representative curves for TG and PG are illustrated.

had been previously reported by Simpson et al. (11). A major difference between the STP and NHA (12) was the concentrations of reagents in the trigger mixture (TF and tPA), and it appeared that this might be critical for this assay. The effects of various concentrations of TF and tPA were examined, therefore, as described in *Methods*. Optimal conditions for low concentrations of trigger reagents similar to those used in the NHA were determined. TF and tPA increased both the TG and PG in dose-dependent manners, and the optimal concentrations determined by LT, Peak, and ttPeak of thrombin and plasmin generation were 1 pM for TF and 3.3 nM for tPA, respectively (data not shown). The concentration of TF and tPA was intermediate and less, respectively, compared to those used in the NHA (TF/tPA: 0.28 pM/5.4 nM [12]) and in the STP (TF/tPA: 5 pM/7.5 nM [11]). The individual curves for TG and PG obtained using normal plasma under these conditions are shown in ▶Figure 1A. The first derivative data from the raw curves demonstrating thrombin and plasmin generation (T/P-G) are illustrated in ▶Figure 1B. The four parameters derived from the TG and PG are summarised in ▶Table 1.

Intra-assay variation was determined from eight separate assays, and demonstrated a similar coefficient of variation (CV) for the TG (0.75-7.8%) and the PG parameters (1.1-7.0%). In addition, inter-assay variation was examined using a single sample per day measured over seven different days. The CV in these experiments ranged between 3.7-17.6% (TG) and 6.6-15.7% (PG), respectively (▶Table 2). These results were similar to those reported previously (11, 12).

Table 2: Intra- and inter-assay coefficient variation (CV).

	Intra-assay CV		Inter-assay CV	
	TG	PG	TG	PG
	%		%	
LT (min)	7.8	7.0	17.6	15.7
Peak (nM)	6.0	1.9	9.7	10.2
ttPeak (min)	3.9	4.8	7.2	6.6
EP (nM*min)	0.75	1.1	3.7	9.1

The intra-assay variation experiments were evaluated from eight times samples. The inter-assay was calculated using a single sample per day measured over 7 different days.

Interplay between coagulation and fibrinolysis in the modified T/P-G

To investigate whether the modified T/P-G assay reflected the sequential interplay of coagulation (represented by the TG) and fibrinolysis (represented by the PG), generation of the key enzymes was evaluated in the presence of argatroban (a thrombin-specific inhibitor), activated protein C (APC), and thrombomodulin (TM). It was anticipated that these agents could induce different responses in coagulation and fibrinolysis. Normal plasma was incubated with increasing concentrations of argatroban, APC, and TM, prior to the T/P-G measurements. The addition of argatroban resulted in a significant impairment in TG in a dose-dependent manner, and demonstrated a >90% decrease in peak thrombin at a concentration of 10 μM (therapeutic blood level). In contrast, the lagtime in PG as well as in TG was prolonged, but the peak level of plasmin was elevated in the presence of up to 10 μM argatroban and reached a plateau at >10 μM (▶Figure 2A). The effects of APC and TM, known to be central in anti-coagulant mechanisms, were different from those of argatroban. The addition of APC (0-100 nM, ▶Figure 2B) and TM (0-5 nM, ▶Figure 2C) decreased the coagulation capacity in TG in similar dose-dependent manners. Moreover, the addition of TM also decreased the PG dose-dependently, with an ~50% level of peak plasmin at a concentration of 1 nM. The addition of APC, however, resulted in little decrease in PG. The findings showed that TG was depressed by all three agents. PG was delayed by TM but was enhanced by argatroban, the thrombin inhibitor. Our assay seemed to be particularly sensitive, therefore, to thrombin activatable fibrinolysis inhibitor (TAFI)-mediated effects (15). Furthermore, the addition of CPI, a competitive inhibitor of TAFI, mediated a slight dose-dependent increase in PG (▶Figure 2D).

Evaluation of individual factor-deficient plasma samples in the modified T/P-G assay

To investigate the role of individual coagulation factors in the interaction between coagulation and fibrinolysis, we examined individual, specific factor-deficient plasmas (-def) in the modified T/P-G assay. ▶Figure 3 illustrates representative curves from TG (*inset*) and PG with these plasma samples. In the TG assays with FVII-def, the lag-time alone was prolonged. With FVIII-def and FIX-def (components in the tenase assembly), the levels of peak thrombin were significantly diminished as previously reported (16). A mild reduction in peak thrombin was observed with FXIII-def and fibrinogen-def. With common pathway-related components, however, especially FV-def and FX-def (relevant to prothrombinase assembly), and with FII-def very little TG was evident. Similarly, there appeared to be very little PG with FV-def and FX-def. PG was modestly decreased with FII-def compared to normal plasma, but it was of interest that the capacity for PG was much greater than TG with this specific deficient plasma. PG with fibrinogen-def was markedly impaired, confirming the importance of fibrin formation as a scaffold to drive PG. With the other coagulant deficient-plasmas PG was equal to that with normal plasma. There was no PG with plasminogen-def, whereas with α2-PI-def, PG was enhanced by ~3-fold compared with normal plasma.

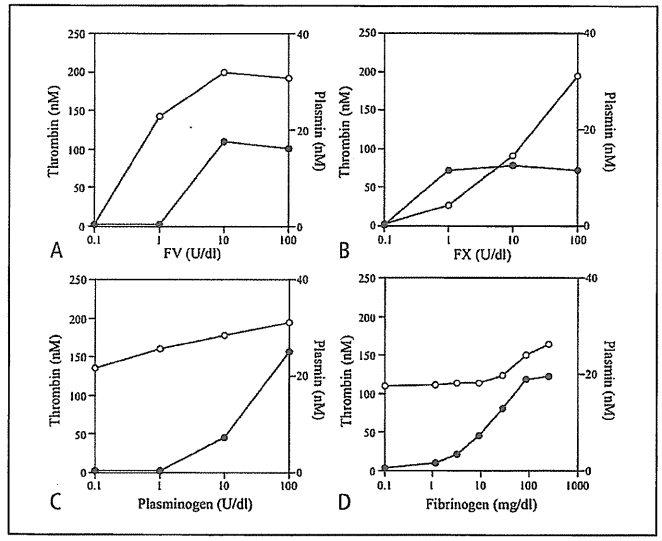

Figure 4: Effects of purified individual factors on T/P-G assays. Various amounts of purified coagulant proteins FV (panel A), FX (panel B), plasminogen (panel C), or fibrinogen (panel D) were added to the respective factor-deficient plasma, prior to measurements of TG (□) and PG (□) as described in *Methods*. The parameters (peak thrombin and peak plasmin) were plotted as functions of protein concentrations.

Effects of individual coagulant factors in the modified T/P-G assay

The experiments described above indicated that the common pathway-related factors (FV and FX), fibrinogen, and plasminogen uniquely influenced TG and PG. These effects were further examined, therefore, by the addition of purified exogenous coagulation factors to the respective deficient plasma. The addition of various amounts of FV and FX to FV-def and FX-def respectively, resulted in significant improvements in both TG and PG (▶Figure 4A, B), suggesting that these factors were essential to both mechanisms. However, 1 U/dl of FX possessed the same potential capacity for PG as 100 U/dl, whilst FV at 1 U/dl level did not mediate PG. It seemed possible, therefore, that the influence of FV on PG was greater than that of FX. With plasminogen-def, the addition of purified plasminogen increased the TG as well as PG dose-dependently, and it appeared that PG exerted a somewhat positive effect on TG (▶Figure 4C). This finding tended to confirm the close inter-relationship between coagulation and fibrinolysis. Interestingly, the addition of small amounts of fibrinogen (10 mg/dl) induced little change in TG (▶Figure 4D) but significant improvements in PG, demonstrating that small amounts of generated fibrin are likely to be sufficient for PG.

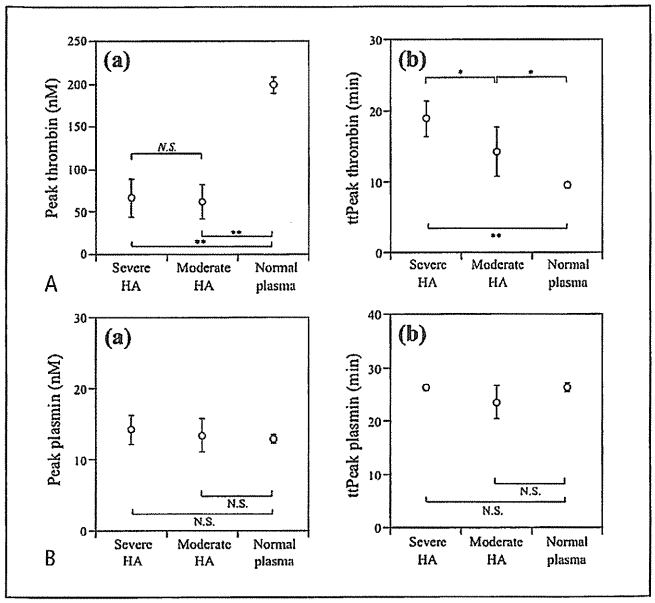

Figure 5: T/P-G in plasmas from patients with various phenotypes of haemophilia A. The trigger reagents were added to haemophilia A plasma (severe or moderate type) or normal plasma prior to evaluating TG (A) and PG (B) as described in *Methods*. The peak level (a) and ttPeak (b) of thrombin or plasmin were derived from the T/P-G data obtained. In all instances, results are shown as mean ± SD. HA: haemophilia A. *; $p<0.05$, **; $p<0.01$, N.S.; not significant.

Simultaneous T/P-G in patients with haemophilia A

The balance between coagulation and fibrinolysis in relation to levels of FVIII activity was examined in haemophilia A patients with various phenotypes. The ttPeak parameter in TG was significantly different among severe type (<0.2 U/dl), moderate type (1-5 U/dl), and normal plasma, but the peak thrombin in severe type was similar to that in moderate type (▶Figure 5Aa-b), confirming that TF-trigger TG assays are poorly sensitive to very low levels of FVIII activity (17). The peak plasmin parameter in PG with both severe type and moderate type in haemophilia A appeared to be slightly higher than normal, but the differences were not significantly different. The ttPeak parameter in PG with haemophilia A was the same as with normal plasma (▶Figure 5Ba-b). These results were in keeping with the relationship between bleeding symptoms and impairment of coagulation in patients with haemophilia A, and emphasised the imbalance between coagulation and fibrinolysis in these patients.

Discussion

The present study was designed to establish a modified T/P-G technique, based on the STP assay (11) that could provide an enhanced understanding of the relationship between coagulation and fibrinolysis. The previous assay did not appear to be sufficiently sensitive for the assessment of physiological TG and PG interactions. A particularly important point of TG assays is the precise measurement endogenous, generated active-thrombin, but it is difficult to explore normal control mechanism(s) - i.e. dilution of activated factors in flow states, etc. - are not in play in these assays. In addition, the binding of free thrombin and α2-macroglobulin (α2M), a representative thrombin inhibitor in plasma, creates a thrombin-α2M complex that is biologically inactive (18). This complex, however, together with free thrombin reacts with thrombin substrates in fluorometric TG assays, resulting in difficulties in interpretation. Hemker et al. estimated that 30% of thrombin formed was ultimately complexed with α2M (1). The simultaneous STP-assay and NHA reported previously (11, 12), therefore, appeared to highlight an issue of concern regarding the presence of thrombin-α2M complexes. In our studies, although it was difficult to preclude non-specific effects in the coagulation reactions, we utilised EDTA solution in our control buffers, and could preclude the influence of the α2M-thrombin complex that did not play a role in the coagulation reactions during this assay.

The original STP-assay and NHA offered advantages for evaluating individual TG and PG simultaneously in separate microtitre wells and in single wells, respectively (11, 12). The NHA provided data that identified the tight interplay between coagulation and fibrinolysis, but the STP assay did not. The single well system of the NHA, however, could be affected by untoward interference and interactions with signal detection. A major difference between these assays was the concentrations of reagents (TF and tPA) used in trigger mixtures, and we focused on the low concentrations of TF and tPA used in the NHA in an attempt to advance the STP assay using separate wells. We confirmed the potential capability of our T/P-G assay to identify the interplay between coagulation and fibrinolysis by demonstrating increases in TG caused by the addition of plasminogen, together with unique and opposite responses in TG and PG caused by argatroban, APC and TM (see ▶Figure 2). In particular, similar to the NHA, our modified assay was also sensitive to TAFI-mediated effects on fibrinolysis (15). Fundamentally, it appeared that the decrease in TG, mediated by argatroban and APC, reduced TAFI activation, and resulted in an increase in profibrinolytic activity (and no decrease in APC). In contrast, in keeping with current concepts, the addition of TM appeared to result in the formation of thrombin-TM complexes that significantly catalysed both the PC and TAFI activation, resulting in the impairment of TG and PG, respectively. Furthermore, enhanced fibrinolysis was observed in association with CPI-mediated inhibition of TAFI activation. From these data, our modified simultaneous T/P-G assay reflected the interplay between coagulation and fibrinolysis, and appeared to offer a relatively straightforward means to assess the important these balances.

In this respect, our assay revealed a significant role for individual coagulation factors in interacting mechanisms of coagulation and fibrinolysis. In particular, i) TG appeared to be responsible for the initiation of PG, and even trace amounts of TG promoted sufficient PG. ii) The absence of PG impaired TG (e.g. in fibrinogen-def), and PG was governed by the concentration of fibrinogen. iii) FV and FX, components of the prothrombinase assembly, were absolutely essential for the expression of both TG and PG, and FVIII

(and FIX), components of the tenase assembly, enhanced TG but did not affect PG. iv) The deficiency of anti-fibrinolytic factor, α2-PI, enhanced PG but had little effect on TG. These findings confirm that haemostasis is governed by a controlled balance between coagulation and fibrinolysis. In particular, unlike FVIII and FIX-def (haemophilia A and B), FV-def rarely results in major bleeding. It may be that both TG and PG are markedly compromised in FV-def, but TG alone is severely impaired in FVIII-def. Likewise, in α2-PI-def and Plg-def, enhanced PG and depressed PG, respectively, confer haemorrhagic or thrombotic tendencies.

We further demonstrated that haemorrhagic symptoms in patients with haemophilia A depended on significant impairment of coagulation and were not likely to be associated with modified fibrinolysis. Previous reports (19, 20) have suggested that patients with severe haemophilia A have enhanced fibrinolytic capacity, mediated by reduced TG and the subsequently decreased TAFI activation. We clearly demonstrated the opposite effects of APC and TM on PG but there were no differences in fibrinolytic capacity in patients with severe haemophilia A. It may be that our assay lacks sensitivity in this group of patients, and the relationship between bleeding tendency and phenotype is often not consistent, but nevertheless it seems unlikely that the haemophilia phenotype can be attributed to the imbalance between coagulation and fibrinolysis.

Our current findings provide challenging data for further studies. i) Although the modified T/P-G assay was designed to examine haemostasis *in vivo*, other factors that are likely to contribute to physiological haemostasis, including the vascular endothelium, platelets, and leucocytes, must be considered. ii) Our assay was performed using platelet-poor plasma, and it may be that the use of platelet-rich plasma or whole blood would provide important additional information. iii) A single assay condition was utilised for all variable coagulation deficiencies. Broader consideration of anti-coagulant pathways might be useful. iv) The local effects of TG and PG at sites of vascular injury could be pivotal for complete assessment of clinical disorders of haemostasis.

In conclusion, the control of haemorrhage and thrombosis is based on a sensitive balance between coagulation and fibrinolysis. Our assay would appear to be clinically helpful for the screening of patients with defective haemostasis in congenital disorders. Wider application of techniques of this nature could help to advance knowledge of the complex, functional interacting mechanisms of coagulation and fibrinolysis.

What is known about this topic?

- Simultaneous evaluation of thrombin and plasmin facilitates an overall understanding of normal and pathological haemostasis.
- Combined thrombin and plasmin generation assays have become to be adapted as the technique to evaluate the haemostatic state in patients with various haemostatic disorders.

What does this paper add?

- We established a simultaneous thrombin and plasmin generation (T/P-G) measurement using separate microtitre wells to retain sensitivity with the interplay between coagulation and fibrinolysis.
- The most evident impairments in TG were observed with deficiencies of coagulation factors participating in the prothrombinase complex. Defects in PG were observed with deficiencies of factor (F)V, FX, fibrinogen, and plasminogen.
- TG appeared to be a prerequisite for the initiation of PG, and overall PG was governed by fibrinogen concentration.
- TG, but not PG, in haemophilia A correlated with levels of FVIII:C, confirming that the abnormal haemostasis in haemophilia A results in a severe imbalance between coagulation and fibrinolysis.
- The modified T/P-G assay could provide an enhanced understanding of haemorrhage and thrombosis in clinical practice.

Conflicts of interest

None declared.

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Elevated plasma factor VIII enhances venous thrombus formation in rabbits: Contribution of factor XI, von Willebrand factor and tissue factor

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Summary

Elevated plasma levels of factor VIII (FVIII) are associated with increased risk of deep venous thrombosis. The aim of this study is to elucidate how elevated FVIII levels affect venous thrombus formation and propagation *in vivo*. We examined rabbit plasma FVIII activity, plasma thrombin generation, whole blood coagulation, platelet aggregation and venous wall thrombogenicity before and one hour after an intravenous infusion of recombinant human FVIII (rFVIII). Venous thrombus induced by the endothelial denudation of rabbit jugular veins was histologically assessed. Thrombus propagation was evaluated as indocyanine green fluorescence intensity. Argatroban, a thrombin inhibitor, and neutralised antibodies for tissue factor (TF), factor XI (FXI), and von Willebrand factor (VWF) were infused before or after thrombus induction to investigate their effects on venous thrombus formation or propagation. Recombinant FVIII (100 IU/kg) increased rabbit plasma FVIII activity two-fold and significantly enhanced whole blood coagulation and total plasma thrombin generation, but did not affect initial thrombin generation time, platelet aggregation and venous wall thrombogenicity. The rFVIII infusion also increased the size of venous thrombus 1 hour after thrombus induction. Argatroban and the antibodies for TF, FXI or VWF inhibited such enhanced thrombus formation and all except TF suppressed thrombus propagation. In conclusion, elevated plasma FVIII levels enhance venous thrombus formation and propagation. Excess thrombin generation by FXI and VWF-mediated FVIII recruitment appear to contribute to the growth of FVIII-driven venous thrombus.

Keywords

Deep venous thrombosis, factor VIII, factor XI, von Willebrand factor

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Received: January 28, 2013
Accepted after minor revision: April 12, 2013
Prepublished online: May 2, 2013
doi:10.1160/TH13-01-0069
Thromb Haemost 2013; 110: 62–75

Introduction

Venous thromboembolism (VTE) that comprises deep venous thrombosis (DVT) and pulmonary embolism (PE) has become a major medical problem, with an overall age- and sex-adjusted annual incidence of > 1:1,000. The incidence of VTE noticeably increases with advancing age, and PE represents an increasing proportion of total VTE with high morbidity and mortality rates (1). Many acquired and inherited risk factors have been identified including surgery, long-term immobilisation, age, malignancy, and deficiencies of antithrombin, protein C and protein S (2). In addition to these, recent epidemiological studies suggest that high plasma levels of factor VIII (FVIII) are associated with increased risk of DVT (3-5).

Factor VIII (FVIII) is a glycoprotein that is an essential cofactor for blood coagulation, and it circulates in plasma as a complex with von Willebrand factor (VWF). This complex is protected from proteolysis by activated protein C (6), and thrombin causes FVIII to dissociate from VWF (7). Activated FVIII (FVIIIa) binds to activated factor IX on negatively charged phospholipids such as the surface of activated platelets (8), where it subsequently activates factor X. The regulation of plasma FVIII levels and activity is complex, and FVIII coagulation activity ranges from < 50% in controls to > 300%, and 500% in patients with VTE (9).

Venous thrombus is initiated by endothelial injury and/or slow or static blood flow. It is generally recognised that the initiation phase is mainly modulated by procoagulant activity of the venous wall, and the propagation phase is regulated by blood flow and procoagulant factors in circulating blood. We previously demonstrated a close association among FVIII, platelets, VWF and fibrin in venous thrombi derived from human DVT, and that FVIII contributes to platelet aggregation and fibrin formation on a collagen surface under low shear conditions *in vitro* (10). Animal studies have also shown that FVIII plays a significant role in venous thrombus formation (11, 12). However, whether elevated FVIII levels promote venous thrombus formation and/or propagation,

and its association with other coagulation factors *in vivo* remain unclear.

The present study investigates this issue in a rabbit model of venous thrombosis.

Materials and methods

Affinity of human rFVIII for rabbit VWF

The affinity between recombinant human FVIII (rFVIII, Kogenate-FS, Bayer Healthcare, Leverkusen, Germany) and rabbit VWF was examined using an enzyme-linked immunosorbent assay (ELISA) (13). Rabbit plasma (100 µl) with or without rFVIII and diluted 10,000-fold was added to microtitre plates coated with anti-rabbit VWF antibody and incubated at 37°C for 2 hours (h). The contents of each well were removed, the plates were incubated with 100 µl of horseradish peroxidase (HRP)-conjugated anti-human FVIII antibody for 1 h at room temperature, washed and then perborate/3, 3', 5, 5'-tetramethylbenzidine substrate (100 µl) was added. After 20 minutes (min) at room temperature, the enzymatic reaction was stopped by adding 0.5 N sulfuric acid. The amount of FVIII bound to rabbit VWF was measured at 450 nm.

Coagulation parameters

Blood samples were collected from the central ear arteries of rabbits into 3.8% sodium citrate (9:1, v/v). Plasma samples were prepared by centrifugation at 2,580 g for 10 min at room temperature, and incubated with rFVIII (final concentrations: 0, 0.025, 0.25, 2.5 IU/ml) for 10 min. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured using a coagulation timer (Thrombotrack, AXIS-SHIELD; PoC AS, Oslo, Norway).

Coagulant activity of FVIII in rabbit plasma

The FVIII coagulant activity in rabbit plasma was measured using a one-stage clotting assay with a coagulation timer (Thrombotrack). Plasma samples were collected before, 30, 60 and 120 min after an intravenous infusion of rFVIII. The FVIII activity was assessed by measuring aPTT in diluted human FVIII-deficient plasma (Haematologic Technologies Inc., Essex Junction, VT, USA) (13). Pooled rabbit plasma served as the standard.

Thrombin generation assay

Thrombin generation in rabbit plasma was measured by calibrated automated thrombography as follows. Rabbit plasma collected before and 30 and 60 min after the rFVIII infusion was incubated with (final concentrations) 0.3 µM ellagic acid (Sysmex, Kobe, Japan), 0.5 pM recombinant human TF (Innovin®; Dade, Marburg, Germany) and 4 µM synthetic PL vesicles comprising phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine at a ratio of 1:6:3 as trigger reagents. Thrombin generation was started by adding $CaCl_2$ and the fluorogenic substrate Z-Gly-Gly-Arg-AMC. Lag time (LT), peak thrombin (Peak Th), time to peak (ttPeak) and endogenous thrombin potential (ETP), were calculated using Thrombinoscope software (Thrombinoscope BV, Maastricht, Netherlands) (14).

Thromboelastogram assay

Whole blood haemostatic parameters before, 30 and 60 min after rFVIII infusion were measured using a ROTEM analyser (Pentapharm GmbH, Munich, Germany). Blood samples (300 µl) in 3.8% sodium citrate (9:1, v/v) were transferred into the ROTEM reaction chamber. The blood re-calcified with 20 µl of 0.2 M $CaCl_2$ before clot formation was measured in duplicate using the standard NATEG evaluation parameters provided by the manufacturer (15). We assessed clotting time (CT) as the elapsed time from re-calcification to the start of clot formation, maximum clot firmness (MCF) and alpha angle, and the slope of clot formation. These parameters describe the following phases of the clotting process: initiation (CT), termination/final clot strength (MCF), and reaction velocity of clot formation (alpha angle).

Measurement of platelet aggregation

Blood samples in 3.8% sodium citrate (9:1, v/v) were separated by centrifugation at 130 g for 10 min and at 2,580 g for 10 min for platelet-rich plasma (PRP) and -poor plasma (PPP), respectively. We measured thrombin-induced platelet aggregation by mixing blood samples with acid citrate dextrose solution (9:1, v/v), and adding the mixture to acid citrate dextrose and modified suspension buffer (137 mM NaCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 2.7 mM KCl, 1.1 mM $MgCl_2$, 5.6 mM dextrose, pH 7.3). The mixtures were separated by centrifugation at 100 g for 10 min (16). Platelets sedimented from PRP by centrifugation at 1,010 g for 10 min with 0.7 µM (final concentration) of prostaglandin I_2 (Sigma-Aldrich Corp., St. Louis, MO, USA) were re-suspended in modified suspension buffer.

The number of platelets was adjusted to 2×10^5 μl^{-1} with PPP (for PRP aggregation) or suspension buffer (for washed platelet aggregation). Adjusted plasma was incubated with rFVIII (1.25 IU/ml) or with distilled water (control) for 10 minutes (min). Thereafter, collagen (Nycomed Austria GmbH, A-4020 Linz, Austria), adenosine 5'-diphosphate (ADP, Sigma-Aldrich Corp.), botrocetin (American Diagnostica Inc., Stamford, CT, USA) or thrombin (Sigma-Aldrich Corp.) was added as agonists. Platelet aggregation was measured using a PA-20 aggregation analyser (Kowa, Aichi, Japan). Changes in light transmittance caused by each agonist were recorded for 10 min (5 min for thrombin-induced platelet aggregation) and maximal aggregation was estimated. The extent of aggregation was expressed as a ratio (%) of the maximum light transmittance obtained with PPP or suspension buffer.

Tissue factor (TF) activities in normal jugular veins

To evaluate TF activities in the vascular walls, rabbit plasma clotting time initiated by the vessel homogenate was measured using a coagulation timer (Thrombotrack) (17). Jugular veins removed

from rabbits that had been infused with rFVIII (1.25 IU/ml) or saline were homogenised in Tris-buffered saline (pH 7.4) containing 10 mM $CaCl_2$ and 0.1% Triton X-100 (Nacalai Tesque Inc., Kyoto, Japan) using a Polytron PT3100 (Kinematica, Littau, Switzerland). After centrifugation at 2,580 g for 10 min, the supernatant (vessel sample; 100 µl containing 100 µg protein) was incubated with rabbit plasma (100 µl) for 1 min. Clotting assays were then started by adding 20 mM CaCl2 (100 µl). Protein concentrations were determined using bicinchoninic acid (BCA) protein assay kits (Pierce, Rockford, IL, USA). We also measured TF protein levels in the walls of rabbit veins and in plasma using a TF ELISA kit (USCN Life Science Inc., Houston, TX, USA).

Preparation of anti-rabbit TF antibody, R37

A monoclonal anti-rabbit TF neutralising antibody, R37, was generated using a standard procedure. Briefly, lymph node cells were isolated from five female SD rats (Japan Charles River, Yokohama, Japan) which had been immunised with recombinant rabbit TF (American Diagnostica Inc.), and fused with murine myeloma SP2/0 cells to establish hybridoma clones. Monoclonal hybridomas that secreted an antibody binding to rabbit TF were selected by an ELISA assay. Monoclonal antibodies were respectively purified with recombinant Protein A from the culture supernatants of the selected hybridoma clones. R37 exhibited a potent neutralising activity against TF/factor VII-catalysed factor X activation which was measured by an enzymatic assay using purified rabbit coagulation factors (American Diagnostica Inc. and Enzyme Research Laboratories, Swansea, UK) and S-2222 (Chromogenix Co., Milan, Italy). The neutralising ability of R37 was assessed using a diluted PT assay with modification (18). The PT reagent Thromboplastin C plus (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) was diluted 100-fold with 25 mM $CaCl_2$ and R37 was incubated with the dilute PT reagent or the rabbit plasma for 10 min at 37°C prior to the clotting experiments. R37 dose-dependently prolonged PT under both conditions (see Suppl. Figure 1, available online at www.thrombosis-online.com).

Thrombus formation in rabbit jugular veins

The Animal Care Committee of the University of Miyazaki (No.2010-511-3) approved the study protocols. Fifty male Japanese white rabbits weighing 2.5 - 3.0 kg were fed with a conventional diet. All surgical operations proceeded under aseptic conditions and general anesthesia was induced via an intravenous infusion of pentobarbital (25 mg/kg body weight). Thrombi were induced in the jugular veins by endothelial denudation using a 3F balloon catheter (Edwards Lifesciences, Irvine, CA, USA) (19). Saline (control) or rFVIII (100 IU/kg) was infused into the ear vein before balloon injury. Sixty minutes thereafter, the rabbits were infused with heparin (500 U/kg, i.v.) and then sacrificed with an overdose of pentobarbital (60 mg/kg, i.v.). The animals were perfused with 50 ml of phosphate buffered saline (0.01 mol/l) and perfusion-fixed with 50 ml of 4% paraformaldehyde to evaluate thrombus size and content.

We investigated the roles of thrombin, TF, factor XI (FXI) and VWF in thrombus formation by infusing 50 µg/kg/min of the thrombin inhibitor, argatroban (Argaron, Nichiiko Co. Ltd., Toyama, Japan), 3.0 mg/kg of anti-TF antibody R37 prepared by us, 3.0 mg/kg of XI-5108 anti-FXIa antibody (19, 20), or 1.0 mg/kg of AJW200 anti-VWF antibody (a gift from Ajinomoto Pharmaceutical Co. Ltd., Tokyo, Japan) (21) immediately before endothelial denudation. Argatroban was continuously infused i.v. because it has a short half-life (22).

Fluorescent imaging of venous thrombus

We observed thrombus formation and propagation in the jugular vein in real time by exposing the rabbit jugular vein and then infusing 3 µg of indocyanine green (ICG; Sigma-Aldrich Corp.) into the ear vein before, and 5, 15, 30, 45 and 60 min after endothelial denudation (23). A PDE-neo C10935-11 near-infrared camera (Hamamatsu Photonics Co. Ltd., Shizuoka, Japan) was placed 5 cm over the vein. Four regions of interest (ROIs, 20 × 20 pixels) were established at regular intervals on the vessel with one adjacent ROI as background (▶Figure 6A). The signal intensity was measured in all images from all animals. The vessels were washed with saline 1 min after ICG infusion and the average fluorescence intensity in each ROI was analysed using dedicated U11437 software (Hamamatsu Photonics Co. Ltd.). Data are expressed as ratios of the signal intensity of the vein to the adjacent background. The removed venous thrombi emitted fluorescence. To assess the effect of antithrombotic agents on thrombus propagation under elevated FVIII levels, argatroban, anti-TF, anti-FXI or anti-VWF antibodies were infused to the point where the average fluorescence intensity of ICG in each ROI exceeded > 3-fold the background (when mural thrombus developed).

Immunohistochemistry of rabbit venous thrombus

At 1 h after thrombus induction, jugular veins were fixed in 4% paraformaldehyde for 24 h at 4°C and embedded in paraffin. Sections (3-µm thick) were stained with hematoxylin and eosin and immunohistochemically examined using antibodies against glycoprotein (GP) IIb/IIIa (Affinity Biologicals Inc., Ancaster, CA, USA), fibrin (a gift from Takeda Chemical Industries Ltd., Osaka, Japan) and human FVIII (VIII-3776, Chugai Pharmaceutical Co. Ltd., Shizuoka, Japan) (10). The sections were stained with Envision (Dako, Glostrup, Denmark) or donkey anti-sheep IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Horseradish peroxidase activity was visualised using 3, 3'-diaminobenzidine tetrahydrochloride and the sections were faintly counterstained with Mayer's hematoxylin. Areas of venous thrombus and positive immunostaining for GPIIb/IIIa and fibrin were analysed using a color imaging morphometric system (WinROOF, Mitani, Fukui, Japan) (17).

Statistical analysis

All data are expressed as means ± standard error. Differences between or among individual groups were compared using paired or unpaired t-tests, respectively, or ANOVA with the Bonferroni post-hoc test (GraphPad Prism 5, GraphPad Software Inc., San Diego, CA, USA). A p-value of < 0.05 was considered to indicate statistical significance.

Results

Recombinant human FVIII binds rabbit VWF and shortens aPTT, but not PT in rabbit plasma

We found that rFVIII dose-dependently bound rabbit VWF (▶Figure 1). Adding rFVIII (final concentration, 0.025-2.5 IU/ml) to rabbit plasma significantly and dose-dependently shortened aPTT, but did not affect PT (▶Figure 2A). We infused rabbits

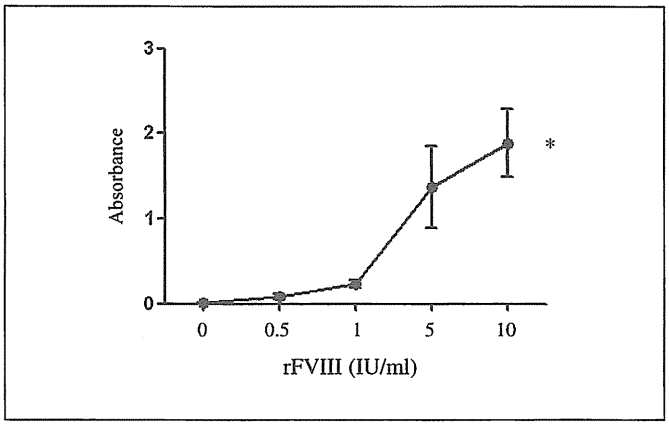

Figure 1: Affinity of rFVIII for rabbit VWF. Affinity between rFVIII and rabbit VWF was measured by ELISA. Diluted rabbit plasma with or without rFVIII was added to microtitre plates coated with anti-rabbit VWF antibody. The rFVIII dose-dependently bound to rabbit VWF and was detected using anti-human FVIII antibodies. (*$p < 0.0001$; $n = 5$ each).

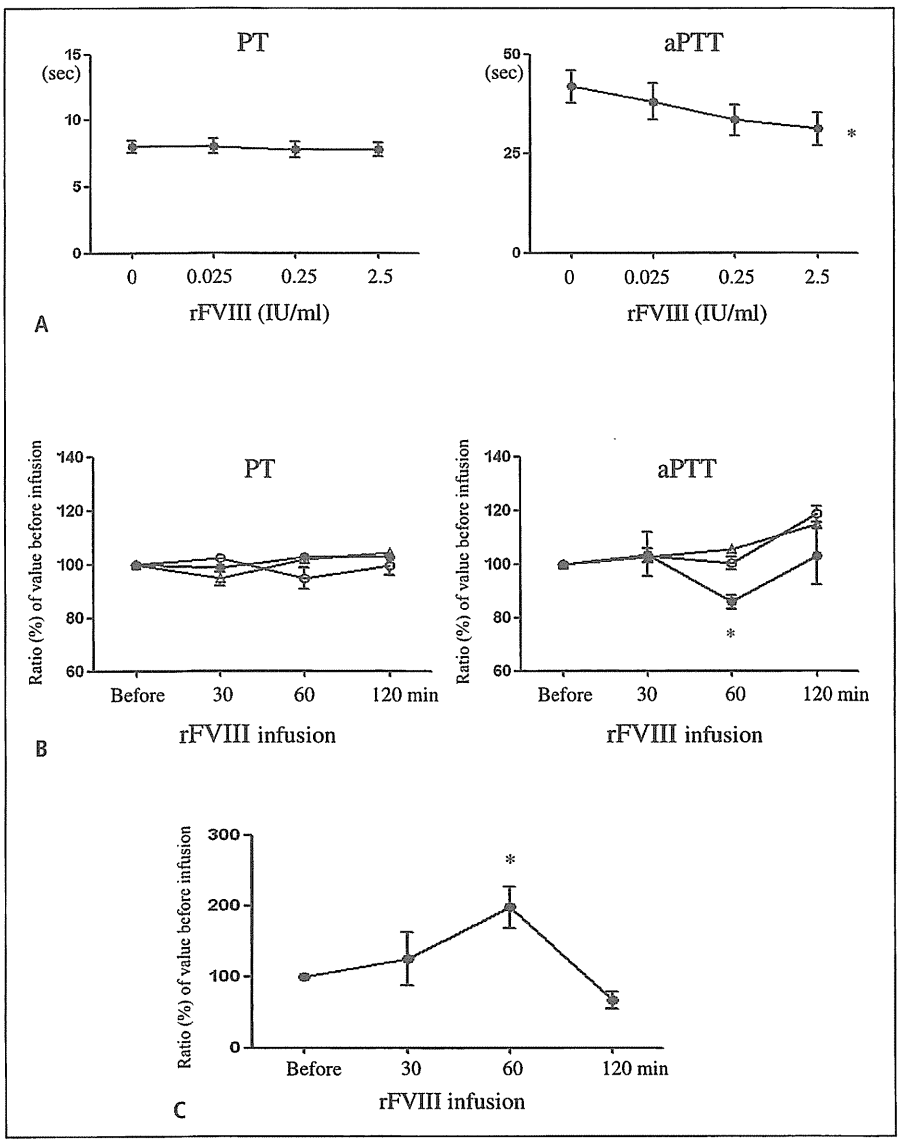

Figure 2: Effects of rFVIII on PT and aPTT *in vitro* and *ex vivo*, and plasma FVIII activity. A) Recombinant human FVIII shortened aPTT *in vitro* (*$p < 0.0001$), but did not affect PT ($p = 0.37$; $n = 4$ each). B) Three doses of rFVIII (20, 40 or 100 IU/kg) were infused into rabbits. Blood samples were collected before, 30, 60 and 120 min after rFVIII infusion. Infused rFVIII at 100 (filled circles) but not at 20 (open circles) or 40 (open triangles) IU/kg shortened aPTT within 1 h (*$p < 0.05$ vs before rFVIII infusion). However, no rFVIII doses affected PT ($p = 0.18$; $n = 4$ each). C) Plasma FVIII activity was measured using one-stage clotting assays in rabbit plasma diluted with human FVIII-deficient plasma. Blood samples were collected before and after rFVIII (100 IU/kg) infusion. Infused rFVIII increased plasma FVIII activity for up to 60 min ($n = 4$ each; *$p < 0.05$ vs before rFVIII infusion).

Table 1: Thrombin generation in rabbit plasma *ex vivo* before and after rFVIII infusion.

Parameters	Before (n = 6)	30 min after (n = 6)	60 min after (n = 6)
LT (min)	3.4 ± 0.2	3.2 ± 0.2	3.2 ± 0.1
Peak Th (nM)	250 ± 23	295 ± 26	313 ± 40*
ttPeak (min)	6.4 ± 0.3	5.6 ± 0.2†	5.6 ± 0.3†
ETP (nM x min)	1243 ± 116	1291 ± 100	1485 ± 139‡

ETP, endogenous thrombin potential; LT, lag time; Peak Th, peak Thrombin;. ttPeak, time to peak. *p < 0.05, †p<0.001 ‡p < 0.01 vs before rFVIII infusion. Data are shown as means ± standard error.

Table 2: Whole blood coagulation in rabbit plasma *ex vivo* before and after rFVIII infusion.

Parameters	Before (n = 6)	30 min after (n = 6)	60 min after (n = 6)
CT (sec)	1034 ± 74	519 ± 46†	299 ± 58†
MCF (mm)	59 ± 1	66 ± 1*	67 ± 1†
Alpha angle (°)	47 ± 3	65 ± 3*	75 ± 2†

CT, clotting time; MCF, maximum clot firmness. *p < 0.01, †p < 0.001 vs. before rFVIII infusion. Data are shown as means ± standard error.

with 20, 40 or 100 IU/kg of rFVIII and measured PT and aPTT *ex vivo*. We found that 100, but not 20 or 40 IU/kg of rFVIII significantly shortened aPTT one hour after the infusion (▶ Figure 2B).

Recombinant human FVIII enhanced rabbit plasma FVIII activity one hour after infusion

Plasma FVIII activity in rabbit plasma measured using a one-stage clotting assay before, and at 30, 60 and 120 min after rFVIII (100 IU/kg) increased about two-fold for up to 1 h after, compared with that before rFVIII infusion (198 ± 58 %, p < 0.05; ▶ Figure 2C).

Recombinant human FVIII enhances thrombin generation and whole blood coagulation

▶ Table 1 and ▶ Table 2 show thrombin generation parameters triggered by ellagic acid, TF and PL and whole blood coagulation parameters initiated by $CaCl_2$. The infusion of rFVIII enhanced peak Th and ETP and shortened ttPeak, but did not affect LT. The results of whole blood coagulation assays showed that the infusion of rFVIII significantly shortened CT, and increased MCF and alpha angle. These findings indicate that the rFVIII infusion enhanced whole blood coagulability via increased thrombin generation.

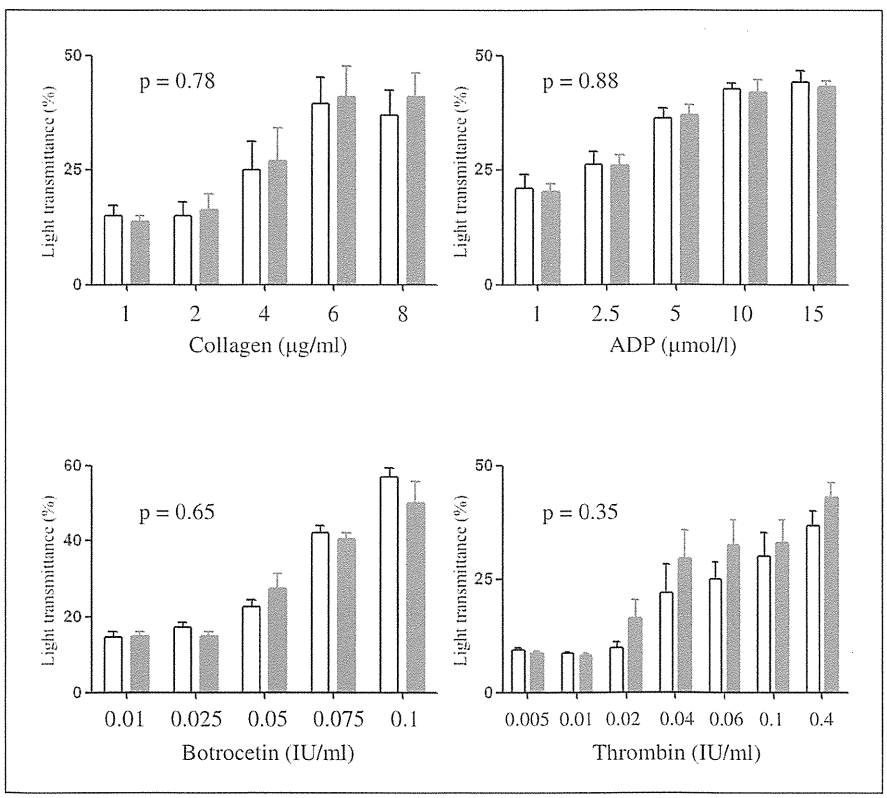

Figure 3: Platelet aggregation with or without rFVIII *in vitro*. Rabbit PRP and washed platelets with (filled columns) or without (opened columns) rFVIII were prepared as described in *Materials and methods*. Platelet aggregation was initiated by adding collagen, ADP, botrocetin or thrombin. Infused rFVIII did not affect platelet function (n = 4 each).