処方のポイント

● 病型によって治療方針が異なるので，治療前の病型診断が重要である（表1）。
● 酢酸デスモプレシン（DDAVP）は血管内皮細胞などに貯蔵されたvon Willebrand factor
（VWF）を血漿中に放出し，それによる止血を目的としているため，VWFを完全に欠い
ている（Type 3型）あるいは，異常分子を産生している患者（Type 2型）では止血効果は期
待できない。
● DDAVPは使用する前に前負荷試験を行い，効果を確かめておく。
● VWFは血漿中で第Ⅷ因子（FⅧ）と結合しているため，一般的には血漿由来FⅧ製剤には
VWFが含まれている。しかし，クロスエイトMは血漿由来の製剤であるが，モノクロー
ナル抗体で精製されているためVWFはほとんど含まれていない。
● FⅧ/VWF製剤はすべての病型に有効であるが，その投与量や投与期間は出血の程度に
よって異なる。多機能を有するVWFの止血モニターはさまざまであるが，血漿VWF：
RCoを用いるのが一般的である。
● 正常血漿中のVWF：RCoを100％とすると，FⅧ/VWF製剤をVWF：RCoとして1単
位/kgの輸注によって生体内では1〜2％のVWF：RCoの上昇が期待される。

使用上の注意

● DDAVPは頻脈，頭痛，顔面発赤，ナトリウム貯留などの副作用があるため，心疾患の
ある患者や妊婦などには慎重な投与が必要である。
● VWFは特有なマルチマー構造をとっており，マルチマーサイズが活性に影響する。F
Ⅷ/VWF製剤のロットによって含まれるVWFマルチマーが破壊されているものもあるの
で注意を要する。

禁忌

● DDAVPは，止血効果が著しく減弱するため，短期間での連続使用は禁忌である。
● DDAVPは，Type 2，3型には無効であるが，とくにType 2Bには投与禁忌である。
Type 2B型は，VWFの血小板膜GP1bに対する親和性が亢進することで血漿高分子VWF
マルチマーが消失する。DDAVPは血小板凝集を引き起こすので禁忌とされている。

［松本雅則］

7

血
液

日本人の血栓性素因

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POINT

- 血管，血小板，血液凝固関連の多くの遺伝子が動脈血栓性素因として報告されている．
- 遺伝子によっては研究間に結果のばらつきがある．
- メタアナリシスにより過去の研究結果の統合が行われている．
- 単独の遺伝子多型では，後天的要因ほど強力な危険因子ではない．

はじめに

血栓性疾患は，動脈血栓と静脈血栓に分けて考える必要がある．深部静脈血栓症や肺血栓塞栓症のような静脈血栓症では，血液凝固因子や凝固制御因子の関与が大きく，血栓性素因として複数の先天性凝固・凝固制御因子欠乏症が知られている．一方，脳梗塞や冠動脈疾患にみられる動脈血栓では，血小板凝集が血栓形成の主体であり，動脈硬化のような血管内壁の質的変化も深く関与している．すなわち，加齢，喫煙，高血圧，糖尿病，肥満などの後天的要因が，複雑に絡み合って動脈血栓症の危険因子として大きく寄与している．このことが動脈血栓症での先天的血栓性素因の同定を困難にしている．実際，静脈血栓症に比べ動脈血栓症の遺伝的素因はいまだ明快に語られていない．

このように動脈血栓症には後天的要因の関与が大きいと考えられるが，遺伝的要因もある程度関与していることが，双生児を用いた研究から確かめられた[1]．Flossmann らは，これまでに報告された研究結果を統合し，脳梗塞の発症率が一卵性または二卵性双生児のあいだで，あるいは家族集積性によって違いがあるか否かを検討した．その結果，一卵性双生児は二卵性双生児より高率に発症の一致を認め（オッズ比 1.65；95 ％信頼区間

1.2～2.3），遺伝的要因の寄与を検出している．彼らの研究は個々の遺伝子の寄与までを検討しているわけではないが，過去の研究では数々の遺伝子多型が危険因子として報告されている．

以下に，虚血性脳血管障害を含む動脈閉塞症の遺伝的素因として，報告されてきた数々の遺伝子多型のなかでも特に広く研究されている多型を紹介する．

血管へ影響を及ぼす因子

1. アンジオテンシン変換酵素（ACE）イントロン16 挿入(I)/欠失(D)多型

アンジオテンシン変換酵素（angiotensin converting enzyme；ACE）はレニン-アンジオテンシン系で働くカルボキシペプチダーゼであり，アンジオテンシン II の生成やブラジキニンの分解に働く．生理的には循環調節に重要であるが，高血圧・動脈硬化などの病態や血管リモデリングとも密接にかかわっている．*ACE* 遺伝子にはイントロン 16 に Alu 様配列（287 bp）の I/D 多型が存在する．この多型が ACE の血中濃度に大きく影響し，D アレルをもつヒトの血中 ACE レベルが高いことが報告された[2]．その後，虚血性心疾患や脳梗塞との関連が多くの研究によって検討され，D アレルをもつことは脳梗塞の独立した危険因子であると報告された[3]．日本人を対象にし

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た研究でも，D アレルは高血圧患者における脳梗塞の危険因子であるとされている[4]．日本人における DD 遺伝子型の出現頻度は 8 ％である[4]．

2. メチレンテトラヒドロ葉酸還元酵素（MTHFR）C677T 多型

ホモシステインは 5-メチルテトラヒドロ葉酸からメチル基を受け，メチオニンへ変換される．この反応で生成した葉酸は，メチレンテトラヒドロ葉酸還元酵素（methylenetetrahydrofolate reductase；MTHFR）により 5-メチルテトラヒドロ葉酸へとリサイクルされる．MTHFR の機能が低下すると，ホモシステインからメチオニンへの代謝が低下し，血中ホモシステイン濃度が上昇する．ホモシステインは，血小板の活性化，凝固因子の活性上昇，ヘパラン硫酸の発現低下，トロンボキサンの誘導などの多様な作用により，血管内皮の機能障害を起こすと考えられる．*MTHFR* 遺伝子の C677T（Ala222Val）変異は，MTHFR の単量体への解離と FAD の解離が速く，その結果活性が低下し，ホモシステインの代謝速度が低下する[5]．TT 遺伝子型は，軽度の高ホモシステイン血症を呈し，欧米や日本の研究で脳梗塞や冠動脈疾患の危険因子であるとの報告もあるが[6,7]，危険因子ではないとする報告もある．このような結果の一因として，血中の葉酸濃度が，C677T 多型と疾患の相関解析における交絡因子となっている可能性が指摘されている．日本人における TT 遺伝子型の出現頻度は 10～11 ％である[8]．

3. 内皮型一酸化窒素合成酵素（eNOS〈NOS3 ともいわれる〉）

内皮型一酸化窒素合成酵素（endothelial NO synthase；eNOS）から合成される NO には，血管平滑筋の弛緩や増殖抑制，内皮への白血球の接着抑制，抗血栓性の維持など，さまざまな生理機能がある．*eNOS* 遺伝子には，プロモーター活性に影響を及ぼす T-786C，イントロン 4 にある 27 bp 配列の 4 回または 5 回の繰り返し，酵素の安定性に影響を及ぼす Glu298Asp 変異が知られている．これらの多型は，主に日本人を対象とし

た研究で心疾患との関連が報告されている[9]．しかし他の集団を用いた研究の結果は必ずしも一致していない[10]．最近，脳梗塞のサブタイプと多型との相関解析により，ラクナ梗塞と Glu298Asp 変異（$p = 0.008$）[11]，イントロン 4 にある多型（$p = 0.01$）[12] との関連が報告された．このことは脳梗塞を病型分類して解析することにより，より明確な疾患との関連が見出される可能性があることを示唆している．

血液凝固・線溶に関与する因子

1. factor V Leiden（Arg506Gln）変異，プロトロンビン G20210A 多型

factor V Leiden は，活性化プロテイン C による分解に対して抵抗性を示し，その結果凝固能が亢進する[13]．Leiden 変異は，欧米人の血栓性素因として最も確立されたものであり，一般人口における出現頻度も約 3 ％と高いが，日本人ではこれまで検出されていない．プロトロンビン G20210A 多型は，mRNA の 3′ 非翻訳領域に位置しており，mRNA のプロセシングに影響を及ぼす[14]．欧米では血栓症患者での出現頻度が有意に高いと報告されているが，factor V Leiden 同様日本人には認められていない（表 1）．

2. フィブリノーゲンβ鎖 G-455A 多型

血中フィブリノーゲン量の増加と動脈血栓症との関連は，多くの報告により確かめられている．β鎖の多型のなかでは G-455A が最もよく研究されており，AA 遺伝子型保有者は GG 型保有者より血中フィブリノーゲン濃度が高い．しかし，G-455A 多型と冠動脈疾患や脳梗塞との関連については，一致した結果が得られていない[15,16]．このことは，フィブリノーゲンが，インターロイキン 6 に応答して発現上昇する急性期反応蛋白であることと関係しているかもしれない．フィブリノーゲンの血中濃度は，年齢，肥満，喫煙の影響で大きく変動することが知られており，これらの要因に起因する動脈硬化病変の炎症反応を反映し

表1　日本人・欧米人における血栓性素因となる遺伝子多型のアレル頻度比較

多型	リスクとなるアレル	日本人	欧米人
factor V Leiden (Arg506Gln)	Gln	0	0.02～0.03
プロトロンビン G20210A	A	0	0.01～0.02
フィブリノーゲンβ鎖 G-455A	A	0.13	0.19～0.26
PAI-1 4G/5G	4G	0.63	0.55
プラスミノーゲン Tochigi (Ala601Thr)	Thr	0.02	0

数値は一般人口での頻度.

ているとする説がある.

3. プラスミノーゲンアクチベーター阻害因子（plasminogen activator inhibitor ; PAI）-1 4G/5G 多型

　PAI-1 は組織中でも多様な機能を発揮するが，血中においては血栓の溶解を制御している．*PAI-1* 遺伝子プロモーターの-675 位には，G 塩基の欠失・挿入による 4G/5G 多型が存在する．4G アレル保有者の血中 PAI-1 濃度は，5G アレル保有者より高い．この多型は動脈閉塞症との関連で多くの報告があるが，結論は統一的ではない．

4. 凝固制御因子欠乏症

　凝固制御因子の欠損症は，静脈血栓症の危険因子としてとらえられることが多いが，脳梗塞の危険因子としても認識されるべきである．他の危険因子が陰性の若年発症例，血栓症患者の家族内発症例，静脈血栓の合併例などでは，凝固制御因子欠乏症の可能性が疑われる．日本人では，プロテイン S，プロテイン C，アンチトロンビン欠乏症がみられる．凝固制御因子は活性測定値が 50 % 以下になると血栓傾向が出現する．

血小板機能に関与する因子

　血小板糖蛋白（glycoprotein ; GP）は，血小板の活性化により高次構造変化を起こし，血小板血栓の形成過程で中心的役割を担う．フィブリノーゲン受容体である GP IIb/IIIa 複合体の *GP IIIa* 遺伝子上の Leu33Pro 変異は，冠動脈疾患や脳梗塞との関連で多数の報告があるが結果は一致していない．Leu33Pro 変異の頻度は日本人では非常に低い．フォンビルブランド因子（von Willebrand factor）受容体である GP Ib/IX/V 複合体の *GP Ibα* 遺伝子上の多型（Thr145Met 変異）についても，動脈閉塞性疾患との関連で複数の報告がある．統一的な結果ばかりではないが，日本人を対象とした研究で脳梗塞との関連が検出されている（$p = 0.0005$）[17]．

脳梗塞に関係する遺伝子多型のメタアナリシス—これまでに報告された遺伝的素因の検証

　脳梗塞の遺伝的素因を探索する研究から，多数の遺伝子多型が遺伝的素因になりうるとして報告されてきた．しかし，それらのいくつかは結果の再現性が乏しく，いまだ危険因子として確実視されているわけではない．研究間で結果の再現性が乏しい原因として，いくつかの可能性が考えられる．

　① 多型の出現頻度が低い，あるいは被験者数の不足のために，疾患と多型との相関を評価するための統計的検出力が不足していた可能性．

　② 試験のデザイン（エンドポイントの設定）や被験者集団（特に患者群）の特徴が，試験によって異なっている可能性．

　③ 人種的背景がヘテロな集団を対象として試験が施行された可能性（多型の出現頻度は人種間で大きく異なる）．

　これらの要因を排除するために，同じ仮説を検

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表2 脳梗塞の素因候補遺伝子

遺伝子（報告数）	多型	モデル	危険因子アレルの頻度（%）	患者数	対照数	オッズ比（95%信頼区間）	p_{Het} value
factor V Leiden (26) *	Arg506Gln	優性	6.5	4,588	13,798	1.33 (1.12-1.58)	0.03
MTHFR (22)	C677T	劣性	13.7	3,387	4,597	1.24 (1.08-1.42)	0.22
プロトロンビン (19) *	G20210A	優性	2.9	3,028	7,131	1.44 (1.11-1.86)	0.91
ACE (11)	I/D	劣性	26.4	2,990	11,305	1.21 (1.08-1.35)	0.47
factor XIII (6) *	Val34Leu	劣性	6.6	2,166	1,950	0.97 (0.75-1.25)	0.08
アポリポ蛋白E (10)	ε4, ε3, ε2	アレルε4 vs 他アレル	29.3	1,805	10,921	0.96 (0.84-1.11)	0.02
GP IIIa (9)	Leu33Pro	優性	27.3	1,467	2,537	1.11 (0.95-1.28)	0.76
eNOS (3)	Glu298Asp	劣性	12.5	1,086	1,089	0.98 (0.76-1.26)	0.40
PAI1 (4)	4G/5G	劣性	18.9	842	1,189	1.47 (1.13-1.92)	0.75
GP Ibα (3)	VNTR	D/D vs 他アレル	2.08	816	719	0.81 (0.39-1.70)	0.78
GP IIb (3)	Ile→Ser	劣性	13.9	770	1,090	0.99 (0.74-1.32)	0.42
GP Ibα (4)	Thr→Met	優性	13.4	564	962	1.55 (1.14-2.11)	0.68
factorVII (3)	A1/A2	優性	24.8	545	504	1.11 (0.83-1.48)	0.20
GP Ibα (3)	Kozak 配列	T/T vs C/C＋C/T	71.7	350	549	1.88 (1.28-2.76)	＜0.001
LPL (3)	Asn291Ser	優性	4.78	452	8,879	1.27 (0.80-2.01)	0.73

p_{Het} value：報告間におけるオッズ比のばらつきの p 値（値が小さい場合，有意なばらつきがある），VNTR：variable number tandem repeat
＊ 日本人には認められない多型
（Casas JP, et al. Arch Neurol 2004；61：1652-61[18] より）

証した試験を収集し，結果を統計的に統合して再検証するメタアナリシスが行われる．脳梗塞に関するメタアナリシスはすでにいくつかの報告があるが，最近報告された Casas らの研究[18] はこれまでで最大規模である．彼らは，2003 年 1 月までに発表されたすべての脳梗塞に関するケース・コントロール研究について検索を行い，画像による脳梗塞の確定診断があり，白人のみを対象にしている 120 の報告について解析を進めた（対象が 18 歳未満，中間形質での検討は除外）．

　約 18,000 人の患者と約 58,000 人の健常者について 32 遺伝子を解析した．結果を表2[18] に示す．これまで広く研究されてきた 4 つの多型，factor V Leiden，MTHFR C677T，プロトロンビン G20210A，ACE I/D は脳梗塞との相関が認められた．これに対して，同様に多数の報告がある factor XIII，アポリポ蛋白 E，血小板 GP IIIa などの遺伝子は今回の解析では有意ではなかった．有意な相関が検出された多型であっても，強力な危険因子というわけではなかった（オッズ比 1.21～1.44）．ここでは単独の多型と脳梗塞との関連

について単点のみで解析されたが，ハプロタイプ解析も有効であると思われる．

ゲノムワイド解析による最近の成果

　最近，脳梗塞に相関する新しい遺伝子として，ホスホジエステラーゼ 4D（PDE4D）[19] と 5-リポキシゲナーゼ活性化蛋白（ALOX5AP）[20] が報告された．これらは，ゲノム全域に分布する多型マーカーと疾患との相関解析により同定された．これらの遺伝子は，別の集団で疾患との相関が検証されること，疾患の成因と遺伝子機能との関連が解明されることが，今後の研究に委ねられている．今後，さらに多型データベースが充実し，タイピング技術が進歩すれば，ゲノムワイドな解析により新たな疾患関連遺伝子が同定される可能性がある．

おわりに

　脳梗塞のなかには，単一の遺伝子異常で発症

し，その原因遺伝子が同定されているものもある．しかし大多数の脳梗塞は，ここまで述べてきたように，後天的要因と遺伝的素因との相互作用の結果，発症するものと考えられる．これまでに同定された遺伝子の危険因子としての寄与率は，それぞれ単独では後天的要因ほど強いものではないようである．このような遺伝的素因を同定する

ためには，検出力を十分に維持していることに配慮して解析が行われなければならない．遺伝子多型には，人種間で出現頻度に違いがあるものが多いことからも，厳密な病型分類に基づいた大規模なメタアナリシスが，日本人集団について行われることが期待される．

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7章 脳梗塞

ADAMTS 13

A Disintegrin and Metalloprotease on Thrombospondin-1 Motif, 13

小亀浩市

ADAMTS 13 は von Willebrand 因子を切断する血漿酵素である. その活性測定は, 血栓性血小板減少性紫斑病の確定診断, あるいは特発性血小板減少性紫斑病や播種性血管内凝固症候群の除外診断に有用である.

検査の目的

ADAMTS 13(a disintegrin-like and metalloprotease with thrombospondin-1 motif, 13)は, 血漿に存在する蛋白質分解酵素であり, **von Willebrand 因子**(von Willebrand factor ; VWF)を特異的に切断する. VWF は, 超高分子量のホモマルチマーとして血管内皮細胞から分泌される蛋白質であり, 分子量が大きいほど血小板粘着凝集活性が高い. したがって, ADAMTS 13 は VWF を適度に切断することで止血機能を調節していると考えられる.

ADAMTS 13 の活性が低下すると, 血液中に超高分子量の VWF が蓄積するため, 血小板が凝集しやすい状態になる. その結果, さまざまな臓器の細小血管で血小板血栓が生じ, 多様な症状をもたらす. これが**血栓性血小板減少性紫斑病**(thrombotic thrombocytopenic purpura ; TTP)の発症機序である[1)2)].

TTP の臨床症状は, 溶血性尿毒症症候群(hemolytic uremic syndrome ; HUS)と酷似しており, 播種性血管内凝固症候群(disseminated intravascular coagulation ; DIC)との類似点も多い. ADAMTS13 活性の著減を特徴とする TTP を類似疾患と鑑別するために, ADAMTS 13 活性の測定が有用である.

検査法の実際

ADAMTS 13 の実体が判明する以前から, VWF 切断酵素活性を測定する方法は開発されてきた. 最初は, Furlan 博士らによる SDS アガロースゲル電気泳動法と, Tsai 博士による SDS-ポリアクリルアミドゲル電気泳動法である. いずれも, 精製ヒト VWF マルチマーを血漿と反応させ, 電気泳動後にウェスタンブロットを行う.

これらの方法は TTP 研究の突破口を開いたが, 操作が煩雑であり, 臨床検査レベルで普及するには無理がある. のちにコラーゲン結合能測定法, サンドイッチ ELISA 法, リストセチンコファクター活性測定法など簡素化が図られたが, より簡便な方法が望まれていた[3)].

著者らは, 種々の VWF 断片を作製し, ADAMTS 13 との反応性を調べた. その結果, Asp^{1596} から Arg^{1668} までの 73 アミノ酸残基からなるペプチドが特異的基質となる最小領域であることが判明した[4)]. この領域を VWF 73 と呼んでいる.

さらに, VWF 73 の 2 残基を修飾した合成ペプチド FRETS-VWF 73 を開発した[5)]. これは, 蛍光共鳴エネルギー転移(fluorescence resonance energy transfer ; FRET)技術を利用した消光性蛍光基質であり, ADAMTS 13 による切断に比例して蛍光強度が増加する. 手順は, 96 穴プレート上で反応用緩衝液に希釈したクエン酸血漿に基質溶液を添加し, 蛍光プレートリーダーで経時的に測定するのみである(図). 測定は 1 時間で終了し, 蛍光値の増加速度を標準血漿と比較検量する. 操作が

用語解説————von Willebrand 因子
血漿に存在する超高分子量の糖蛋白質. コラーゲンや血小板表面蛋白質に結合し, 一次止血における血小板の粘着や凝集を仲介する.

用語解説————血栓性血小板減少性紫斑病
血小板減少と溶血性貧血を主症状とし, しばしば動揺性精神神経症状, 腎障害, 発熱を併発する難病. 先天性と後天性に分類される.

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反応用緩衝液（96μL）＋血漿（4μL）
        ↓
FRETS-VWF73溶液（4μM，100μL）
        ↓
蛍光プレートリーダー（励起340nm，測定440nm）
        ↓  5分ごとに1時間測定
蛍光増加速度を標準血漿と比較検量
```
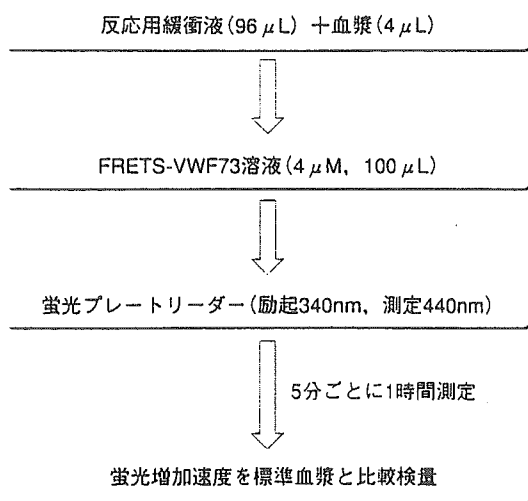
図　FRETS-VWF 73 を用いた血漿 ADAMTS 13 活性の測定

簡便であるため，臨床検査レベルでの普及が期待される．FRETS-VWF 73 は㈱ペプチド研究所から販売されており，受託検査として㈱エスアールエルが受け付けている．

一方，ADAMTS 13 抗原量の測定に関しては，㈱三菱化学ヤトロンからキットとして販売されている．病態との関連はまだ活性ほど詳細に調べられていない．

診断的意義

TTP は，*ADAMTS 13* 遺伝子の変異による先天性と，ADAMTS 13 に対する自己抗体の出現による後天性に大別されるが，いずれにおいても早期の診断と治療開始が重要である．病態の悪化が速く，致死的な経過をたどることも多いため，できるだけ早く治療（新鮮凍結血漿輸注あるいは血漿交換）を行うべきである．迅速かつ適切な治療を開始するための判断基準のひとつとして，ADAMTS 13 活性の測定は重要である．現在のところ，カットオフ値は標準血漿の 5 ％といわれている．

治療においても，活性測定は有用であると考えられる．例えば治療経過の観察や治療用血漿の選択などに奏効するかもしれない．

TTP の確定診断だけでなく，特発性血小板減少性紫斑病や DIC の除外診断にも有用である．TTP 患者への血小板輸注は禁忌であるため，判断が難しい症例では ADAMTS 13 活性を測定することが重要である．

その他注意すべき点

TTP 様の臨床症状があり，さらに ADAMTS 13 活性が著減していれば，TTP と確定できる．しかし，臨床上 TTP と診断するのが妥当であっても，低値でない活性が認められる症例も存在する．それが活性測定法に依存するのか，あるいは ADAMTS 13 以外の要因が潜んでいるのか，現時点で明確な解答はない．臨床症状から定義される TTP と，活性測定から定義される ADAMTS 13 欠損症は，1 対 1 で対応しない．鑑別困難な症例があるのが現状であり，診断ガイドラインの作成が望まれる．

ADAMTS 13

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FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay

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Summary

A plasma metalloprotease, ADAMTS13, cleaves von Willebrand factor (VWF) multimers and downregulates their activity in platelet aggregation. Functional ADAMTS13 deficiency leads to the accumulation of hyperactive large VWF multimers, inducing a life-threatening disease, thrombotic thrombocytopenic purpura (TTP). Although measuring ADAMTS13 activity is important in TTP diagnosis, existing methods require time and skill. Here, we report a fluorescence resonance energy transfer (FRET) assay for ADAMTS13 activity. We developed a synthetic 73-amino-acid peptide, FRETS-VWF73. Cleavage of this substrate between two modified residues relieves the fluorescence quenching in the intact peptide. Incubation of FRETS-VWF73 with normal human plasma quantitatively increased fluorescence over time, while ADAMTS13-deficient plasma had no effect. Quantitative analysis could be achieved within a 1-h period using a 96-well format in commercial plate readers with common filters. The FRETS-VWF73 assay will be useful for the characterization of thrombotic microangiopathies like TTP and may clarify the importance of ADAMTS13 activity as a predictive marker for various thrombotic diseases.

Keywords: ADAMTS13, von Willebrand factor, platelet, thrombotic thrombocytopenic purpura, fluorescence resonance energy transfer.

Thrombotic thrombocytopenic purpura (TTP), a syndrome characterized by thrombocytopenia and microangiopathic haemolytic anaemia, is often associated with neurological dysfunction, renal failure and fever (Moschcowitz, 1924; Moake et al, 1982). Although most patients with TTP experience these crises idiopathically throughout adulthood, some patients present with neonatal onset and frequent relapses, also called Upshaw–Schulman syndrome (USS). Recent genetic studies have revealed that the majority of USS patients are homozygous or compound heterozygous for a critical mutation of the ADAMTS13 gene (Levy et al, 2001; Kokame et al, 2002; Antoine et al, 2003; Assink et al, 2003; Savasan et al, 2003; Schneppenheim et al, 2003; Matsumoto et al, 2004; Pimanda et al, 2004). ADAMTS13 encodes a plasma metalloprotease of the ADAMTS family (Levy et al, 2001; Soejima et al, 2001; Zheng et al, 2001; Plaimauer et al, 2002; Banno et al, 2004). Many patients with acquired TTP possess inhibitory auto-antibodies against ADAMTS13 (Furlan et al, 1998; Tsai & Lian, 1998). As the clinical characteristics of TTP are similar to those of other microangiopathic haemolytic anaemias, such as haemolytic uremic syndrome (HUS) and disseminated intravascular coagulation (DIC), an assay measuring ADAMTS13 activity would be an useful tool for appropriate diagnosis and treatment of TTP.

ADAMTS13 cleaves the peptidyl bond between Y1605 and M1606 in the A2 domain of von Willebrand factor (VWF) (Dent et al, 1990; Tsai et al, 1994; Furlan et al, 1996; Tsai, 1996), which circulates in plasma as large multimeric forms, ranging in size from 500 to 20 000 kDa. Functional ADAMTS13 deficiency can lead to the accumulation of large, hyperactive VWF multimers. A method to measure VWF-cleavage activity of ADAMTS13 was originally developed by Furlan et al (1996) and Tsai (1996), in which purified human VWF multimers were incubated with plasma in the presence of either urea or guanidine. The reaction products were separated by sodium dodecyl sulphate (SDS)-agarose (Furlan et al, 1996) or SDS-polyacrylamide (Tsai, 1996) gel electrophoresis, followed by Western blotting analysis with anti-VWF antibodies. Although these methods have significantly increased our understanding of the role of ADAMTS13 in TTP pathogenesis, they are not widely used at the clinical level because of technical complications.

Several groups have attempted to develop more simple and rapid diagnostic procedures for clinical use, including a

collagen-binding assay (Gerritsen *et al*, 1999), an immunora-diometric assay using two site-directed VWF antibodies (Obert *et al*, 1999) and a ristocetin-cofactor assay (Böhm *et al*, 2002). Multicentre comparison studies of these different assays showed varied performance but supported the usefulness of the ADAMTS13 assay for TTP diagnosis (Studt *et al*, 2003; Tripodi *et al*, 2004). These assays, however, still demand complicated procedures and highly specialized materials. Therefore, a more rapid, reliable and convenient method of measuring VWF activity is eagerly awaited.

As chromogenic substrate assays are used in the clinical measurement of protease activities, initial studies were sought to identify a short oligopeptide that can be specifically cleaved by ADAMTS13 (Furlan & Lämmle, 2002). As these attempts have systematically failed, the cleavage at Y1605–M1606 of VWF probably depends on both the specific residues in the vicinity of the scissile bond and more remote sequences. Recently, we have succeeded in creating a recombinant substrate encompassing the shortest region of VWF that serves as a specific substrate for ADAMTS13 (Kokame *et al*, 2004). The peptide substrate, designated VWF73, contains 73-amino-acid residues of VWF from D1596 to R1668. In this study, we have chemically modified VWF73 to facilitate the quantitative measurement of ADAMTS13 activity in a single-step procedure.

Materials and methods

Materials

The fluorogenic substrate, FRETS-VWF73, was chemically synthesized by Thermo Electron GmbH (Sedanstrasse, Ulm, Germany) and the Peptide Institute, Inc. (Osaka, Japan). It was dissolved in 25% dimethyl sulphoxide/water to prepare the 100-μmol/l stock solution. Human plasma was obtained by centrifugation from whole blood that was treated with a 1/10 volume of 3·8% sodium citrate as an anti-coagulant. A protease inhibitor cocktail (Sigma, St Louis, MO, USA) used in the cleavage experiments contained 1 mmol/l 4-(2-aminoethyl) benzenesulphonyl fluoride, 15 μmol/l pepstatin A, 14 μmol/l *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane, 36 μmol/l bestatin, 21 μmol/l leupeptin and 0·8 μmol/l aprotinin at a final concentration.

Fluorescent assay to measure the ADAMTS13 activity

Pooled human plasma (a range of 0–8 μl as a standard), or 4 μl of each test plasma, was diluted in 100 μl of assay buffer (5 mmol/l Bis–Tris, 25 mmol/l CaCl$_2$, 0·005% Tween-20, pH 6·0) in a 96-well white plate (Sumitomo Bakelite, Tokyo, Japan). Then, 100 μl of 4 μmol/l FRETS-VWF73 in the assay buffer was added to each well. Fluorescence was measured at 30 C in a Wallac 1420 ARVO multilabel counter (PerkinElmer Japan, Yokohama, Japan) equipped with a 340-nm excitation filter and a 450-nm emission filter. Fluorescence was measured every 5 min. The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 to 60 min using the prism software (GraphPad Software, San Diego, CA, USA).

Preparation of recombinant ADAMTS13 (rADAMTS13)

HeLa cells were cultured in Dulbecco's minimal essential medium (Invitrogen, Carlsbad, NM, USA) supplemented with 10% fetal bovine serum in humidified air with 5% CO$_2$ at 37 C. To produce rADAMTS13, the human ADAMTS13-expression plasmid was transfected into the subconfluent cells using FuGENE6 (Roche Diagnostics, Indianapolis, IN, USA), as described previously (Kokame *et al*, 2002; Matsumoto *et al*, 2004). Following a 4-h incubation, the culture medium was replaced with serum-free OPTI-MEM I medium (Invitrogen) and the culture was incubated for 44 h. The medium was collected and concentrated to one-eighth the original volume using Centricon YM-30 (Millipore, Billerica, MA, USA). As a negative control, a series of operations was performed in parallel as for the untransfected cells.

Subject population

The Suita study participants were arbitrarily selected from the municipality population registry of Suita city, stratified by gender and 10-year age groups. The basic sampling of the population started in 1989 with a cohort study base (Mannami *et al*, 1997). In the present study, 100 consecutive samples were selected from this population as a control group. This study was approved by the ethical committee on human research of the National Cardiovascular Centre. Written informed consent was obtained from all subjects prior to testing.

Results

Design of the fluorogenic substrate for ADAMTS13

To utilize fluorescence resonance energy transfer (FRET) to measure ADAMTS13 activity, we chemically synthesized a fluorogenic peptide, FRETS-VWF73 (Fig. 1), containing the 73-amino acids from D1596 to R1668 of VWF. Within this peptide, the Q1599 residue at the P7 position was converted to a 2,3-diaminopropionic residue (A2pr) modified with a 2-(N-

NH$_2$-DRE-A2pr-APNLVYMVTG-A2pr-PASDEIKRLPGDIQVVP
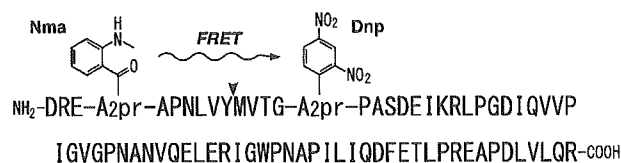IGVGPNANVQELERIGWPNAPILIQDFETLPREAPDLVLQR-COOH

Fig 1. Structure of FRETS-VWF73. Within the 73-amino-acid peptide sequence, corresponding to the region from D1596 to R1668 of von Willebrand factor (VWF), Q1599 and N1610 were substituted with A2pr(Nma) and A2pr(Dnp) respectively. The arrowhead indicates the site cleaved by ADAMTS13.

methylamino)benzoyl group (Nma). The N1610 residue of the P5' position was converted to A2pr modified with a 2,4-dinitrophenyl group (Dnp). When the Nma group is excited at 340 nm, fluorescence resonance energy is transferred to the neighbouring quencher, Dnp. If the bond between Y1605 and M1606 is cleaved, the energy transfer quenching the fluorescence does not occur, allowing the emission of fluorescence at 440 nm from Nma.

Cleavage of FRETS-VWF73 by plasma ADAMTS13

To explore the cleavage activity present in plasma, FRETS-VWF73 was incubated with normal human plasma in a fluorescent plate reader. Emission at 450 nm increased with time, indicating that FRETS-VWF73 was cleaved between the two A2pr residues by a plasma component (Fig. 2A). The increase of fluorescence was not inhibited by the addition of a protease inhibitor cocktail (mixed inhibitors effective against a broad range of serine proteases, cysteine proteases, aminopeptidases and acid proteases), but was completely inhibited by a divalent cation chelator (EDTA), suggesting that cleavage was mediated by the plasma metalloprotease, ADAMTS13, with minimal contribution of other plasma proteases. In fact, neither thrombin nor plasmin (5 μg/ml each, Sigma) increased fluorescence of FRETS-VWF73 (data not shown). The incubation of FRETS-VWF73 with plasma from an ADAMTS13-deficient patient showed no increase of fluorescence (Fig. 2A). The addition of ADAMTS13-deficient plasma to the normal plasma did not interfere with the cleavage of FRETS-VWF73 by the normal plasma (data not shown).

To verify further the cleavage by ADAMTS13, the substrate was incubated with the conditioned medium of cultured HeLa cells (Fig. 2B). Incubation with the medium of ADAMTS13-transfected cells showed the time-dependent increase of fluorescence, whereas the incubation with the medium of untransfected cells did not. All these data supported the conclusion that ADAMTS13 specifically cleaved FRETS-VWF73.

Plasma-dose dependency

FRETS-VWF73 cleavage was quantitatively dependent on plasma dosage (Fig. 3). We monitored fluorescence increase in the presence of variable volumes of normal plasma to the reaction mixture. The fluorescence over time increased with increasing plasma in a dose-dependent manner (Fig. 3A). To compensate for any differences in background fluorescence derived from plasma itself and to calculate the initial reaction rate, we estimated the slopes of the fluorescence over time using time points 0 and 60 min from a linear regression. These slopes (reaction rates) were then plotted against the plasma dosage (Fig. 3B). The data points fitted to a non-linear regression, indicating that ADAMTS13 activity in sample plasma could be estimated from the fluorescence reaction rate.
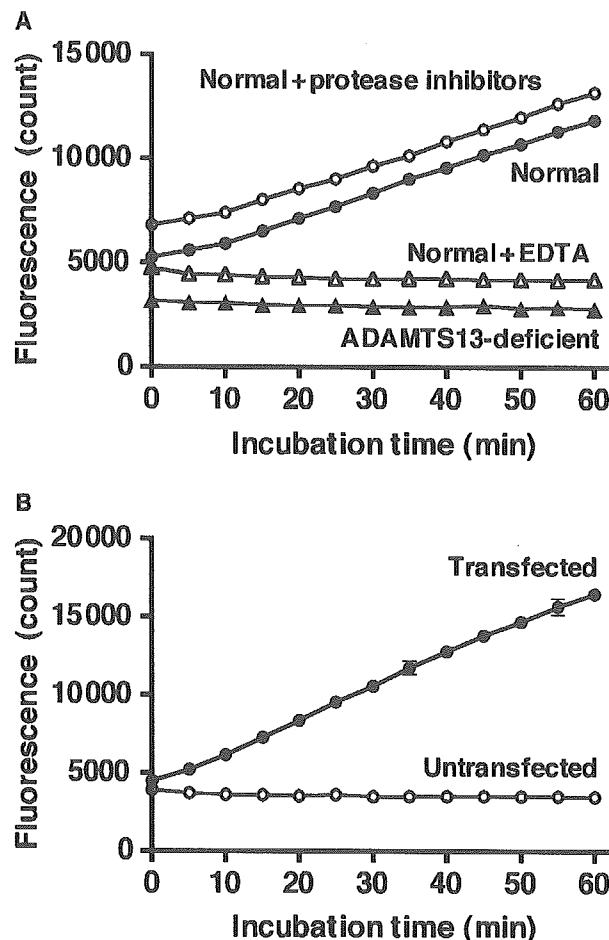

Fig 2. Cleavage of FRETS-VWF73 by ADAMTS13. (A) Fluorescence changes in FRETS-VWF73 during incubation with plasma. FRETS-VWF73 was incubated with normal plasma from a single donor in the absence (closed circles) or presence of either protease inhibitors (open circles) or EDTA (open triangles). The substrate was also incubated with ADAMTS13-deficient plasma from a congenital thrombotic thrombocytopenic purpura patient (closed triangles). Fluorescent emission at 450 nm was measured at the indicated times. A representative of three repetitive experiments is shown. (B) Fluorescence changes in FRETS-VWF73 during incubation with recombinant ADAMTS13 (rADAMTS13). FRETS-VWF73 was incubated with the conditioned medium of HeLa cells transfected with (closed circles) or without (open circles) ADAMTS13-expression plasmid DNA. Values shown are the mean and SD ($n = 3$).

Optimization of the FRETS-VWF73 assay

We next optimized reaction conditions to increase both the sensitivity and rapidity of measurement (Fig. 4). As ADAMTS13 requires divalent metal ions for proteolytic activity, we monitored the cleavage of FRETS-VWF73 by plasma in the presence of various metal ions (Fig. 4A). Ca^{2+} and Ba^{2+} ions were the most favourable for the reaction, although Mg^{2+} and Zn^{2+} also enhanced ADAMTS13 activity. In contrast, Mn^{2+} and Ni^{2+} could not activate the reaction, consistent with previous reports (Furlan et al, 1996; Tsai, 1996). Testing of various Ca^{2+} ion concentrations revealed that a range of
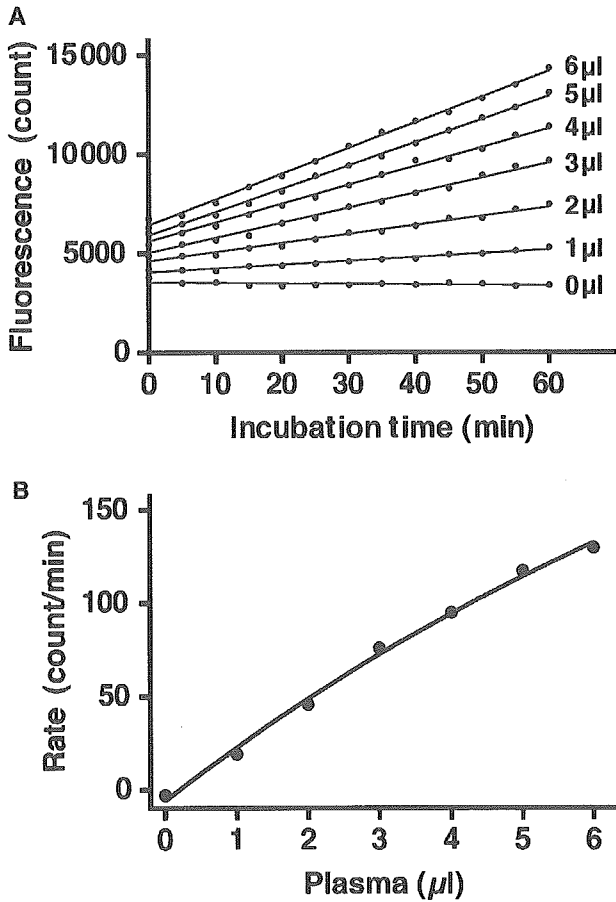
A

B

Fig 3. Plasma-dose dependency of FRETS-VWF73 cleavage. (A) Fluorescence was measured at 5-min intervals after the addition of FRETS-VWF73 to 0–6 µl normal plasma. (B) The reaction rates of time points 0 and 60 min were plotted against plasma dosage. Values were fit to a non-linear regression curve.

10–50 mmol/l Ca^{2+} was optimal for the reaction (Fig. 4B). We also examined the effect of differing NaCl concentrations, determining that lower concentrations provided more rapid cleavage (Fig. 4C), as seen in previous reports (Furlan *et al*, 1996; Kokame *et al*, 2004). The pH optimum for the FRETS-VWF73 assay was approximately 6·0 (Fig. 4D), which differed from previous studies reporting an optimal pH of 8·0–10·0 for the cleavage reaction (Furlan *et al*, 1996). This inconsistency may be a result of different reaction conditions, such as the presence or absence of denaturants. In addition, substitution of Q1599 and N1610 to A2pr(Nma) and A2pr(Dnp), respectively,

Fig 4. Optimization of the FRETS-VWF73 assay. (A) To determine metal ion dependency, FRETS-VWF73 was incubated with normal plasma from a single donor in the presence of the indicated divalent ions. (B) To measure Ca^{2+}-concentration dependency, FRETS-VWF73 was incubated with plasma in the presence of 0–100 mmol/l $CaCl_2$. (C) Ion-strength dependency was determined by incubating FRETS-VWF73 with plasma in the presence of 0–1000 mmol/l NaCl. (D) To measure pH dependency, FRETS-VWF73 was incubated with plasma in the indicated pH buffer. The reaction rates of time points 0 and 60 min are shown with the mean and SD ($n = 3$).

A

B

C

D
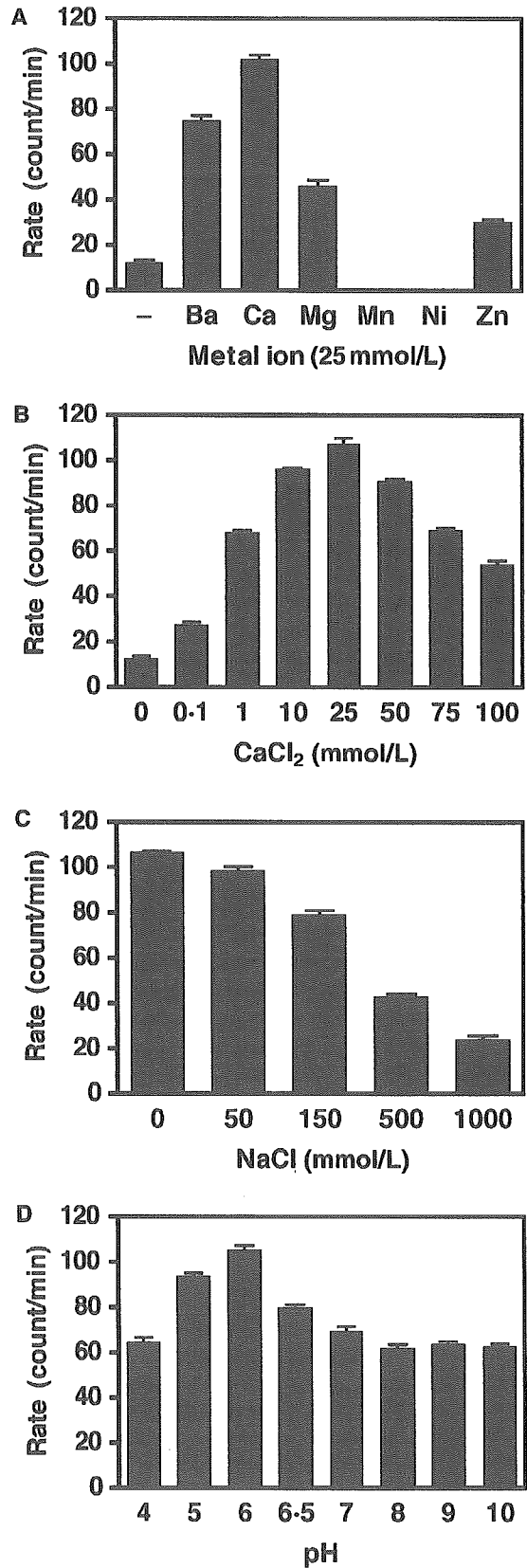
may affect the cleavage pH dependency. Alternatively, pH dependency of the assay might be affected not only by the cleavage efficiency, but also fluorescence emission, because most fluorescence reactions are highly pH dependent. Regardless, these data indicated that the FRETS-VWF73 assay was most efficient in the reaction buffer containing 5 mmol/l Bis–Tris, 25 mmol/l $CaCl_2$ and 0·005% Tween 20 at pH 6·0.

Reproducibility

We examined inter-run reproducibility of the FRETS-VWF73 assay. Plasma-dose dependency in the optimized condition was observed independently seven times. Each regression curve corresponded well with the other curves, indicating that the assay was obviously reproducible (data not shown). The relative ADAMTS13 activities of three different plasma samples were also measured independently seven times, where the activity of pooled plasma was normalized as 100%. The mean ± standard deviation (SD) values of the three samples were 113·9 ± 2·4, 62·5 ± 2·1 and 22·3 ± 1·4% ($n = 7$), respectively, indicating that the inter-assay variation was significantly small. The coefficients of variation of the three samples were 2·1, 3·4 and 6·3% ($n = 7$) respectively.

Plasma ADAMTS13 activity of patients and healthy individuals

To evaluate the FRETS-VWF73 assay for potential clinical use, we measured the relative ADAMTS13 activity in 78 plasma samples from various patients and 100 healthy individuals (Fig. 5A). The relative activities were estimated from the activity of pooled plasma prepared from all the 100 healthy individuals (66·0 ± 11·7 years old). Plasma samples from congenital TTP patients, homozygotes or compound heterozygotes of critical *ADAMTS13* mutations (Kokame *et al*, 2002; Matsumoto *et al*, 2004), all exhibited very low (<1%) or undetectable activities. The majority (33 samples) of plasma samples obtained from 41 patients with idiopathic TTP also showed low (<5%) or undetectable activities. The most

possible explanation would be a deficiency of plasma ADAMTS13 level or generation of auto-antibodies against ADAMTS13, although there may be some other factor, such as auto-antibodies, that bind to the substrate and protect it from being cleaved. In contrast, plasma from parents or siblings of
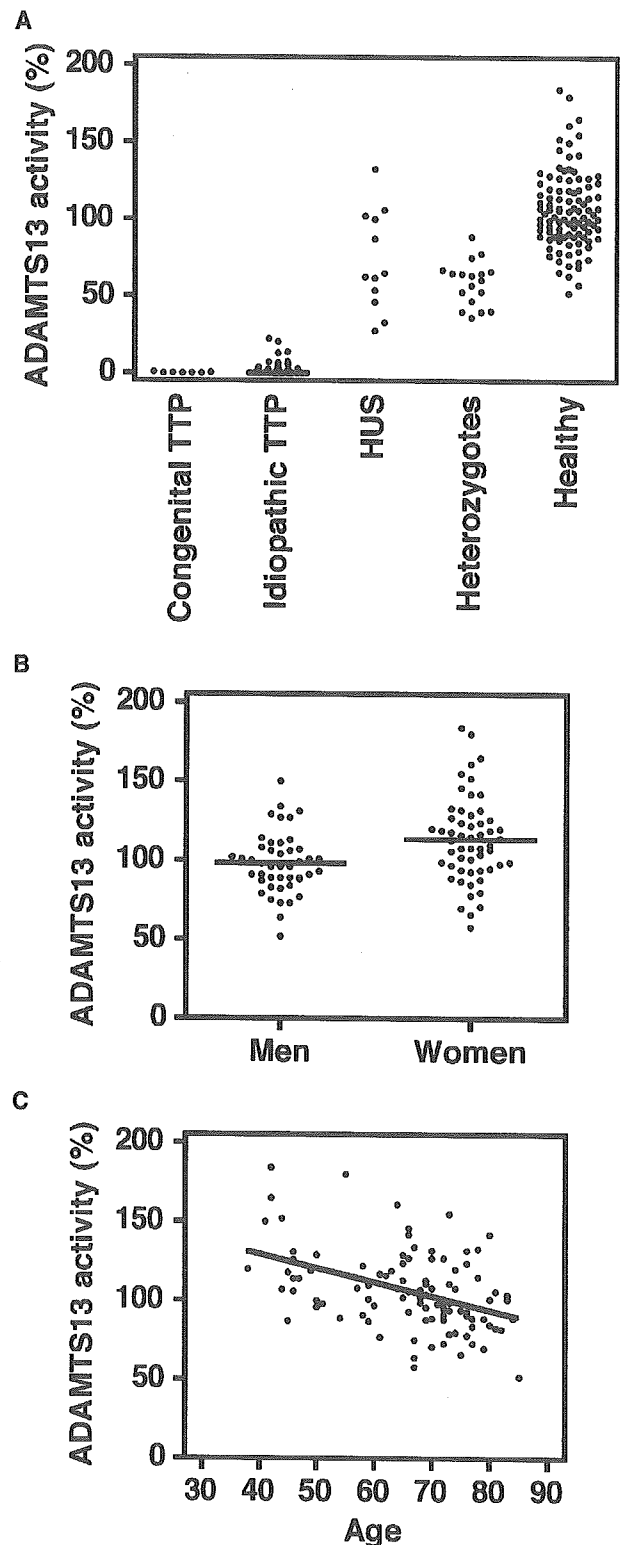

Fig 5. ADAMTS13 activity in plasma from patients and healthy individuals. (A) Relative ADAMTS13 activity was measured using the FRETS-VWF73 assay. FRETS-VWF73 was incubated with 4 μl of plasma from seven congenital thrombotic thrombocytopenic purpura (TTP) patients, 41 idiopathic TTP patients, 12 haemolytic uremic syndrome patients and 18 heterozygotes of ADAMTS13 with critical mutations. Plasma samples from randomly selected 100 healthy individuals were also examined. The relative ADAMTS13 activities were estimated from the standard curve, which was drawn up on the basis of the reaction rates of the pooled plasma prepared from all the 100 healthy individuals. (B) Association of ADAMTS13 activity with gender. Plasma ADAMTS13 activity in the healthy individuals was plotted by gender (45 men and 55 women). Bars indicate the means. Unpaired *t*-test identified significant differences between men and women ($P = 0·0016$). (C) Association of ADAMTS13 activity with age. The plasma ADAMTS13 activity correlated significantly with age ($P < 0·0001$). A line of best fit is indicated.

congenital TTP patients, heterozygotes of *ADAMTS13* mutations, exhibited on average approximately half the activity (59·0 ± 14·4%) of healthy individuals, while the plasma of patients with HUS showed substantial activity (73·2 ± 32·3%). Thus, the FRETS-VWF73 assay can be used to measure ADAMTS13 activity for TTP diagnosis in clinical samples.

Association of ADAMTS13 activity with gender and age

The measured ADAMTS13 activities of plasma samples from 100 healthy individuals (45 men aged 67·4 ± 11·5 years old and 55 women aged 64·9 ± 11·8 years old) were plotted according to gender (Fig. 5B). Comparison of the ADAMTS13 activities between men (97·9 ± 19·2%) and women (113·5 ± 27·1%) using the unpaired *t*-test demonstrated a significant difference between these groups ($P = 0·0016$), suggesting that the ADAMTS13 activity of women should be significantly higher than that of men. Examination of the effect of age on ADAMTS13 activities using Spearman's rank correlation revealed a significant correlation ($r = -0·396$, $P < 0·0001$) (Fig. 5C). The slopes of best fit in linear regression analysis were $-0·894 ± 0·196$, with R^2 values of 0·175 ($P < 0·0001$), suggesting that plasma ADAMTS13 activity should decrease with advancing age, at least after the early 40s.

Discussion

The Y1605–M1606 bond is inaccessible in native VWF and made sensitive to ADAMTS13 by denaturation and shear force. Structural modelling has suggested that the bond is buried in the core β-sheet of the VWF A2 domain (Jenkins *et al*, 1998; Sutherland *et al*, 2004). This partially explains the requirement for denaturants or shear force in the hydrolysis of the Y1605–M1606 bond by ADAMTS13. VWF73, corresponding to the C-terminal two-fifths of the A2 domain, can be efficiently cleaved by ADAMTS13 in the absence of denaturants and shear force (Kokame *et al*, 2004), suggesting that the N-terminal three-fifths of the A2 domain may prevent ADAMTS13 from accessing the cleavage site. A recent study indicated that the VWF A1 domain inhibits cleavage of the A2 domain by ADAMTS13; binding of platelet glycoprotein Ibα to the A1 domain appears to relieve the inhibition (Nishio *et al*, 2004). As VWF73 is a relatively small substrate, cleavage is less likely to be affected by other molecules. Therefore, VWF73 is an appropriate core for the convenient single-step fluorogenic assay for ADAMTS13 activity developed in this study.

Being a chemically modified version of VWF73 containing A2pr(Nma) and A2pr(Dnp), FRETS-VWF73 was a good substrate for ADAMTS13 cleavage, suggesting that Q1599 at the P7 position and N1610 at the P5′ position are not essential for the cleavage. We also examined the substitution of N1602 at the P4 position to A2pr(Nma). Although the peptide could be cleaved by plasma ADAMTS13, the efficiency was lower than that of the original FRETS-VWF73 (data not shown). The

shorter distance of the modified residue from the cleavage site may interfere with efficient cleavage by ADAMTS13.

Enzymatic studies of ADAMTS13 will progress using FRETS-VWF73 as a model substrate in the future. The previously established substrate, purified plasma VWF, is comprised of non-uniform multimers with multiple cleavage sites. In contrast, FRETS-VWF73 is a monomeric molecule with a single cleavage site, facilitating the determination of cleavage kinetic parameters. No denaturants are required for the reaction, making this assay more closely reflect the physiological conditions. Although the optimal cleavage of FRETS-VWF73 still requires a hypotonic environment, isotonic solution gives approximately 80% of the activity observed in NaCl-free conditions (Fig. 3C) for kinetic analyses. VWF73, however, is not suitable for studying the functions of the other VWF domains, such as A1 and A3.

The greatest impact of the FRETS-VWF73 assay will be as a potential clinical diagnostic test. Unlike previous assays, the assay is a simple procedure, requiring no special reagents or equipment except a fluorescence spectrophotometer. These advantages may popularize ADAMTS13-activity measurement at the clinical level. The best possible application will be the appropriate diagnosis of TTP. The FRETS-VWF73 assay could be useful also for selecting curative plasma before administration to patients, as ADAMTS13 activity in the general population varies widely (Fig. 5). The selection of high-titre plasma may improve the responses of patients to plasma infusion or exchange treatment.

The relationship between ADAMTS13 deficiency and TTP is more complicated than originally thought (George & Vesely, 2004; Zheng *et al*, 2004); the problem may be because of symptomatical and pathological variety and diagnostic criteria of TTP. Not all patients with TTP present the classical five features of disease, thrombocytopenia, microangiopathic haemolytic anaemia, neurological dysfunction, renal failure and fever. Although severe ADAMTS13 deficiency is observed in most patients with idiopathic TTP without pre-existing medical conditions (Furlan *et al*, 1998; Tsai & Lian, 1998), the association between ADAMTS13 deficiency and TTP is unclear in less highly selected patient groups (Veyradier *et al*, 2001; Vesely *et al*, 2003). ADAMTS13 measurement cannot be used to predict exactly response to plasma exchange in patients that are clinically diagnosed with TTP (Vesely *et al*, 2003). An accurate ADAMTS13 assay may help to categorize TTP patients into subgroups and help establish objective diagnostic criteria.

What should be the cut-off value of ADAMTS13 activity for the diagnosis of TTP or ADAMTS13 deficiency? The present and previous studies (Mannucci *et al*, 2001; Veyradier *et al*, 2001; Böhm *et al*, 2002) showed a wide distribution of the ADAMTS13 activity in the healthy population. Further, we showed that the ADAMTS13 activity was associated with gender and age. As we used pooled plasma that was derived from relatively older individuals as a standard, the apparent ADAMTS13 activity of patient plasma may be over estimated

in the present study. To determine the universally applicable cut-off value, the definition of standard plasma will be of primary importance. The availability of purified or recombinant ADAMTS13 may help the standardization of ADAMTS13 assay. The gender- and age-oriented distribution of ADAMTS13 activity will need to be determined in the general population. Although the FRETS-VWF73 assay detected significant activity in some idiopathic TTP patients, the value was evidently lower than the lowest activity of 100 healthy individuals (Fig. 5A). Therefore, the cut-off value (for instance, the mean −2 SD % of normal activity) may have to be determined considering gender and age. The FRETS-VWF73 assay, suitable for high-throughput measurement, would accelerate such a population study.

Acknowledgements

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Brief report

Localization of ADAMTS13 to the stellate cells of human liver

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Although the chromosomal localization (9q34) of the gene encoding the human form of ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13) and its exclusive expression in the liver have been established, the cells that produce this enzyme are yet to be determined. We investigated the expression of ADAMTS13 mRNA and protein in fresh frozen specimens obtained during liver biopsies of 8 patients with liver diseases. In situ hybridizations to localize ADAMTS13 mRNA showed positive signals exclusively in perisinusoidal cells with irregularly elongated dendritic processes extending between hepatocytes. Furthermore, ADAMTS13 was detected immunohistochemically in perisinusoidal cells, whereas no staining was observed in hepatocytes. The positive cells varied in shape from unipolar to dendritic with irregularly elongated cyto-plasmic processes, features common to hepatic stellate cells (HSCs). Double-labeling experiments revealed that the ADAMTS13-positive cells also expressed α-smooth muscle actin, confirming that these cells were activated HSCs. These results suggest that HSCs may be major cells producing ADAMTS13 in human liver. (Blood. 2005;106:922-924)

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Introduction

ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13) is a metalloproteinase that specifically cleaves the multimeric von Willebrand factor (VWF) between Tyr1605 and Met1606 within the VWF A2 domain.[1-4] VWF is synthesized in vascular endothelial cells, and released into the plasma as unusually large VWF multimers (UL-VWFMs).[5,6] Usually, UL-VWFMs are rapidly degraded into smaller VWF multimers by ADAMTS13. Deficiency of ADAMTS13 caused either by mutations of the *ADAMTS13* gene[1,7] or by inhibitory autoantibodies against ADAMTS13[8,9] increases the plasma levels of UL-VWFMs, which leads to platelet clumping and/or thrombi under high shear stress, resulting in thrombotic thrombocytopenic purpura (TTP).[5-9]

Northern blot analysis indicated that the 4.6-kilobase ADAMTS13 mRNA was exclusively expressed in the liver, and a 2.4-kilobase ADAMTS13 mRNA was also expressed in placenta and skeletal muscle.[2] In situ hybridization analysis revealed that the mRNA signals were expressed exclusively in the perisinusoidal cells,[10] but without addressing the type of cells expressing ADAMTS13. Moreover, a substantial decrease of plasma ADAMTS13 activity in patients with chronic liver disease has been associated with its disease progression, but not always with the serum levels of enzymes produced by hepatocyte.[11] Thus, specification and/or localization of the cells that produce this enzyme in the liver should have clinical impor-tance, and may help elucidate the pathogenesis of sinusoidal microcirculatory disturbances and/or thrombotic complications in patients with liver diseases.

In this study, we have clearly shown that ADAMTS13 is produced specifically in hepatic stellate cells, formerly called Ito cells, by both in situ hybridization techniques and immunohisto-chemical analysis using 2 novel mouse monoclonal antibodies specific for ADAMTS13.

Study design

Patients

This study examined 8 patients with liver disease (6 women and 2 men; mean age, 54.6 years; range, 43-72 years) including 4 patients with hepatitis C virus (HCV)–related chronic hepatitis, one patient with hepatitis B virus–related chronic hepatitis, one patient with primary biliary cirrhosis, one patient with autoimmune hepatitis, and one patient with a drug-induced liver injury who had undergone laparoscopies or percutaneous needle biopsies. Laboratory findings of these patients showed well-preserved functional liver capacity and platelet counts (mean, $18.3 \times 10^4/mm^3$; range, 8.8-$32.3 \times 10^4/mm^3$). Informed consent was provided by the patients and their families before the biopsies. The protocol used in this study was approved by the Nara Medical University Hospital Ethics Committee, Nara, Japan.

Production and characterization of anti-ADAMTS13 murine monoclonal antibodies

Full-length wild-type recombinant (r) ADAMTS13 that was purified by anti-FLAG (fludarabine, cytarabine, and granulocyte colony–stimulating factor) M2 agarose affinity chromatography (Sigma, Saint Louis, MO) was used as an immunogen[12] to produce monoclonal antibodies (A10 and C7) against ADAMTS13 in mice following standard procedures.[13] The immunoglobulin subclasses of A10 and C7 were IgG2b-κ and IgG1-κ, respectively. These antibodies were purified with a Protein A column (Amersham Biosciences, Uppsala, Sweden). These 2 monoclonal antibodies were able to detect endogenous plasma ADAMTS13 as a 190-kDa/180-kDa doublet band by Western blot analysis under nonreducing conditions. The epitopes recognized by the A10 and C7 antibodies were determined to reside in the disintegrin-like domain, and the seventh and eighth thrombospondin type-1 domains, respectively. Detailed characterizations of these antibodies are shown as supplementary data (Figure S1, available on the *Blood* website; see the Supplemental Figure link at the top of the online article).

Immunohistochemistry

Fresh liver specimens obtained from the 8 patients were fixed in 4% paraformaldehyde solution before frozen sections were prepared. The sections were incubated with primary antibodies (A10 and C7) overnight at 4°C. Bound primary antibodies were subsequently immunodetected using a standard avidin-biotin-peroxidase complex technique. To simultaneously detect A10- and anti–α-smooth muscle actin (α-SMA) immunoreactivity, sequential incubations using the following reagents were performed: A10 antibodies and anti–α-SMA antibodies (DAKO, Kyoto, Japan) followed by Alexa 488–conjugated anti–mouse immunoglobulin G (Invitrogen, Carlsbad, CA) and Alexa 546–conjugated anti–rabbit IgG (Molecular Probes). These labeled sections were observed with a Nikon Labphoto-2 fluorescent microscope and imaged with an MRC-600 confocal laser-scanning microscope system (Bio Rad Laboratories, Tokyo, Japan). Figures were assembled using Confocal Assistant software (Bio Rad Laboratories).

In situ hybridization

The cDNA encoding human ADAMTS13 was kindly provided by Dr Kenji Soejima (Chemo-Thero-Therapeutic Institute, Kumamoto, Japan). Digoxygenin (DIG)–labeled cRNA probes (sense and antisense) were transcribed using either T3 (sense) or T7 (antisense) RNA polymerase and a plasmid with an insert corresponding to nucleotides 3710-4237 of the full-length human ADAMTS13 transcript (Genbank accession no. AB069 698). All prehybridization procedures have been previously described.[14] To visualize the DIG-labeled probes, the sections were incubated with alkaline-phosphatase–conjugated anti-DIG antibodies (Roche Diagnostics KK, Tokyo, Japan) followed by 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate solution (Roche).

Results and discussion

When immunostaining was performed with A10 antibodies on liver specimens from a patient with HCV-related chronic hepatitis, dense brown staining was observed in perisinusoidal cells inside the lobule (Figure 1A). Furthermore, when the same liver section was stained with C7 antibodies, staining patterns were similar to those of A10 antibodies (Figure 1B). The varied morphologic features of these positive cells were consistent with those previously described for perisinusoidal stellate cells (Figure 1C-G).[15,16] Control specimens treated with unspecific mouse IgG instead of primary antibodies displayed no significant staining (data not shown). In situ hybridizations using antisense probes for ADAMTS13 revealed strongly positive labeling only in perisinusoidal cells with irregularly elongated cytoplasmic processes extending between hepatocytes (Figure 1H), whereas control specimens treated with
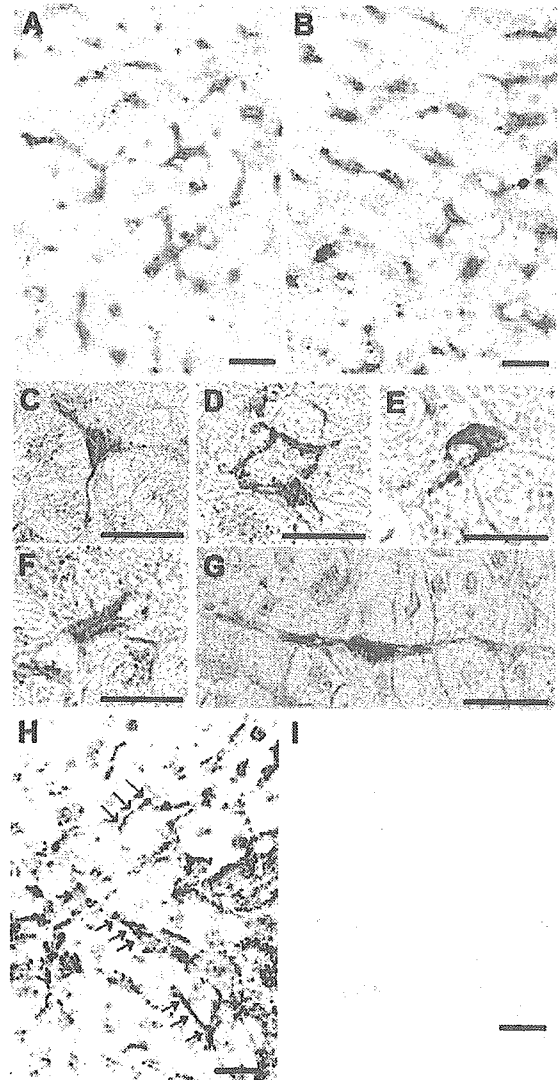

Figure 1. ADAMTS13 protein and mRNA expression in frozen sections of a liver specimen from a patient with hepatitis C–related chronic hepatitis. Immunostaining using ADAMTS13-specific monoclonal antibodies (A10) showed dense brown staining in perisinusoidal cells, but not in hepatocytes, inside the lobule (A). When the same liver section was stained with C7 antibodies, staining patterns were similar to those of A10 antibodies (B). The positive cells had a variety of morphologic forms. Examples shown here include a tripolar cell with long processes extending between hepatocytes (C), combined cells surrounding a hepatocyte (D), a dome-shaped cell (E), a bipolar cell with short processes (F), and a unipolar cell with long processes (G). Panels A-B: original magnification, × 200; panels C-G: original magnification, × 400. ADAMTS13 mRNA expression was examined using in situ hybridization. Strongly positive labeling was seen only in perisinusoidal cells, which were unipolar to dendritic in shape with irregularly elongated cytoplasmic processes extending between hepatocytes (H, arrows; original magnification, × 200). When sense probes were used, we observed no significant staining (I; original magnification, × 200). (Bar = 30 μm in A-I.)

sense probes showed no significant staining (Figure 1I). These ADAMTS13 mRNA–positive cells resembled HSCs morphologically, suggesting that HSCs produce ADAMTS13. In order to evaluate whether the ADAMTS13-positive cells truly would be HSCs, we performed double immunofluorescence immunohistochemistry with A10 anti-ADAMTS13 antibodies and anti–α-SMA antibodies. The microfilament protein α-SMA has been recognized as a specific marker for stellate cells[17]; activated stellate cells in pathologic livers are strongly α-SMA–positive, whereas in normal adult human liver, some stellate cells are α-SMA–positive.[18] Immunofluorescence labeling with anti-ADAMTS13 antibodies showed intense green fluorescence in perisinusoidal cells that
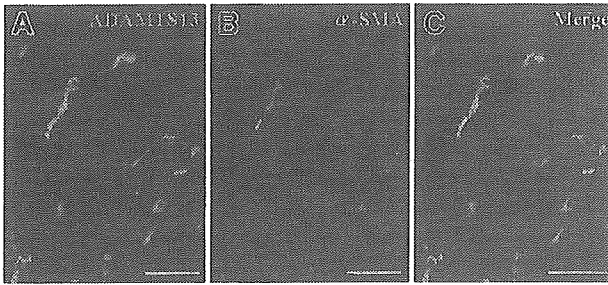
Figure 2. Confocal laser-scanning immunofluorescence microscopy using frozen sections of a liver specimen from a patient with hepatitis C–related chronic hepatitis. Immunofluorescence labeling with anti-ADAMTS13 antibodies showed intense green fluorescence in the perisinusoidal cells, which were irregular in shape with spotty, oval, unipolar, and bipolar cytoplasmic processes extending between the hepatocytes (A). Red fluorescence indicated α-SMA immunoreactivity, reflecting the fact that these cells were activated HSCs (B). Colocalization of ADAMTS13 (A) and α-SMA (B) in single cells yielded yellow color in the merged figure (C). (Original magnification, × 400; bar = 30 μm in A-C.)

were irregular in shape with spotty, oval, unipolar, and bipolar cytoplasmic processes extending between hepatocytes (Figure 2A). α-SMA antibodies revealed that these cells were also positive for the HSC marker (Figure 2B). Colocalization of ADAMTS13 and α-SMA in single cells clearly indicate that stellate cells produce ADAMTS13 in the liver (Figure 2C). Considering that a similar staining pattern was obtained with

both A10 and C7 antibodies against ADAMTS13 using the same liver sections (Figure 1A-B), it would seem that the HSCs may produce full-length ADAMTS13 protein.

HSCs have many functions, including vitamin A storage, liver fibrogenesis, and regulation of sinusoidal blood flow. These cells are also rich sources of bioactive mediators for maintaining homeostasis in the microenvironment of the hepatic sinusoid.[17] HSCs are located in the space of Disse adjacent to endothelial cells. It is, therefore, of particular interest that HSCs produce ADAMTS13. In patients with liver cirrhosis, a remarkably high level of plasma VWF has been noted.[11,19] Immunostaining with anti-VWF antibodies has shown the presence of this protein in the sinusoidal lining cells and at the scar-parenchyma interface in cases of liver cirrhosis.[20] This is particularly evident in the sinusoids of patients at the early stages of alcoholic liver diseases,[21] indicating the capillarization of the sinusoidal endothelial cells. Considering that ADAMTS13 is synthesized in the HSCs and its substrate, UL-VWFM, is produced in transformed vascular endothelial cells, the deficiency of plasma ADAMTS13 activity in liver diseases may play an important role in sinusoidal microcirculatory disturbances and subsequent development of liver injury. It will be necessary to clarify the intralobular heterogeneity of ADAMTS13 expression in HSCs associated with the activity of plasma ADAMTS13 in different stage of liver diseases.

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Development of ADAMTS13 inhibitor in a patient with hepatitis C virus-related liver cirrhosis causes thrombotic thrombocytopenic purpura

To the Editor:

Deficiency of von Willebrand factor-cleaving protease (VWF-CP/ADAMTS13) increases the plasma unusually large VWF multimers and leads to platelet clumping and/or thrombi under high shear stress [1–3], finally resulting in thrombotic thrombocytopenic purpura (TTP). A few patients who developed TTP during the course of liver diseases have been reported in the previous publications, but without determination of the plasma ADAMTS13 activity. We herein report on a TTP-patient associated with hepatitis C virus (HCV)-related liver cirrhosis, emphasizing the development of anti-ADAMTS13 antibody (IgG inhibitor).

A 65-year-old Japanese man had been treated for HCV-related liver cirrhosis and gastric ulcer in a local hospital since April 1999. He had a history of prophylactic endoscopic injection sclerotherapy because of risky esophageal varices in June 2001. On August 25, he was admitted into our hospital because of a high-grade fever

(38.1 °C) and rapidly increasing ascites. No abnormal neurological findings were noted. Laboratory data revealed severe thrombocytopenia ($15 \times 10^3/mm^3$) and hemolytic anemia (hemoglobin: 9.0 g/dl). The direct Coombs test was weakly positive. Lactate dehydrogenase (LDH) was elevated (823 IU/l, normal range (NR): 230–460 IU/l). The serum levels of the total and indirect bilirubin were 9.7 and 8.4 mg/dl, respectively. The C-reactive protein (CRP) was slightly increased. The level of fibrinogen was decreased (110 mg/dl, NR: 150–450 mg/dl), and the fibrinogen degradation product was increased (13.6 µg/ml, NR: <5 µg/ml). In spite of administration of antibiotics (ceftriaxone 2 g/day, I.V.) and thrombin inhibitor (gabexate mesilate 1500 mg/day, I.V.) for 5 days, no improvements were observed (Fig. 1). No Gram-negative organisms, such as *Escherichia coli* O-157: H7, were detected in the bacterial culture of the patient's feces. The antinuclear antibody was negative. The lupus anticoagulant and anti-cardiolipin antibodies
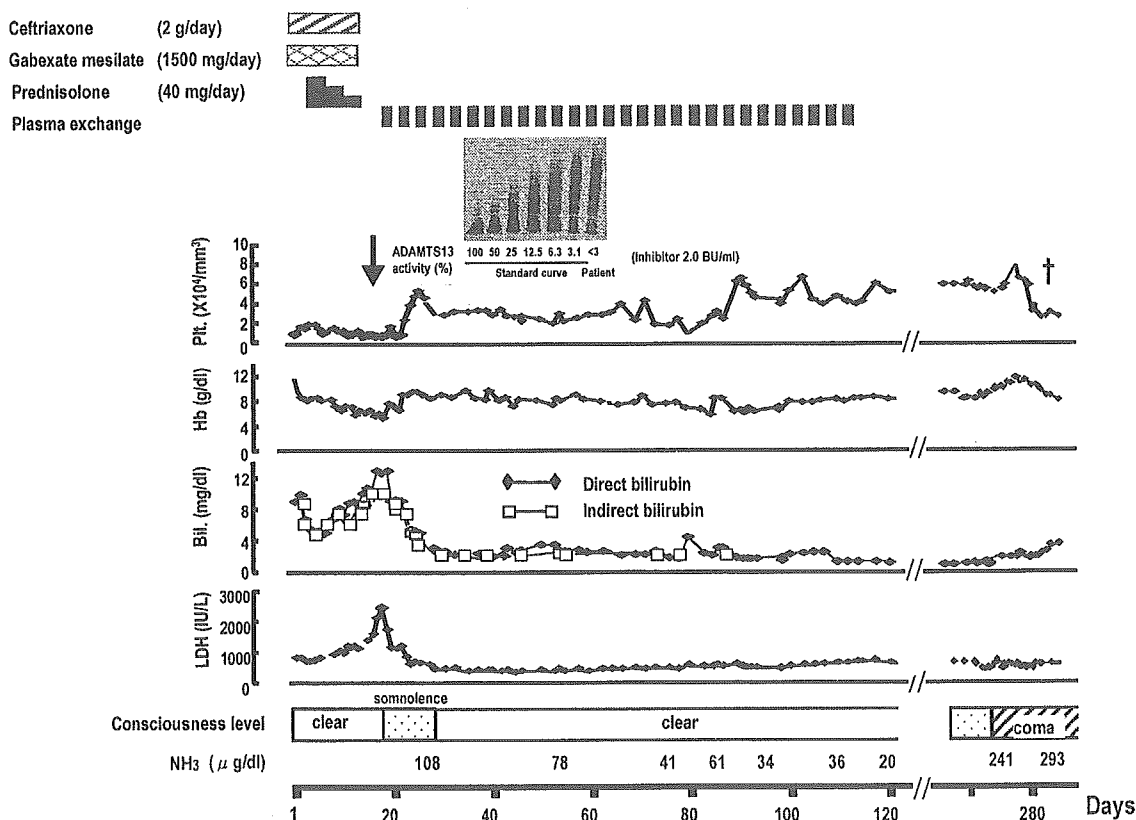

Fig. 1. The clinical course of a cirrhotic patient with thrombotic thrombocytopenic purpura after admission. The inserted luminography includes the standard curve of ADAMTS13 activity from a normal control plasma and the activity of the protease from the patient's plasma. The activity of ADAMTS13 in our patient is less than 3% of the control, which is consistent with the level in the patients with typical thrombotic thrombocytopenic purpura.