表 2 ABO抗体と不規則抗体の差異

	ABO 抗体	不規則抗体
検査時間	5〜10 分	40 分
溶血反応	血管内溶血	血管外溶血
臨床的意義	死にいたる	死亡例はまれ

オンは心筋障害，ひいては心室細動に至ることもある．さらには破砕された赤血球膜成分はリン脂質でできているところから，放出されたADPにより血小板凝集反応が惹起され，ついには血管内凝固症候群(DIC)に陥る．つまりABO不適合輸血は，腎不全，DICが惹起される．

一方，Rh型に対する抗体を含む不規則抗体と反応する抗原が輸血された不規則抗体による不適合輸血ではABO血液型不適合とは異なり，溶血反応は生じるものの血管外(主として脾)溶血であり，腎不全やDICに陥ることはない．

ABO，Rh(D)検査には5分ないしは10分かかるが，不規則抗体検査は40分から約1時間かかるので，緊急輸血時の検査とは患者の緊急度によって変える必要がある．そこで医師は以上に述べた検査時間を考慮して，どの程度の緊急輸血検査を指示して輸血するかを決めなければならない．そのためには緊急輸血のための緊急度をそれぞれの医療機関で決めておき，それに従って検査を行い，製剤を使用するということが必要である．検査ができないほどの緊急時にはO型を，5分または10分待つことができればABO型，Rh(D)型の一致した製剤を，1時間程度の余裕があれば不規則抗体スクリーニングも行った製剤を使用する．

輸血の準備

内科的，あるいは待機的輸血療法のようにかならず輸血を行う場合とは異なり，周術期では準備した血液をかならずしも使用するとは限らない．それゆえ，手術のたびに一定量の血液を準備することはかならずしも必要でなく，むだな準備をすることのないよう，手術ごとにその対応を決めるシステムが必要である．

タイプアンドスクリーニング法(T & S 法：Type and Screening 法)：

待機的手術法を含めて輸血の可能性の低い場合，表1に示したABO血液型，Rh(D)血液型，不規則抗体スクリーニングを行っておく．そして血液型が異なる2回以上の検査で確定し，Rh(D)が陽性で，不規則抗体スクリーニング陰性であれば事前に，いわゆるクロスマッチは行わない．緊急で輸血が必要になった場合には，輸血用血液の血液型のオモテ試験によりABO同型血であることを確認するか，あるいは生食法により主試験が適合の血液を輸血する．あらかじめ輸血血の血液型の確認を購入時すべて行っておけば，その照合はコンピュータにて行うことも可能であり，緊急時の対応はきわめて迅速に行われる．

この方法はあらかじめ準備血を用意しないとなっているが，手術には明らかに輸血が必要になる場合もあるので，そのような手術に際しては医療機関ごとに一定量〔たとえば4単位(800 ml)〕などをクロスマッチを行って払い出し，追加分についてはすべてT & S法で行うことが現実的である．

輸血の実際

1. 輸血用血液製剤の種類と適応

全血製剤は血液保存液（CPD）を混合した全血製剤で，特殊な病態にのみ用いられる．血液成分からなる成分製剤は，大きく赤血球製剤，血小板製剤，血漿製剤に分類されるが，後者二つは止血目的に用いられることから，血液製剤の適応は酸素運搬能の確保と止血目的に限るといってよい．

① 赤血球輸血の目的……赤血球輸血は多くの領域で用いられる基本的な輸血のひとつであるが，その目的とするところは末梢循環系への十分な酸素を補給することである．通常の貧血治療にはもっぱらMAP加赤血球が用いられ，さらに洗浄赤血球，解凍赤血球の代用として使用は十分可能である．以下の血小板とともに，輸血による細菌感染，副作用軽減のために初流血除去と白血球除去が行われている．

② 血小板製剤の適応……血小板の量的・質的異常による出血症状の軽減が投与の目的である．このような病態に血小板輸血が行われるが，有効期間が短いこと，高価な製剤であることから，本製剤の適正使用が強く望まれている．一般的に5

万/μl以上では輸血の適応はなく，2〜5万/μlではときに輸血が必要な場合がある．1〜2万/μlではときに重篤な出血をみることから，輸血が必要であり，1万/μl以下ではしばしば重篤な出血のために輸血が必要となる．血小板が減少した病態でもヘパリン起因性血小板減少症(HIT)や血栓性血小板減少性紫斑病(TTP)では，血小板輸血は行ってはならない．

③ **新鮮凍結血漿**……新鮮凍結血漿(FFP)の適応となるものは凝固因子の欠乏による臨床症状の改善を目的としたものが主であり，いわゆる循環血漿量の保持，改善などには用いない．

凝固因子は血漿1 ml当り平均1単位(100%)の凝固因子を含有していると考えられるが，他の成分に比べて個人差が大きい点を念頭におくべきである．これら凝固因子のうち濃縮製剤が使用できるフィブリノゲン，第Ⅶ，Ⅷ，Ⅸ，ⅩⅢ因子用に用いられることはなく，結局濃縮製剤のない凝固因子の補給用としてのみ意義がある．したがって，FFPの適応となる疾患・病態は凝固因子の補給のみであって他の使用目的はすべて不適切である．使用にあたってまず考えなければならないことは，FFP以外に安全で効果的な血漿分画製剤や代替製剤(リコンビナント製剤など)がない場合にのみ適応があることである．さらに，投与前にプロトロンビン時間(PT)，活性化部分トロンボプラスチン時間(APTT)，フィブリノゲン値を測定することを原則とする．また，投与の目的は凝固因子の欠乏状態による出血傾向の改善であって，単なる検査値の補正ではないことと，また観血処置を伴わない一般的な予防投与についてもその適応ではないことを十分に理解する必要がある．PTでは，(ⅰ)INR2以上，(ⅱ)30%以下，APTTでは(ⅰ)各医療機関における基準の上限の2倍以上，(ⅱ)25%以下とする．一般的に，生理的止血効果を期待するための凝固因子の最小活性値は正常20〜30%である．

④ **輸血の副作用**……輸血によるウイルス感染症，輸血後GVHD，同種免疫など，輸血に関連した副作用に対する対策の進歩は著しいが，輸血による細菌感染症，輸血関連肺傷害や西ナイルウイルス，E型肝炎ウイルス感染，輸血とCreutzfeldt-Jakob病との関連も指摘されている．

表3　出血量と止血に必要な最低濃度の関係

因子	最低濃度	出血量(%)*
platelet	50×10^3/mm^3	230(169〜294)
fibrinogen	1.0 g/l	142(117〜169)
prothrombin	20%**	201(160〜244)
factor Ⅴ	25%**	229(137〜300)
factor Ⅶ	20%**	236(198〜277)

*：正常循環血液量値との割合，**：正常値との割合
(Hiippala, S. T. et al：*Anesth. Analg.*, **81**：360-365, 1995.)

赤十字血液センターから供給される血小板濃厚液，赤血球濃厚液では皮膚穿刺直後の初流血を約20 mlあらかじめ除去することにより採血時の細菌汚染を防ぐとともに，白血球除去フィルターを用いてあらかじめ白血球を除去し抗体産生，免疫抑制，発熱反応などの種々の免疫反応やCMV，EB，HTLV-Ⅰなどのウイルス感染症の予防をいっそう強化している．

大量輸血と凝固障害

術前には出血性素因は認められないにもかかわらず，循環血液量，あるいはそれを超える大量出血の結果として大量輸血を行った場合，血小板数の減少，機能異常や凝固異常がしばしば認められる．**表3**には，出血量と止血に必要な血小板，凝固因子の低下の関係を示す．**表3**でもわかるように，フィブリノゲンに関していえば140%つまり循環血液量の1.4倍の出血に対してフィブリノゲンを含む製剤を投与しなければ，止血に必要な血中濃度，約100 mg/dlを保てなくなることを示している．一方，血小板やその他の凝固因子ではいずれも200%以上，循環血液量の2倍を超える出血で問題となる．本病態では，①フィブリノゲン値の低下を反映して創部からDIC様の，出血点が明らかでなく，まったく凝固塊が得られない出血やいったん止血していた部位からの出血がみられること，②局所的な止血は無効で，適切な輸血療法のみが唯一の対応であることが特徴であり，その病態把握と適切な診断はきわめて重要である．その病態生理としては，第1は大量出血時に輸血される全血製剤あるいは赤血球濃厚液(赤血球MAP)では血小板や凝固因子が十分含有されてい

ないために，赤血球の補充のみで結果的に血小板，凝固因子あるいはその両方が欠乏し希釈性の凝固障害が引き起こされることとなる．第2はDICによる凝固因子の消費による凝固異常が考えられる．そのほかには大量に投与される晶質液，あるいは膠質液による希釈の影響や，低体温による血小板機能低下あるいは凝固反応の低下も考えられる．

1．診断に要する検査

大量出血時には，フィブリノゲン，血小板を含む止血に関する諸因子の状態把握は重要であるので，適切な時期の適切な検査が必要である．術前に明らかな出血性素因のある患者では血液内科医の診断を仰ぐべきであり，周術期の対応についても緊密な連絡が望ましい．以下に周術期における十分理解しておかねばならない止血検査の基本的な点を示すとともに，その意義について述べることにする．

① **スクリーニング検査**……1)血小板数，2)プロトロンビン時間(PT)，3)活性化部分トロンボプラスチン時間(APTT)，4)フィブリノゲン値のスクリーニング検査は必須である．

血小板数はもっとも基本的な検査法のひとつであり，先天的にも後天的にも種々の原因により異常がみられる．

PTは外因系のスクリーニング検査であり，第Ⅶ因子ならびに凝固反応の共通経路(第Ⅹ，Ⅴ因子，プロトロンビンならびにフィブリノゲン)の異常を反映する．さらに，現在では経口抗凝固薬が第Ⅶ，Ⅸ，Ⅹ因子，プロトロンビンを低下させることからのモニターとしても使用されている．

APTTは内因系のスクリーニング検査であり，原理的にはプレカリクレイン，第XII因子，高分子キニノゲン，第XI因子の接触系因子，ならびに第Ⅷ，Ⅸ因子，さらには第Ⅶ因子を除く共通経路の異常を反映する．臨床上，第XI因子を除く接触系因子は出血症状がみられないことからその意義はない．また，共通経路の異常はPTのほうが感度はよいことからPTと合わせて考慮すべきである．したがって，本検査法は，第Ⅷ，Ⅸ因子ならびに第XI因子の異常を検査することがもっとも重要な意義となる．またヘパリン治療のモニターとして，ループスアンチコアグラントのスクリーニングとしても用いられる．

フィブリノゲン定量は高濃度のトロンビンを血漿に加えて得られたトロンビン時間は血漿中のフィブリノゲン濃度の関数であることを原理にした生物学的測定法と，免疫学的に蛋白としての濃度を測定する方法がある．当然のことながら，生物学的法のほうが実際の凝固可能なフィブリノゲン値を示すのでほとんどの施設ではこの方法を用いている．

さて，スクリーニング法ではないが，周術期にヘパリンのモニターとしてもちいられている活性化全血凝固時間法(ACT：Activated Clotting Time)の意義について述べる．本検査は基本的にはヘパリン治療に際してのモニター法であって，十分量のヘパリン量では延長するがこの値が基準値であるからといってヘパリンの効果がないとはいえない．とくに，周術期でヘパリンによる出血が疑われ残存ヘパリンが問題となる場合は，APTTのほうが優れているので，APTTを用いる．もちろん通常の止血のためのスクリーニング方法ではないことはいうまでもない．

② **検体**……止血検査は全血9容に対して，3.8%クエン酸ナトリウム1容の割合で得られたクエン酸加全血を3,000回転15分間遠心して得られた乏血小板血漿を検体とする．したがって，採血に関してはかならず末梢の血管から採血すること，凝固しないように採血すること(穿刺を繰り返さないこと)，採血後はただちに冷却遠心することが必要である．中心静脈から逆流によって得られた検体ではときに使用されているヘパリンなどによって凝固が得られないことや，輸液によって血液が希釈されている可能性があることなどにより，得られた結果が真に患者の異常値を反映しているものかはわからないので，行うべきでない．

2．周術期の出血と輸血療法

周術期にみられる大部分の出血は，全身性の出血性素因に基づくというよりも，むしろ局所的な原因であることから，まず行うべきことは物理的な局所止血である．

周術期に問題となる予期せぬ全身性の出血は，大量出血による凝固障害と，程度の差はあるもの

表 4　周術期における予期せぬ大量出血

1．考えられる病態・疾患
①大量輸血時の希釈性凝固障害
・原因は何であれ，循環血液量を超える大量出血時に主として赤血球製剤の補充を行った場合
・循環血液量の出血が予想されたら止血検査を行い万一に備える
②潜在性 DIC（胸・腹部動脈瘤など）
・検査所見では分子マーカーの上昇程度
・観血的処置により，一挙に顕在化
2．行うべきこと
①物理的止血が可能であれば確実に行う
②血小板数，プロトロンビン時間，活性化部分トロンボプラスチン時間，フィブリノゲン値の測定
③特にフィブリノゲンの補充（クリオ製剤，濃縮フィブリノゲン製剤，新鮮凍結血漿）

の，その基本的な病態に DIC が存在し，術前にはかならずしも顕在化していないが，手術とともに，顕在化し凝固異常を示す DIC 例である（表 4）．このような病態を示すものの代表的疾患・病態としては胸腹部大動脈瘤や乖離性大動脈瘤などの血管性病変がある．これら患者の手術に際しては，まず①このような疾患では思いがけない出血がある可能性があることを十分認識すること，②術前の凝固検査を適切に行い，凝固異常の状態を十分把握する，③術中には適宜凝固検査を行い，不測の事態に備えることが重要である．以下のこのような状態に陥った場合の対応を示す．

① **まず止血検査をそして必要な凝固因子を十分量投与する**……大量輸血時における凝固異常に対しては，フィブリノゲンは循環血液量の 1.5 倍程度で止血に支障をきたす程度まで低下することはすでに述べたが，循環血液量の 50% を超えるような出血が起こったらまず止血検査を行い今後の対応の参考にする必要がある．しかし，止血検査に異常があるからといって予防的な血小板，血漿輸血の適応はない．以後止血が完了するまで適宜行うことが必要であるが，採血はかならず，通常の経皮的採血を行うことの重要性はすでに述べた．

さて，もっとも問題となるのは凝固の最終基質であるフィブリノゲン値であり，100 mg/d*l* 以下であれば濃縮フィブリノゲン製剤，クリオプレシピテート，FFP などの血漿製剤の輸血を行う．クリオ製剤はわが国では現時点供給されていないことは治療上おおいに問題であり，日本赤十字からの供給が望まれる．

クリオ製剤は，新鮮凍結血漿を 4℃の冷蔵庫内にて 1〜2 日間放置した後，遠沈して得られた沈殿部分である．これを無菌的にチューブ接続装置を用いて分離し 37℃で再融解した後，−40℃以下で保存すれば 1 年間は有効で，使用時は 37℃にて速やかに溶解できる．元の新鮮凍結血漿に比べて約 10 倍前後に濃縮されており，通常の新鮮凍結血漿では得ることができないフィブリノゲン値に容易に達することができる．

さて，フィブリノゲン値が 100 mg/d*l* 以上であれば他の因子の補充の目的で血漿製剤の輸血を行う．

② **血小板輸血**……血小板数が 5 万/μ*l* 以下の場合には，かりに出血症状が顕著でなくても血小板輸血を考える．しかし，それ以上であれば，急速に血小板の補充の必要はなく，むしろフィブリノゲン値が重要である．ただし，血小板数が 10 万/μ*l* 前後であるにもかかわらず出血が持続した場合には血小板機能異常あるいは DIC のような凝固異常も考慮する．頭部外傷，動脈瘤破裂，ショック状態が持続し長時間虚血状態が持続した場合が相当する．

③ **赤血球輸血**……異型輸血と不適合輸血は同意義ではない．異型輸血とは文字とおり異なる型の輸血をいい，通常は ABO 血液型の場合を指し，不適合輸血とは患者のもつ抗体と輸血される血液，とくに赤血球との間の抗原抗体反応の結果，溶血反応が生じ死亡に至る場合を含む副作用を生じる輸血をいい，すべて異型輸血である．しかし，異型輸血がすべて不適合輸血とは限らず，たとえば O 型を A 型に，あるいは A 型を AB 型に輸血

する場合などは異型輸血ではあるが，抗原抗体反応は起こらず不適合輸血ではない．大量出血時のような緊急輸血では救命第一で，同型であることに固執して過少輸血になりその結果患者の死を招くことは絶対行ってはいけない．異型ではあっても適合する赤血球製剤を確保して救命をはかるべきであり，もちろん同型の赤血球が入手すればその時点で切り変える．すなわち，緊急輸血時には異型輸血であっても不適合輸血にはならない赤血球輸血を行い，患者の救命を第一とすべきである．

おわりに

周術期の出血には，先天的にせよ，後天的にせよ術前に出血性素因であることが明らかな場合と予期せぬ終結をきたす場合があるが，前者は一部の疾患ではその対応は困難なことはあっても，ほとんどは手術前に対応は可能である．しかし，後者の場合の大部分は局所の止血困難であり，その対応はもっぱら局所的止血を貫徹する以外方法はない．

循環血液量を超える大量出血や胸部外科領域における動脈瘤によって惹起される凝固障害はその原因が凝固因子の欠乏のためであり，局所的な止血法にては解決することは困難である．むしろフィブリノゲンをはじめとする凝固因子，血小板の十分な補充によってのみ止血は可能となる．そのためには，止血凝固のメカニズム，検査の意義およびそれを行うタイミング，そして輸血検査の意義とそのタイミングを十分理解することが求められる．

*　　*　　*

The prevalence of the metabolic syndrome in patients with bipolar disorder is alarmingly high, and the use of the new diagnostic criteria identified a picture that is even more worrisome than we previously reported. In addition to being correlated to a severe cardiovascular and metabolic risk, the metabolic syndrome in general, and obesity in particular, are also correlated with a worse psychiatric outcome. The development and testing of specific interventions to prevent and treat the metabolic syndrome and obesity in patients with bipolar disorder are urgently needed.

Trial Registration: clinicaltrials.gov Identifier: NCT00211263

Dr. Fagiolini is on the speaker bureau of Bristol-Myers Squibb and Pfizer and is a consultant for Bristol-Myers Squibb, Novartis, and Pfizer. Dr. Frank is a consultant for Pfizer Italia and Servier Amerique and has received grant/research support from Forest. Dr. Turkin is on the speaker bureau of GlaxoSmithKline and has received grant/research support from AstraZeneca, Bristol-Myers Squibb, and Novartis. Dr. Soreca is a consultant for Novartis. Dr. Kupfer has been a consultant for Servier Amerique. Ms. Houck has no personal affiliations or financial relationships with any commercial interests to disclose relative to this letter.

REFERENCES

1. Fagiolini A, Frank E, Scott JA, et al. Metabolic syndrome in bipolar disorder: findings from the Bipolar Disorder Center for Pennsylvanians. Bipolar Disord 2005;7(5):424–430
2. Grundy SM, Cleeman JI, Daniels SR, et al. Diagnosis and management of the metabolic syndrome: an American Heart Association/ National Heart, Lung, and Blood Institute Scientific Statement. Circulation 2005;112(17):2735–2752
3. Spearing MK, Post RM, Leverich GS, et al. Modification of the Clinical Global Impressions (CGI) Scale for use in bipolar illness (BP): the CGI-BP. Psychiatry Res 1997;73(3):159–171
4. Global Assessment of Functioning (GAF) Scale. In: American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. Washington, DC: American Psychiatric Association; 1994:32

Andrea Fagiolini, M.D.
Ellen Frank, Ph.D.
University of Pittsburgh
Western Psychiatric Institute and Clinic
Pittsburgh, Pennsylvania
Scott Turkin, M.D.
DuBois Regional Medical Center
DuBois, Pennsylvania
Patricia R. Houck, M.S.H.
Isabella Soreca, M.D.
David J. Kupfer, M.D.
University of Pittsburgh
Western Psychiatric Institute and Clinic
Pittsburgh, Pennsylvania

Effect of Ribavirin, in Combination With Interferon in Patients With Hepatitis C, on the Bleeding Risk Associated With Selective Serotonin Reuptake Inhibitors

Sir: We read with great interest the recent article by Martin et al.[1] reporting that the bleeding risk associated with use of selective serotonin reuptake inhibitors (SSRIs) during antiviral therapy for chronic hepatitis C (CHC) is lower than that previously reported.[2] Several reports had revealed the increasing risk of bleeding, including retinal hemorrhages or gastrointestinal bleeding, by administrating SSRIs to patients receiving interferon-α therapy.[3–6]

Ribavirin was used in combination with interferon in the treatment of patients with CHC in the study by Martin et al.[1] We have reported that ribavirin in combination with interferon, and possibly alone, may reduce the need for clotting factors in hemophilic patients with CHC.[7] In our hospital, we observed a marked reduction in the use of clotting factors in 5 of the 8 hemophilic patients who were being treated for CHC with ribavirin plus interferon. In contrast, no reduction in the use of clotting factors was observed in 47 hemophilic patients previously treated for CHC with interferon alone in our hospital. These findings strongly suggested that the reduced use of clotting factors resulted from the addition of ribavirin.

In the same group of patients, we found that the procoagulant activity of factor VII was elevated in all patients after receiving ribavirin in comparison with activity of factor VII before ribavirin administration.[8] In patients with CHC, the international normalized ratio (INR; prothrombin time) decreased continuously during therapy with peginterferon plus ribavirin in patients both with and without coagulation disorder; INR increased to the pretreatment value after therapy (data not shown). It is possible that ribavirin enhanced coagulation factor activity in both types of patients, reducing the risk of bleeding during therapy. These findings suggest that addition of ribavirin to interferon may be the reason why the risk of bleeding was lower than during antiviral therapy for patients with CHC in the report by Martin and colleagues.[1]

Weinrieb et al.[2] reported that critical upper gastrointestinal bleeding occurred in a patient with CHC being treated with interferon plus ribavirin and an SSRI. However, this patient was suffering end-stage liver disease and was also being given aspirin. We believe that this patient was at risk for bleeding even without an SSRI administration.

Interferon plus ribavirin is now standard therapy for patients with CHC because the efficacy of this combination is higher than that of interferon monotherapy.[9] Morasco et al.[10] reported that use of prophylactic SSRIs to prevent interferon-α–induced depression in patients with CHC was not beneficial. Once a patient develops depressive symptoms, however, SSRIs can be successfully used to treat depression in patients with CHC who are receiving interferon-α therapy and ribavirin treatment. From Martin and colleagues' results[1] and ours, the risk of bleeding due to SSRIs looks likely to be relatively low when ribavirin is administered in addition to interferon for patients with CHC. When treating patients with CHC, physicians have to carefully observe whether bleeding episodes will increase after the reduction or stop of ribavirin due to ribavirin-induced anemia. In addition, when we treat other diseases, including chronic hepatitis B, leukemia, and renal cell carcinoma, with interferon alone, we may have to watch for bleeding during SSRI administration. Further randomized controlled studies of bleeding risk in patients given or not given SSRIs during therapy with interferon plus ribavirin are warranted.

Dr. Martin was shown this letter and declined to comment.

The authors report no financial affiliation or other relationship relevant to the subject of this letter.

REFERENCES

1. Martin KA, Krahn LE, Balan V, et al. Selective serotonin reuptake inhibitors in the context of hepatitis C infection: reexamining the risks of bleeding. J Clin Psychiatry 2007;68:1024–1026
2. Weinrieb RM, Auriacombe M, Lynch KG, et al. A critical review of selective serotonin reuptake inhibitor-associated bleeding: balancing the risk of treating hepatitis C-infected patients.

J Clin Psychiatry 2003;64:1502–1510
3. De Abajo FJ, Rodriguez LA, Montero D. Association between selective serotonin reuptake inhibitors and upper gastrointestinal bleeding: population based case-control study. BMJ 1999;319:1106–1109
4. Loftis JM, Hauser P. Safety of the treatment of interferon-alpha-induced depression. Psychosomatics 2003;44:524–526
5. Hejny C, Sternberg P, Lawson DH, et al. Retinopathy associated with high-dose interferon alfa-2b therapy. Am J Ophthalmol 2001;131:782–787
6. Musselman DL, Lawson DH, Gumnick JF, et al. Paroxetine for the prevention of depression induced by high-dose interferon alfa. N Engl J Med 2001;344:961–966
7. Honda T, Toyoda H, Hayashi K, et al. Ribavirin and use of clotting factors in patients with hemophilia and chronic hepatitis C. JAMA 2005;293:1190–1192
8. Yamamoto K, Honda T, Matsushita T, et al. Anti-HCV agent, ribavirin, elevates the activity of clotting factor VII in patients with hemophilia: a possible mechanism of decreased events of bleeding in patients with hemophilia by ribavirin. J Thromb Haemost 2006;4:469–470
9. McHutchison JG, Gordon SC, Schiff ER, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N Engl J Med 1998;339:1485–1492
10. Morasco BJ, Rifai MA, Loftis JM, et al. A randomized trial of paroxetine to prevent interferon-alpha-induced depression in patients with hepatitis C. J Affect Disord 2007;103:83–90

Takashi Honda, M.D., Ph.D.
Yoshiaki Katano, M.D., Ph.D.
Hidenori Toyoda, M.D., Ph.D.
Kazuhiko Hayashi, M.D., Ph.D.
Masatoshi Ishigami, M.D., Ph.D.
Isao Nakano, M.D., Ph.D.
Hidemi Goto, M.D., Ph.D.
Department of Gastroenterology
Koji Yamamoto, M.D., Ph.D.
Junki Takamatsu, M.D., Ph.D.
Department of Transfusion Medicine
Nagoya University Graduate School of Medicine
Nagoya, Japan

Comments on a Randomized, Double-Blind Comparison of Sertraline and Placebo for Posttraumatic Stress Disorder in a Department of Veterans Affairs Setting

Sir: Selective serotonin reuptake inhibitor antidepressants are first-line pharmacologic treatments of posttraumatic stress disorder (PTSD).[1,2] Robust effects have been seen in previous large studies of sertraline in civilian populations.[3,4] The recent (May 2007) study by Friedman and colleagues[5] failed to find evidence of efficacy of sertraline in a veteran population, despite a comprehensive analysis of potential moderator variables.

There was some evidence, however, of efficacy of sertraline in subjects with non–combat-related traumas, prompting the possibility that sertraline is only effective in persons with civilian trauma. While the authors make a decent case for why this is not true (including an argument for the treatment refractoriness of Vietnam veterans with PTSD), we are not entirely convinced and would like to see ongoing research to address this issue.

Such research into predictors of treatment efficacy in patients with PTSD could complement findings such as those from Davidson and colleagues,[6] which indicate that a marked improvement in anger/irritability after 1 week on sertraline treatment may be a useful prognosticator of eventual response.

In the current academic climate, we are pleased to see a large "negative" study such as this published in a major psychiatric journal, albeit in a delayed manner. Although such negative studies may be challenging to interpret and can raise more questions than they answer, they do add a much needed balanced perspective.

The author reports no financial affiliation or other relationship relevant to the subject of this letter.

References

1. The International Psychopharmacology Algorithm Project. PTSD Algorithm. Available at: http://ipap.org/ptsd/contents.php. Accessed July 7, 2007
2. Ballenger JC, Davidson JR, Lecrubier Y, et al. Consensus statement update on posttraumatic stress disorder from the international consensus group on depression and anxiety. J Clin Psychiatry 2004;65 (suppl 1):55–62
3. Brady K, Pearlstein T, Asnis GM, et al. Efficacy and safety of sertraline treatment of posttraumatic stress disorder: a randomized controlled trial. JAMA 2000 Apr;283(14):1837–1844
4. Davidson JR, Rothbaum BO, van der Kolk BA, et al. Multicenter, double-blind comparison of sertraline and placebo in the treatment of posttraumatic stress disorder. Arch Gen Psychiatry 2001 May;58(5):485–492
5. Friedman MJ, Marmar CR, Baker DG, et al. Randomized, double-blind comparison of sertraline and placebo for posttraumatic stress disorder in a Department of Veterans Affairs setting. J Clin Psychiatry 2007 May;68(5):711–720
6. Davidson J, Landerman LR, Clary CM. Improvement of anger at one week predicts the effects of sertraline and placebo in PTSD. J Psychiatr Res 2004 Sep–Oct;38(5):497–502

Hadi M. Hussain, M.D.
Department of Psychiatry
Military Hospital, Rawalpindi
Rawalpindi, Pakistan

Dr. Friedman and Colleagues Reply

Sir: We certainly agree with Dr. Hussain that more research is needed regarding responsivity of patients with combat-related (compared to non–combat-related) posttraumatic stress disorder (PTSD) to selective serotonin reuptake inhibitors (SSRIs), other medications, and psychotherapy. We also agree that further research on predictors of response would be very useful.

We recognize that negative results among chronic Vietnam veterans in Department of Veterans Affairs (VA) settings have prompted speculation that combat-related PTSD is somehow different and less treatment responsive than non–combat-related PTSD. As reviewed in our article,[1] the preponderance of the evidence suggests to us that it is the chronicity of illness in these VA cohorts rather than something unique about combat trauma that accounts for such negative findings. To this end, we cited some recent studies in which better outcomes to SSRI treatment were observed among (non-VA) participants with combat trauma.[2,3]

Given the fact that a number of medication (and psychotherapy) trials are currently underway with troops and veterans who developed PTSD as a result of combat in Afghanistan and/or Iraq, we will all have much more information on this important question before much longer.

The original study discussed in this letter was supported by funding from Pfizer Inc.

Haemophilia (2008), 14, 556–563 DOI: 10.1111/j.1365-2516.2008.01703.x

ORIGINAL ARTICLE *VWD*

L1503R is a member of group I mutation and has dominant-negative effect on secretion of full-length VWF multimers: an analysis of two patients with type 2A von Willebrand disease

T. KASHIWAGI,* T. MATSUSHITA,† Y. ITO,‡ K. HIRASHIMA,† N. SANDA,* Y. FUJIMORI,* T. YAMADA,* K. OKUMURA,* A. TAKAGI,*§ T. MURATE,*,§ A. KATSUMI,† J. TAKAMATSU,¶ K. YAMAMOTO,¶ T. NAOE† and T. KOJIMA*§

*Departments of *Pathophysiological Laboratory Sciences; †Hematology and Oncology; ‡Division for Medical Research Engineering, Nagoya University Graduate School of Medicine; §Department of Medical Technology, Nagoya University School of Health Sciences; and ¶Division of Transfusion Medicine, Nagoya University Hospital, Nagoya, Japan*

Summary. Type 2A von Willebrand disease (VWD) is characterized by decreased platelet-dependent function of von Willebrand factor (VWF); this in turn is associated with an absence of high-molecular-weight multimers. Sequence analysis of the VWF gene from two unrelated type 2A VWD patients showed an identical, novel, heterozygous T → G transversion at nucleotide 4508, resulting in the substitution of L1503R in the VWF A2 domain. This substitution, which was not found in 60 unrelated normal individuals, was introduced into a full-length VWF cDNA and subsequently expressed in 293T cells. Only trace amount of the mutant VWF protein was secreted but most of the same was retained in 293T cells. Co-transfection experiment of both wild-type and mutant plasmids indicated the dominant-negative mechanism of disease development; as more of mutant DNA was transfected, VWF secretion was impaired in the media, whereas more of VWF was stored in the cell lysates. Molecular dynamic simulations of structural changes induced by L1503R indicated that the mean value of all-atom root-mean-squared-deviation was shifted from those with wild type or another mutation L1503Q that has been reported to be a group II mutation, which is susceptible to ADAMTS13 proteolysis. Protein instability of L1503R may be responsible for its intracellular retention and perhaps the larger VWF multimers, containing more mutant VWF subunits, are likely to be mal-processed and retained within the cell.

Keywords: von Willebrand factor, von Willebrand disease, dominant inheritance, mutation, type 2A, group

Introduction

von Willebrand factor (VWF), one of the largest plasma proteins in mammals, is an essential factor in the primary haemostasis. It forms a bridge between platelet membrane glycoprotein Ib and constituents of the extracellular matrix [1,2], thereby playing a critical role in the adhesion of platelets to sites of vascular injury. Binding of VWF to platelets is a regulated process. VWF is secreted by endothelial cells into either the blood or subendothelial matrix, and VWF in both locations can contribute to platelet adhesion [3,4].

The VWF functions are attributed to its domain structure, which is composed of D1-D2-D'-D3-A1-A2-A3-B1-B2-B3-C1-C2 [5]. The mature protein lacks the D1 and D2 propolypeptides and consists of 2050 amino acid residues. VWF undergoes extensive post-translational modifications including dimerization through multiple intramolecular disulfide bonds between the carboxyl-terminal ends of the protein [6] and once transported to the Golgi, multimerization through interdimer disulfide bonds between the amino-terminal ends [7,8]. Multimers are composed of various lengths but can contain more than 40 VWF subunits and reach sizes of >20 000 kDa.

Correspondence: Tadashi Matsushita, MD, Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, 65-Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan.
Tel.: +81 52 744-2145; fax: +81 52 744 2161;
e-mail: tmatsu@med.nagoya-u.ac.jp

Accepted after revision 6 March 2008

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

Regulating the multimer size is crucial for the balance of haemostasis and thrombosis, because circulation of ultra-large VWF multimers results in the disorder known as thrombotic thrombocytopenic purpura, whereas a reduction in the size of multimers results in the bleeding disorder called von Willebrand disease (VWD) type 2A [9–11]. Type 2A VWD is represented by qualitative defect of VWF multimer size, although plenty of type 2A cases also present quantitative defect of VWF, as revealed by decreased immunoreactive antigens or decreased intensities of lower-sized multimer bands.

The multimer size of VWF is regulated by plasma protease ADAMTS13, which cleaves VWF between Tyr1605 and Met1606 in the A2 domain [12–14]. The A2 domain consists of residues 1480–1672 of VWF and numerous mutations in the A2 domain, which result in type 2A VWD [15–19]. Two distinct pathogenic mechanisms cause type 2A VWD. Group I mutations are characterized by impaired intracellular transport, storage and secretion of high molecular weight multimers, whereas group II mutations are characterized by increased susceptibility of the VWF protein to proteolysis by the ADAMTS13 protease [18]. In this report, we describe a novel mutation in a group I type 2A VWD at which the mutation L1503Q has been described as group II mutation [20]. The amino acid was substituted to arginine and our study indicates that this amino acid position is also important for protein conformation of VWF molecule.

Materials and methods

Patient and sample

Ethical approval for the study was obtained from the Ethics Review Committee of Nagoya University School of Medicine. After obtaining written informed consent, blood samples from the patient were collected in 1/10 volume of 3.13% sodium citrate. Plasma was separated by centrifugation at ×2000 *g* for 20 min, and genomic DNA was extended from peripheral blood leucocytes according to standard procedures [21]. Ristocetin cofactor activity of VWF (VWF:RcoF) was determined by a Latex agglutination test kit (von Willebrand Reagent, Dade Behring, Marburg, Germany). The sensitivity of this test was 0.06 U mL^{-1} and CV was within 20% of five independent assays. Multimer analysis was performed based on a method by Raines *et al.* [22] that uses SDS-1.5% agarose gel electrophoresis and capillary blotting.

VWF mutation analysis

Exon 14, 18, 27, 28 including its exon/intron boundaries of human VWF gene were amplified by PCR in two steps using GeneAmp PCR System 9700 and analysed by ABI PRISM 310 Genetic Analyzer (Applied Biosystems; ABI, Forster City, CA). The fist amplification was performed with primers allowing the specific amplification of the VWF gene and avoiding that of the pseudogene (Table 1) [23]. One

Table 1. Primers.

Name of primer	Length	Sequence	Position*
For PCR and sequencing			
Vi27-u	22	5′-TGTGGGAATATGGAAGTCATTG-3′	Intron27-93
Vi28-d	23	5′-GTATCTTGGCAGATGCATGTAGC-3′	Intron28+69
281 U	19	5′-CTGTCCGAGGCTGAGTTTG-3′	3871
319 L	19	5′-CCGCTCCATCATGTCCACC-3′	3900
672 U	18	5′-CCCATGCCAACCTCAAGC-3′	4263
706 L	18	5′-TTCTCAGGGGCCTGCTTC-3′	4287
1014 U	18	5′-AGGACAGCATCCACGTCA-3′	4605
1080 L	18	5′-CCCCTTTGGACTGTGCCT-3′	4661
For PCR-restriction fragment length polymorphism			
Exon28-SmaI/U			19
Exon28-SmaI/L	20	5′-CAGTGTTGGTCCTGTTGCCG-3′	4716
For mutagenesis			
AU	20	5′-TGCGTTGACCCTGAAGACTG-3′	3568
AL	18	5′-TTTGTCCGATCCTTCCCG-3′	4507
BU	18	5′-CCGGGAAGGATCGGACAA-3′	4509
BL	18	5′-AGAGGTACCGCAGGGCCA-3′	4751

*Nucleotide 1 is an adenine of the ATG-translation initiation codon. Beginning of the intron; a plus sign and the position in the intron. The end of the intron; a minus sign and the position upstream in the intron.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

hundred nanogram of genomic DNA were incubated in 10× *TaKaRa Ex Taq* buffer with 20 pmoles of specific primers, 0.32 nmol dNTP, 0.5 U of *TaKaRa Ex Taq* polymerase in final volumes of 20 mL (Takara, Ohtsu, Japan). After 2 min of denaturation at 94°C, samples were subjected to 30 amplification cycles. Each cycle comprised 30 s denaturation at 94°C, 30 s annealing at 60°C, 2 min extension at 72°C. Reaction was terminated by a final extension for 7 min at 72°C. The second PCR conditions were approximately the same except for annealing temperature (55°C) and extension time (1 min). PCR fragments were purified on QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany) and reaction was with Big Dye Terminator Cycle Sequencing FS Ready Reaction kit (ABI) as described previously [24].

To determine the 4508T → G transition in exon 28 of VWF gene, we used mismatch strategies for PCR–restriction fragment length polymorphism (RFLP) analysis. Briefly, PCR was performed using a partially mismatched sense primer, which introduces *Sma*I site only into mutant allele PCR products, and the antisense primer (Table 1) used for DNA amplification of exon 28 of the VWF gene sequence except for pseudogene. The PCR products were digested with *Sma*I and electrophoresed on a 2% agarose gel.

Plasmid construction

Plasmid pSVHVWF1.1 [25] contain a full length normal human VWF cDNA cloned into the expression vector pSV7D [26] as described [27]. Plasmid pSVHL1503R contains a T → G transition of nucleotide 4508 of pSVHVWF1.1 resulting in a substitution of Leu by Arg at amino acid 1503 of the mature VWF. PCR was performed using the pSVHVWF1.1 vector as template with following primers (Table 1). The two PCR fragments obtained with primers AU-AL and with BU-BL were used as template for an additional PCR with Primer AU and BL. The corresponding PCR product was cloned into pBluescript II KS+ and the DNA sequence of the fragment was confirmed. Finally, and the amprified fragment was inserted into pSVHVWF1.1 that was digested with *Nae*I and *Kpn*I.

Expression of recombinant VWF

Human embryo kidney 293T (HEK293T) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and phenol red (Wako chemicals, Osaka, Japan), supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin B, and 6%(v/v) foetal bovine serum (FBS) at 37°C in 5% CO_2. The cells cultured in 100-mm dishes until they became 50–70% confluent, and then were transiently transfected with plasmids pSVHVWF1.1 (wild type) and pSVHL1503R (mutant) using a calcium phosphate method as described previously [28]. Twenty-four hours after transfection, cells were washed twice with phosphate-buffered saline, and incubated with 10 mL FBS-free DMEM. After 48 h, cell lysates were dissolved in the reporter lysis buffer (Promega, Madison, WI) and collected in line with the cell culture media, followed by centrifugation at ×2500 *g* for 10 min. In transfection experiments using a single construct, 10 μg of DNA was used. Dose-dependent series of co-transfection experiment comprised a total amount of 9 μg DNA with various wild-type and mutant mixture. A pCI plasmid was used to bring the DNA amount to 9 μg. The transfection efficacy was determined for each experiment by measuring the luciferase activity of co-transfected 9.6 μg of pRL-SV40 vector and Dual Luciferase Reporter Assay System (Promega). Antigen levels of recombinant VWF were measured by an enzyme-linked immunosorbent assay (ELISA) using polyclonal rabbit anti-human VWF antibody A082 and peroxidase-conjugated rabbit anti-human VWF antibody P226 (DAKO, Carpinteria, CA) [29]. For each experiment, relative expression level was determined by ELISA and normalized according to the transfection efficacy determined by measuring the luciferase activity.

Molecular dynamics simulation

Molecular dynamics simulations were carried out using the program MOE (Chemical Computing Group Inc., Montreal, QC, Canada) for computer simulation of biomolecules, with the aid of CHARMM27 program for calculation of the force field. The coordinate of the A2 domain of human VWF has been built based on a homology-modelling from the structures of six template proteins [30] and the downloaded PDB file was introduced into our system.

The simulation consisted of molecular mechanical calculation of the minimal energy and heating of the system from the crystalline state at $T = 0$ K to a temperature of 310 K. Coordinates were saved every 0.5 ps and the system was simulated for 10 ns. Comparative analysis of the configurations of wild type and mutant VWF-A2 domain at identical time points and identical environmental conditions was performed during dynamical

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

changes. This yielded a real-time visualization for the whole simulation period, from 0 to 10 ns (Fig. 4). Calculation of the root-mean-squared-deviation (RMSD) was performed using SVL language of MOE program.

Results

Patients

Patient A was a 17-year-old woman suffering from frequent epistaxis and hypermenorrhoea, and has been diagnosed as having VWD. Multimer analysis showed relative loss in high- and middle-sized molecular weight multimers, although smaller-sized multimers; the second to fourth multimer band, were also decreased. (Fig. 1). The mother of patient A has been also diagnosed as having VWD, although detailed analysis has not been performed. Patient B is a 49-year-old man with frequent episodes of epistaxis and his father and son have also been also diagnosed as having VWD. Multimer analysis of patient B has not been available. Patient A and B had reduced VWF ristocetin cofactor activity (VWF:RcoF; 0.10 U mL^{-1} and 0.09 U mL^{-1}, respectively), whereas the VWF antigen levels were decreased by 0.19 U mL^{-1} and 0.28 U mL^{-1}, respectively (Table 2). So far no familial relationship has been identified between the two families.

Fig. 1. Plasma multimer analysis of patient A: plasma from patient A was electrophoresed on SDS-agarose gel and visualized as described in Materials and methods. NP, normal plasma.

Table 2. Plasma VWF concentrations of the patients.

	VWF: Ag (U mL^{-1})	VWF: RcoF (U mL^{-1})	FVIII: C (U mL^{-1})
Patient A	0.19	0.10	0.327
Patient B	0.28	0.09	0.414

VWF: Ag, VWF antigen; VWF: RcoF, VWF ristocetin cofactor activity; FVIII: C, factor VIII procoagulant activity.

In 2006, patient B visited the hospital and complained of uncontrollable epistaxis. 1-8 deamino-D-arginine vasopressin (DDAVP) (28 μg $body^{-1}$: 0.4 μg kg^{-1}) was administrated followed by prompt haemostasis. Unfortunately, the plasma VWF levels were not studied either before or after DDAVP infusion.

Molecular analysis

In both patients, DNA sequence analysis of the VWF exon 28 identified a mutation, a novel T → G transition at nucleotide 4508 of the cDNA that caused amino acid substitution at position 1503 of a leucine with an arginine, L1503R (supplementary Fig. S1). The mismatch PCR–RFLP (*Sma*I-RFLP) method was employed to detect the 4508 T → G transition in exon 28 of the VWF gene of patient A and B (Fig. 2a). The undigested and digested pattern (247 + 228 bp) found in the patients confirmed heterozygous state for the mutation. On the other hand, genomic DNAs from 60 normal individuals presented the homozygous undigested pattern with one single band of 247 bp (Fig. 2a).

Expression of mutant VWF

To determine the effect of the mutation (L1503R) on VWF structure and function, expression vectors pSVHL1503R were transiently transfected into HEK293T cells. VWF antigen levels were assayed in the cell lysates and conditioned media using VWF-specific ELISA (Fig. 3). Relative expression level was normalized according to the transfection efficacy determined by luciferase activity of co-transfected pRL-SV40 vector. Wild-type recombinant VWF was secreted efficiently, achieving a concentration of 1.07 ± 0.41 μg mL^{-1} (range of triplicates) in conditioned media compared with 0.27 ± 0.10 μg mL^{-1} in cell lysates (data not shown). In contrast, rVWF (L1503R) was secreted poorly, reaching a concentration of only 7.2% of wild type in the conditioned media, whereas there was 71.5% of wild type in cell lysates (Fig. 3a).

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

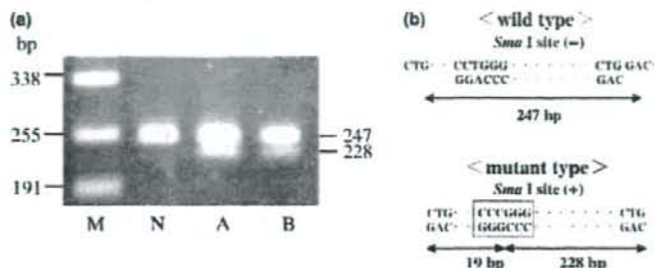

Fig. 2. Mismatch PCR–RFLP analysis: PCR products amplified by two mismatch primers were digested by *Sma*I and analysed by 2% agarose gel electrophoresis as described in the Materials and methods. Lane M, pGEXE/Hinf I digested DNA marker; NP, normal control; A or B is the patient A and B.

Co-expression analysis

Expression analysis of single construct suggested that the mutant protein is not secreted but stored in the cell. Concerning the dominant inheritance of type 2 VWD, it is expected that heterozygous expression of mutant protein may also block the secretion of normal VWF. To mimic the dominant-negative phenotype caused by heterozygosity for the L1503R mutation, plasmid mixtures of wild-type and mutant VWF (L1503R) were co-transfected in HEK293T cells. For a fixed amount of wild-type plasmid (3 μg mL^{-1}), increasing amounts of mutant plasmid caused a dose-dependent inhibition of VWF secretion (Fig. 3b) and a corresponding increase in the level of intracellular VWF (Fig. 3c). Transfection with increasing amounts of only wild-type VWF plasmid did not impair secretion, and intracellular VWF was linearly increased (Fig. 3b, c). These observations indicate that the effects caused by co-transfection with mutant plasmid were specific for the L1503R substitution and are consistent with the proposed

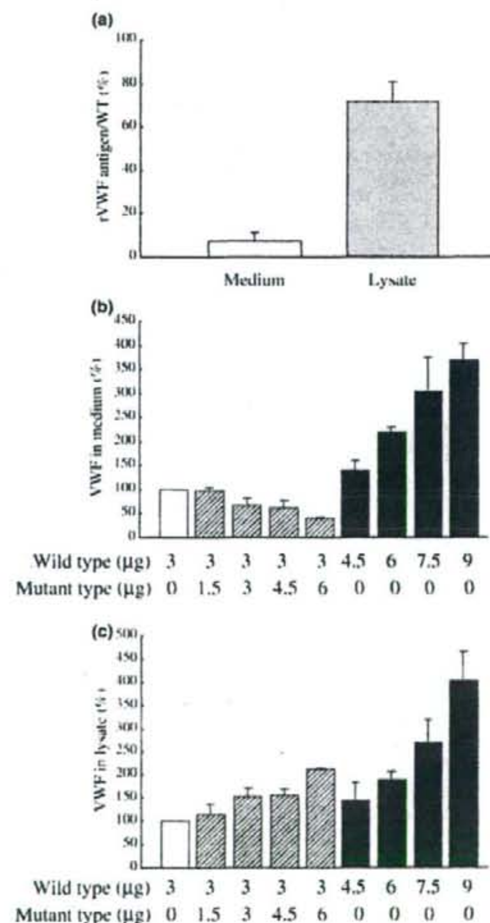

Fig. 3. Transfection experiment of wild type and mutant construct of full-length cDNA of human: VWF wild type and L1503R is expressed in HEK293T cells as described in the Materials and methods. After transfection, cell lysates and media were harvested after 48 h and subjected to ELISA for VWF antigen levels. Expression level was normalized according to the transfection efficacy determined by luciferase activity of co-transfected pRL-SV40 vector. (a), Single transfection analysis; Expression levels of the media (open column) and cell lysates (shaded column) are shown relative to that for expression levels of wild type VWF expressed at the same time. Each column represents the mean ± SD of values obtained in three independent sets of duplicate assays. (b)–(c), Co-transfection of plasmid mixtures of wild type and mutant VWF cDNA; co-transfection was performed by the mixture of varying concentrations (1.5–6 μg mL^{-1}) of mutant plasmids and fixed amount (3 μg) of wild type plasmid (hatched column). As control, varying concentrations (1.5–6 μg mL^{-1}) of wild type plasmid plus 1.5 μg mL^{-1} of same wild type plasmid were transfected (closed column). Relative value of expression is shown relative to that for expression levels of 3 μg of wild type rVWF performed at the same time (open column) as 100%. The VWF antigen levels in the medium (b) and lysate (c) is determined as described above. Each column represents the mean ± SD of values obtained in two independently duplicated transfections.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

dominant-negative effect of the L1503R mutation *in vivo*.

Molecular dynamics simulation

Molecular dynamics simulation was used to study the dynamical effects of the mutation introduced into the structural model. Data simulated from wild type human VWF A2 domain were compared with the two mutant forms L1503R and L1503Q. The mutation L1503Q has been classified as group II mutation that causes the increase in the protein's susceptibility to ADAMTS13 proteolysis [20]. Figure 4 shows the results of calculations of RMSD of the structure of the A2 molecule over a period of 1–10 ns. The mean value of all-atom RMSD of the wild type, L1503R and L1503Q was 3.368 ± 0.057, 3.846 ± 0.109, and 3.449 ± 0.131 respectively. The value of L1503R was in the highest level of energy equalization, although those of the wild type and L1503Q were close to each other and were in lower levels (Fig. 4). It is thus suggested that the presence of L1503R mutation strongly alters the mean square deviation and initiates conformational changes.

Discussion

Our patients' plasma showed markedly reduced levels of VWF ristocetin cofactor activity, with 0.2–0.3 U mL^{-1} of VWF antigen levels (Table 2). Plasma

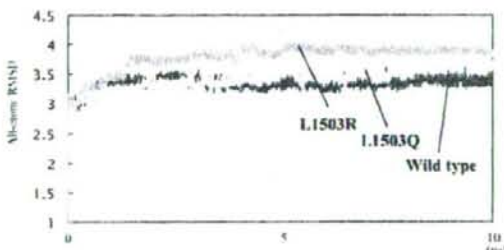

Fig. 4. All-atom root-mean-square deviation (RMSD) from the model of VWF-A2 domain as a function of time: plots of mean square deviations in the configuration of the VWF A2 molecule during the 10 ns of simulation of molecular dynamics. Molecular dynamics simulations were carried out using the program MOE with the aid of CHARMM27 program for calculation of the force field. Detailed methods are described in the Materials and methods. The coordinate of the A2 domain of human VWF has been built based on a homology-modelling from the structures of six template proteins [29] and the downloaded PDB file was introduced into our system. RMSD value of wild type (black), L1503Q (light grey), and L1503R (dark grey) were plotted over time of 10 ns.

multimer analysis of patient A indicated that larger-sized multimer was relatively decreased. Currently, the patient is diagnosed as type 2A VWD, although any pathological mechanism leading to quantitative VWF defect is not excluded.

By determining the nucleotide sequence of exon 28 of the mutant VWF gene, a single candidate mutation was identified: a T>G substitution at 4508 of the VWF gene, thereby substituting leucine at 1503 to arginine of the VWF A2 domain. This missense mutation was not found in 60 normal individuals. The results of expression analysis of the mutant construct revealed that VWF antigen of mutant L1503R is markedly reduced by <10% in the medium but normal (80%) in the lysates, suggesting that substantial levels of mutant subunit were synthesized but appeared to have been retained within the cell. Thus, the mutation is sufficient to cause reduced secretion of functional VWF and thus it appears to belong to group I of VWD type 2A.

The defect in biosynthesis and secretion of VWF L1503R suggests a clue on the general mechanism by which mutations may be causing VWD that has been inherited in autosomal dominant way. Co-transfection experiment confirmed the dominant-negative mechanism of disease development; as the concentration of transfected mutant DNA is increased, secreted VWF protein is decreased by ~40%. In contrast, VWF antigen was accumulated in the lysates depending on mutant concentrations. Therefore, the mutation L1503R inhibits the secretion of a co-inherited normal allele, thereby causing a dominant form of VWD.

Although detailed VWF level has not been determined, clinical symptom of patient B was improved by DDAVP administration. As DDAVP appears to increase VWF secretion from cell storage, it is suggested that haemostasis had been accomplished by increased secretion of VWF multimers inherited from normal allele.

Structural changes induced by mutations can be studied with molecular dynamics simulations. The all-atom RMSD with respect to the model is shown in Fig. 4 for the simulations of wild type A2 domain, along with that for L1503Q and L1503R. The simulations of three molecules indicated that no dramatic unfolding occurred. However, the mean values of all-atom RMSD of the L1503R were shifted from those with wild type and L1503Q (Fig. 4), suggesting that L1503R is in the higher energy equalization level. This finding suggests that the group I mutation L1503R cause significant deviations, coupled to thermal fluctuations, when compared with group II mutation L1503Q or wild type

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

A2 domain. Sutherland *et al.* found that the mutation L1503Q caused no significant structural change [30], being consistent with our finding that there were little difference of RMSD values between wild type and L1503Q (Fig. 4). Apparently, protein instability of L1503R may be responsible for its intracellular retention leading to a group I disease phenotype.

On the other hand, it was shown that Leu1503 and the physiological proteolytic cleavage site for ADAMTS13 (Y1605-M1606) are localized close together in two adjacent parallel β-sheets [20]. The proteolysis site for ADAMTS13 is not present on the surface of the protein, suggesting that proteolysis appears to occur only when the VWF protein is partially unfolded under conditions of high shear stress (*in vivo*) or through denaturant exposure (*in vitro*) [30].

In VWD type 2A, it can also been seen that the two different substitutions at the same amino acid position have resulted in group I and group II mutations respectively; G1505E were a group II mutation, whereas G1505R resulted in a representative phenotype of group I mutations [30]. By molecular dynamics simulation of a series of group I and II mutations, G1505R was found to cause significant structural deviations over multiple regions of the A2 domain, confirming that protein instability of VWF with group I mutations may be responsible for the intracellular retention [30].

There are limitations that must be considered when interpreting the results of molecular dynamics simulation. First, a homology model has been used as no real crystal structure is available [30] and errors in the model might affect the outcome of simulations. Second, conformational changes that may occur beyond 10 ns simulation time could not be observed. Finally, simulations could not interpret where the mutant protein is mainly mal-processed and retained, during the transport from ER to the Golgi apparatus. In fact, multimerization takes place in the Golgi apparatus, and a class of group I type 2A VWD mutations is compatible with the transport of mutant subunits to the Golgi, where they inhibit multimer assembly [18]. On the other hand, subunits bearing a class of type 1 mutation accumulate in the endoplasmic reticulum and would reduce the transport of wild type subunits to the Golgi apparatus [31], and those arriving in the Golgi could assemble into full multimers resulting in multimers typical in type 1 VWD. As our case also showed decreased lower molecular weight multimers, it may be considered to have intermediate properties, which are identified among patients with indeterminate phenotypes.

Acknowledgements

We are thankful to Ms Chika Wakamatsu for her excellent techniques in molecular biology. A part of our work was supported by Sankyo Foundation of Life Sciences.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

References

1 Tschopp TB, Weiss HJ, Baumgartner HR. Decreased adhesion of platelets to subendothelium in von Willebrand's disease. *J Lab Clin Med* 1974; **83**: 296–300.
2 Sakariassen KS, Bolhuis PA, Sixma JJ. Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-Von Willebrand factor bound to the subendothelium. *Nature* 1979; **279**: 636–8.
3 Stel HV, Sakariassen KS, de Groot PG, van Mourik JA, Sixma JJ. von Willebrand factor in the vessel wall mediates platelet adherence. *Blood* 1985; **65**: 85–90.
4 Turitto VT, Weiss HJ, Zimmerman TS, Sussman II. Factor VIII/ von Willebrand factor in subendothelium mediates platelet adhesion. *Blood* 1985; **65**: 823–31.
5 Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem* 1998; **67**: 395–424.
6 Purvis AR, Gross J, Dang LT *et al.* Two Cys residues essential for von Willebrand factor multimer assembly in the Golgi. *Proc Natl Acad Sci USA* 2007; **104**: 15647–52.
7 Marti T, Rösselet SJ, Titani K, Walsh KA. Identification of disulfide-bridged substructures within human von Willebrand factor. *Biochemistry* 1987; **26**: 8099–109.
8 Voorberg J, Fontijn R, Calafat J, Janssen H, van Mourik JA, Pannekoek H. Assembly and routing of von Willebrand factor variants: the requirements for disulfide-linked dimerization reside within the carboxy-terminal 151 amino acids. *J Cell Biol* 1991; **113**: 195–205.
9 Sadler JE, Moake JL, Miyata T, George JN. Recent advances in thrombotic thrombocytopenic purpura. *Hematology (Am Soc Hematol Educ Program)* 2004: 407–23.
10 Matsushita T, Dong Z, Sadler JE. von Willebrand's factor and von Willebrand's diesease. *Curr Opin Hematol* 1994; **1**: 362–8.
11 Hassenpflug WA, Budde U, Obser T *et al.* Impact of mutations in the von Willebrand factor A2 domain on ADAMTS13-dependent proteolysis. *Blood* 2006; **107**: 2339–45.
12 Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. *Blood* 2001; **98**: 1662–6.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

13 Gerritsen HE, Robles R, Lammle B, Furlan M. Partial amino acid sequence of purified von Willebrand factor-cleaving protease. *Blood* 2001; **98**: 1654–61.

14 Soejima K, Mimura N, Hirashima M *et al.* A novel human metalloprotease synthesized in the liver and secreted into the blood: possibly, the von Willebrand factor-cleaving protease? *J Biochem (Tokyo)* 2001; **130**: 475–80.

15 Batlle J, Lopez Fernandez MF, Campos M *et al.* The heterogeneity of type IIA von Willebrand's disease: studies with protease inhibitors. *Blood* 1986; **68**: 1207–12.

16 Dent JA, Berkowitz SD, Ware J, Kasper CK, Ruggeri ZM. Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand factor. *Proc Natl Acad Sci U S A* 1990; **87**: 6306–10.

17 Gralnick HR, Williams SB, McKeown LP *et al. In vitro* correction of the abnormal multimeric structure of von Willebrand factor in type IIa von Willebrand's disease. *Proc Natl Acad Sci U S A* 1985; **82**: 5968–72.

18 Lyons SE, Bruck ME, Bowie EJ, Ginsburg D. Impaired intracellular transport produced by a subset of type IIA von Willebrand disease mutations. *J Biol Chem* 1992; **267**: 4424–30.

19 Zimmerman TS, Dent JA, Ruggeri ZM, Nannini LH. Subunit composition of plasma von Willebrand factor. Cleavage is present in normal individuals, increased in IIA and IIB von Willebrand disease, but minimal in variants with aberrant structure of individual oligomers (types IIC, IID, and IIE). *J Clin Invest* 1986; **77**: 947–51.

20 O'Brien LA, Sutherland JJ, Hegadorn C *et al.* A novel type 2A (Group II) von Willebrand disease mutation (L1503Q) associated with loss of the highest molecular weight von Willebrand factor multimers. *J Thromb Haemost* 2004; **2**: 1135–42.

21 Matsushita T, Tanimoto M, Yamamoto K *et al.* DNA sequence analysis of three inhibitor-positive hemophilia B patients without gross gene deletion. Identification of four novel mutations in factor IX gene. *J Lab Clin Med* 1990; **116**: 492–7.

22 Raines G, Aumann H, Sykes S, Street A. Multimeric analysis of von Willebrand factor by molecular sieving electrophoresis in sodium dodecyl sulphate agarose gel. *Thromb Res* 1990; **60**: 201–12.

23 Zhang ZP, Lindstedt M, Falk G, Blomback M, Egberg N, Anvret M. Nonsense mutations of the von Willebrand factor gene in patients with von Willebrand disease type III and type I. *Am J Hum Genet* 1992; **51**: 850–8.

24 Okada H, Takagi A, Murate T *et al.* Identification of protein Salpha gene mutations including four novel mutations in eight unrelated patients with protein S deficiency. *Br J Haematol* 2004; **126**: 219–25.

25 Matsushita T, Sadler JE. Identification of amino acid residues essential for von Willebrand factor binding to platelet glycoprotein Ib. Charged-to-alanine scanning mutagenesis of the A1 domain of human von Willebrand factor. *J Biol Chem* 1995; **270**: 13406–14.

26 Burke RL, Pachl C, Quiroga M *et al.* The functional domains of coagulation factor VIII:C. *J Biol Chem* 1986; **261**: 12574–8.

27 Wagner DD, Saffaripour S, Bonfanti R *et al.* Induction of specific storage organelles by von Willebrand factor propolypeptide. *Cell* 1991; **64**: 403–13.

28 Sobue S, Hagiwara K, Banno Y *et al.* Transcription factor specificity protein 1 (Sp1) is the main regulator of nerve growth factor-induced sphingosine kinase 1 gene expression of the rat pheochromocytoma cell line, PC12. *J Neurochem* 2005; **95**: 940–9.

29 Tuley EA, Gaucher C, Jorieux S, Worrall NK, Sadler JE, Mazurier C. Expression of von Willebrand factor "Normandy": an autosomal mutation that mimics hemophilia A. *Proc Natl Acad Sci USA* 1991; **88**: 6377–81.

30 Sutherland JJ, O'Brien LA, Lillicrap D, Weaver DF. Molecular modeling of the von Willebrand factor A2 domain and the effects of associated type 2A von Willebrand disease mutations. *J Mol Model* 2004; **10**: 259–70.

31 Eikenboom JC, Matsushita T, Reitsma PH *et al.* Dominant type 1 von Willebrand disease caused by mutated cysteine residues in the D3 domain of von Willebrand factor. *Blood* 1996; **88**: 2433–41.

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. DNA sequence analysis of exon 28 of patient A. DNA sequence analysis of exon 28 of patient A reveals a T > G transversion at nucleotide 4508. The patient is heterozygous for this transition, as indicated by the double chromatogram peak. The result of patient B was identical and is not shown.

This material is available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2516.2008.01703.x.

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

Original Paper

Intervirology

Intervirology 2008;51:21–25
DOI: 10.1159/000118792

Received: October 16, 2007
Accepted after revision: January 2, 2008
Published online: February 29, 2008

Prevalence of Hepatitis E Virus IgG Antibody in Japanese Patients with Hemophilia

Hidenori Toyoda[a] Takashi Honda[b] Kazuhiko Hayashi[b] Yoshiaki Katano[b] Hidemi Goto[b] Takashi Kumada[a] Kazuaki Takahashi[d] Natsumi Abe[d] Shunji Mishiro[d] Junki Takamatsu[c]

[a]Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Departments of [b]Gastroenterology and [c]Transfusion Medicine, Nagoya University School of Medicine, Nagoya, and [d]Department of Medical Science, Toshiba General Hospital, Tokyo, Japan

Key Words

Hemophilia · Hepatitis E virus · Transfusion transmission

Abstract

Objective: We investigated the prevalence of antibody against hepatitis E virus (HEV) in Japanese patients with hemophilia. ***Methods:*** IgG antibody against HEV was measured in serum of 80 Japanese patients with hemophilia by enzyme-linked immunosorbent assay. The prevalence of HEV antibody was compared with the reported prevalence of HEV antibody in Japanese patients undergoing hemodialysis and in Japanese healthy blood donors. Characteristics of patients and coinfection with other transfusion-transmissible viruses were compared in patients with and without HEV antibody. ***Results:*** Anti-HEV IgG antibody was detected in 13 of 80 patients (16.3%). The prevalence was far higher than that reported in Japanese blood donors (3.7%) and was higher than that in Japanese patients undergoing hemodialysis (9.4%). The patients with HEV antibody were significantly older than those without. HEV antibody was not detected in patients <20 years of age and in patients who had received only virus-inactivated coagulation factors. No association was observed between positivity for anti-HEV antibody and severity of hemophilia or coinfection with other parenterally transmissible viruses. ***Conclusion:*** Our results suggest that the parenteral transmission of HEV may have occurred in Japanese patients with hemophilia via non-virus-inactivated coagulation factors.

Copyright © 2008 S. Karger AG, Basel

Introduction

Infection with hepatitis E virus (HEV), which can cause acute hepatitis E, is an important public health concern in many developing countries, where sanitation is suboptimal; large epidemics of hepatitis E have been reported from Asia, Africa, and Latin America [1]. Although only sporadic cases of acute hepatitis E have been reported in many industrialized countries including the United States, Europe, and Japan [1–5], some healthy individuals in industrialized countries are seropositive for HEV antibodies [6, 7].

A relatively recent report [8] described a patient who was infected with HEV via transfused blood from a vol-

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2008 S. Karger AG, Basel
0300–5526/08/0511–0021$24.50/0

Accessible online at:
www.karger.com/int

Hidenori Toyoda, MD, PhD
Department of Gastroenterology, Ogaki Municipal Hospital
4-86 Minaminokawa
Ogaki 503-8502 (Japan)
Tel. +81 584 81 3341, Fax +81 584 75 5715, E-Mail tkumada@he.mirai.ne.jp

untary blood donor, and the potential risk of posttransfusion hepatitis E even in non- or low-endemic countries including Japan was suggested. The parenteral route of HEV transmission, however, remains controversial; some studies have suggested parenteral transmission of HEV, but others have shown no parenteral transmission of this virus [9–20]. Patients with hemophilia are at high risk of infection by transfusion-transmissible viruses due to the frequent use of coagulation factors. High rates of infection by hepatitis C virus (HCV), human immunodeficiency virus (HIV), and GB virus C (GBV-C) have been reported in patients with hemophilia [21–26]. If HEV infection could also have occurred in patients with hemophilia via coagulation factors, the prevalence of seropositivity for HEV antibodies would be high.

We previously investigated the prevalence of IgG antibody against hepatitis A virus (HAV) in Japanese patients with hemophilia [26] and reported a higher prevalence of HAV antibody in Japanese patients with hemophilia in comparison with normal Japanese subjects, suggesting the association between the use of coagulation factor and HAV infection. As for HEV, that is the other hepatitis virus with nonparenteral transmission, we did not investigate its prevalence in hemophilia patients because it had been considered to be rare in Japan. However, it was reported that zoonotic food-borne transmission of HEV to humans sometimes occurs; HEV infection is not so rare in Japan. In the present study, we evaluated the prevalence of antibody against HEV in Japanese patients with hemophilia to investigate the possibility of parenteral transmission of HEV by means of coagulation factors.

Patients and Methods

Patients

Eighty Japanese patients with hemophilia were involved in the study. These patients were selected from among 188 hemophilia patients who were followed up at Nagoya University Hospital and because they had sufficient stored serum samples after 2003. The patient group comprised 80 males, 59 with hemophilia A and 21 with hemophilia B. Fifty-one patients had received both non-virus-inactivated and virus-inactivated coagulation factors, and 29 had received only virus-inactivated coagulation factors. Fifty-four patients had received both domestic and imported coagulation factors that had been manufactured in the United States or in Europe, and 26 had received only domestic coagulation factors. Patients were 39.0 ± 14.4 years of age. No patient had a history of travel abroad. Written informed consent was obtained from all patients before serum samples were obtained. The study was approved by the University Ethics Committee and carried out in compliance with the Helsinki Declaration.

Serologic and Virologic Analyses of HAV, Hepatitis B Virus (HBV), HCV, GBV-C, HIV, and HEV

HAV antibody was measured in serum samples with a commercially available enzyme immunoassay kit (Dainabot, Tokyo, Japan) according to the manufacturer's instructions. HBV surface antigen (HBsAg), HBV surface antibody (HBsAb), and HBV core antibody (HBcAb) were measured with Architect HBsAg QT, Architect HBs, and Architect HBc, respectively (all Abbott Japan, Tokyo). HCV was analyzed by HCV antibody assay (third-generation assay kit; Dainabot), and HCV RNA was analyzed with the Amplicor HCV test, version 2.0 (Roche Diagnostics, Branchburg, N.J., USA). GBV-C RNA was measured by RT-PCR with nested primers deduced from conserved blocks in the 5'-untranslated region by a method described previously [27]. HIV1 infection was confirmed by anti-HIV1 antibody detection achieved with a particle agglutination test (Serodia-HIV; Fuji Rebio, Tokyo, Japan). IgG antibody against HEV was measured in serum by enzyme-linked immunosorbent assay as described by Li et al. [28].

Statistical Analysis

Differences in the proportion of patients with and without HEV antibody were analyzed by χ^2 test. Differences in quantitative variables were analyzed by Mann-Whitney U test. All p values were derived from two-tailed tests, and $p < 0.05$ was accepted as statistically significant.

Results

IgG antibody against HEV was detected in 13 of the 80 patients (16.3%) with hemophilia. The clinical characteristics of patients with and without HEV antibody are shown in table 1. The patients in whom HEV antibody was detected were significantly older than those in whom HEV antibody was not detected (46.9 ± 17.9 vs. 37.4 ± 13.1 years, p = 0.0346). No patient <21 years of age had HEV antibody. All patients with HEV antibody had started coagulation factor therapy before 1985. No patient who had received only virus-inactivated coagulation factors was positive for HEV antibody, whereas 13 of 51 patients (25.5%) who had received non-virus-inactivated coagulation factors were positive for HEV antibody. In contrast, HEV antibody was detected in similar percentages of patients who received only domestic and those who had received both domestic and imported coagulation factors (15.4 vs. 16.7%).

The prevalences of HAV, HBV, HCV, HIV, and GBV-C in patients with and without HEV antibody are shown in table 2. No differences were observed in the prevalence rates of these viruses between the two groups.

Table 1. Characteristics of the patients with and without HEV antibody

	HEV antibody positive (n = 13)	HEV antibody negative (n = 67)	p
Age, years (mean ± SD)[a]	46.8 ± 17.1	36.0 ± 13.5	0.0345
Type of hemophilia			
A	9 (15.3)	50 (84.7)	0.9519
B	4 (19.0)	17 (81.0)	
Severity of hemophilia			
Mild	3 (23.1)	10 (76.9)	0.6476
Moderate	0	2 (100)	
Severe	10 (15.4)	55 (84.6)	
Coagulation factors			
Virus-inactivated only	0	29 (100)	
Both non-virus-inactivated and virus-inactivated	13 (25.5)	38 (74.5)	0.0079
Domestic	4 (15.4)	22 (84.6)	
Both domestic and imported	9 (16.7)	45 (83.3)	0.8842

Numbers (and percentages) of patients are shown unless otherwise indicated.
[a] Age at the time of measurement of HEV antibody.

Table 2. Prevalence rates of other transfusion-transmissible viruses in patients with and without HEV antibody

Positive for	HEV antibody positive (n = 13)	HEV antibody negative (n = 67)	p
HAV IgG antibody	6 (46.2)	15 (22.4)	0.1505
HBV surface antigen	1 (7.7)	0	0.3572
HBV surface antibody	8 (61.5)	40 (59.7)	0.9015
HBV core antibody	10 (76.9)	52 (77.6)	0.9566
HCV antibody	13 (100.0)	63 (94.2)	0.8348
HCV RNA	13 (100.0)	63 (94.2)	0.8348
HIV1 antibody	5 (38.5)	27 (40.3)	0.9015
GBV-C RNA[a]	4 (44.4)	12 (30.8)	0.6949

Numbers (and percentages) of patients are shown.
[a] Among 48 patients in whom GBV-C RNA was measured (9 with HEV antibody and 39 without HEV antibody).

Table 3. Reported prevalence rates of HEV antibody in patients with hemophilia and in patients undergoing hemodialysis

Authors (year of publication)	Country	Prevalence (%) of patients with HEV antibody
Patients with hemophilia		
Mannucci et al. [12] (1994)	Italy	0/60 (0)
Barzilai et al. [13] (1995)	Israel	16/188 (8.5)
Klarmann et al. [14] (1995)	Germany	1/37 (2.7)
Zaaijer et al. [15] (1995)	Netherlands	4/296 (1.4)
Buffet et al. [16] (1996)	France	5/63 (7.9)
Our study (2007)	Japan	13/80 (16.3)
Patients undergoing hemodialysis		
Courtney et al. [10] (1994)	Ireland	0/45 (0)
Halfon et al. [11] (1994)	France	16/147 (10.9)
Psichogiou et al. [17] (1996)	Greece	27/420 (6.4)
Fabrizi et al. [18] (1997)	Italy	6/204 (2.9)
Mitsui et al. [20] (2004)	Japan	39/416 (9.4)

Table 4. Prevalence rates of HEV antibody among patients with hemophilia, patients on hemodialysis, and healthy blood donors in Japan

Age, years	Patients with hemophilia (n = 80)	Patients undergoing hemodialysis (n = 416) [20]	Healthy blood donors (n = 5,343) [29]
≤19	0/6		7/812 (0.9)
20–29	1/18 (5.6)	1/33 (3.0)[a]	19/1,043 (1.8)
30–39	5/24 (20.8)		28/1,146 (2.4)
40–49	2/17 (11.8)	3/40 (7.5)	53/966 (5.5)
50–59	3/10 (30.0)	10/109 (9.2)	54/744 (7.3)
≥60	2/5 (40.0)	25/234 (10.7)	39/632 (6.2)

Numbers (and percentages) of patients are shown.
The age range was 16–84 years in patients with hemophilia, 23–91 years in patients undergoing hemodialysis, and 16–69 years in healthy blood donors.
[a] Patients with an age range of 23–39 years.

Discussion

Whether HEV is transmitted parenterally remains controversial (table 3); the existence of transfusion transmission of HEV is still unclear. A high prevalence of anti-HEV antibody was reportedly observed among hemodialysis patients, the majority of whom had a history of blood transfusions [11, 20]. However, other investigators found only a few HEV antibody-positive patients in larger groups of hemodialysis patients [10, 17, 18]. In industrialized countries, the prevalence of positivity for HEV antibody in patients with hemophilia seems to differ between countries [12–16], whereas a higher prevalence in patients with hemophilia than in volunteer blood donors has been reported in nonindustrialized countries [19].

This is the first report that investigated the prevalence of HEV antibody in hemophilia patients in Japan. The prevalence of HEV antibody in our study patients (16.3%) was higher than that previously reported in Japanese blood donors (3.7%) [29] and in Japanese patients undergoing hemodialysis (9.4%) [20] (table 4). The gradual increase in the prevalence of HEV antibody between healthy blood donors, patients undergoing hemodialysis, and patients with hemophilia suggests a possible role of parenteral transmission of HEV. Because coagulation factors that are currently used in Japan are very unlikely to contain IgG antibodies [30, 31], the HEV antibodies that were detected in our patients cannot have been passively acquired from recently used coagulation factors.

The absence of HEV antibody in patients <20 years of age and in patients who had received only virus-inactivated coagulation factors suggests that HEV infection might have occurred by means of non-virus-inactivated coagulation factors that had been used before the mid-1980s. In contrast, we did not find a difference in the prevalence of HEV antibody between patients who received only domestic coagulation factors and those who had a history of using imported coagulation factors, unlike the difference in the prevalence of HIV or HCV genotype 1a infections between Japanese hemophilia patients with and without the use of imported coagulation factors [23]. Recent studies have indicated that hepatitis E is a zoonosis [2, 4, 32–39], and it has been shown that zoonotic food-borne transmission of HEV to humans may play an important role in the occurrence of HEV infection in Japan [38–41]. In addition, silent viremia due to HEV, i.e., the presence of HEV in the bloodstream but without acute hepatitis, has been reported [19, 42, 43]. HEV infection, therefore, may not be rare in Japan [44, 45]. Contamination of domestic, non-virus-inactivated coagulation factors by HEV is, therefore, not unlikely, and the use of domestic coagulation factors could have caused HEV infection in Japanese patients with hemophilia. In addition, the use of coagulation factors that had been manufactured from plasma of individuals from an area where HEV infection is endemic could have caused HEV infections in our patients.

We found no association of HEV antibody with infection by transfusion-transmissible viruses (HBV, HCV, HIV, and GBV-C) or with the presence of HAV antibody. It was difficult to elucidate the characteristics of patients with HEV antibody on the basis of the coinfecting viruses because of the high prevalence of infection by these viruses.

The marked differences between countries in the prevalence of HEV antibody in patients with hemophilia, which sparks controversy over the possibility of parenteral transmission of HEV, might be due to differences in the origins and methods of manufacturing coagulation factors, especially during the period in which non-virus-inactivated coagulation factors were used. Unfortunately, the prevalence of HEV antibodies in volunteer blood donors in the various countries has not been reported, and the origins of the plasma used for manufacturing coagulation factors in these countries are unknown. It is well known that the prevalence of HEV varies widely throughout the world. Plasma of individuals from high-prevalence areas might have had more chance of contamination during the period when non-virus-inactivated coagulation factors were used.

In summary, the high prevalence of HEV antibody in our patients with hemophilia suggests the possibility of a parenteral route of HEV transmission. However, the coagulation factors used in our patients are not now available for examination, so we cannot prove that the coagulation factors used in Japan were contaminated by HEV. Further studies are needed to clarify whether HEV has been transmitted by means of coagulation factors. Tracing the origin of the plasma used for coagulation factors would be helpful in understanding the association between the prevalence of HEV antibody and the use of coagulation factors in patients with hemophilia.

References

1 Purcell RH, Emerson SU: Hepatitis E virus; in Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B, Straus SE (eds): Fields Virology, ed 4. Philadelphia, Lippincott Williams & Wilkins, 2001, pp 3051–3061.

2 Harrison TJ: Hepatitis E virus – an update. Liver 1999;19:171–176.

3 Schlauder GG, Mushahwar IK: Genetic heterogeneity of hepatitis E virus. J Med Virol 2001;65:282–292.

4 Smith JL: A review of hepatitis E virus. J Food Protect 2001;64:572–586.

5 Okamoto H, Takahashi M, Nishizawa T: Features of hepatitis E virus infection in Japan. Intern Med 2003;42:1065–1071.

6 Mast EE, Kuramoto IK, Favorov MO, Schoening VR, Burkholder BT, Shapiro CN, Holland PV: Prevalence of and risk factors for antibody to hepatitis E virus seroreactivity among blood donors in Northern California. J Infect Dis 1997;176:34–40.

7 Thomas DL, Yarbough PO, Vlahov D, Tsarev SA, Nelson KE, Saah AJ, Purcell RH: Seroreactivity to hepatitis E virus in areas where the disease is not endemic. J Clin Microbiol 1997;35:1244–1247.

8 Matsubayashi K, Nagaoka Y, Sakata H, Sato S, Fukai K, Kato T, Takahashi K, Mishiro S, Imai M, Takeda N, Ikeda H: Transfusion-transmitted hepatitis E caused by apparently indigenous hepatitis E virus strain in Hokkaido, Japan. Transfusion 2004;44:934–940.
9 Wang CH, Flehmig B, Moeckli R: Transmission of hepatitis E virus by transfusion? Lancet 1993;341:825–826.
10 Courtney MG, O'Mahoney M, Albloushi S, Sachithanandan S, Walshe J, Carmody M, Donoghue J, Parfrey N, Shattock AG, Fielding J: Hepatitis E virus antibody prevalence. Lancet 1994;344:1166.
11 Halfon P, Ouzan D, Chanas M, Khiri H, Feryn JM, Mangin L, Masseyef MF, Salvadori JM: High prevalence of hepatitis E virus antibody in haemodialysis patients. Lancet 1994;344:746.
12 Mannucci PM, Gringeri A, Santagostino E, Romano L, Zanetti A: Low risk of transmission of hepatitis E virus by large-pool coagulation factor concentrates. Lancet 1994;343:597–598.
13 Barzilai A, Schulman S, Karetnyi YV, Favorov MO, Levin E, Mendelson E, Weiss P, Fields HA, Varon D, Martinowitz U: Hepatitis E virus infection in hemophiliacs. J Med Virol 1995;46:153–156.
14 Klarmann D, Kreuz W, Kornhuber B: Low prevalence of hepatitis E virus antibodies in hepatitis C virus-positive patients with coagulation disorders. Transfusion 1995;35:969–970.
15 Zaaijer HL, Mauser-Bunschoten EP, ten Veen JH, Kapprell HP, Kok M, van den Berg HM, Lelie PN: Hepatitis E virus antibodies among patients with hemophilia, blood donors, and hepatitis patients. J Med Virol 1995;46:244–246.
16 Buffet C, Laurent-Puig P, Chandot S, Laurian Y, Charpentier B, Briantais MJ, Dussaix E: A high hepatitis E virus seroprevalence among renal transplantation and haemophilia patient populations. J Hepatol 1996;24:122–125.
17 Psichogiou M, Vaindirli E, Tzala E, Voudiclari S, Boletis J, Vosnidis G, Moutafis S, Skoutelis G, Hadjiconstantinou V, Troonen H, Hatzakis A: Hepatitis E virus (HEV) infection in haemodialysis patients. The Multicentre Haemodialysis Cohort Study on Viral Hepatitis. Nephrol Dial Transplant 1996;11:1093–1095.
18 Fabrizi F, Lunghi G, Bacchini G, Corti M, Pagano A, Locatelli F: Hepatitis E virus infection in haemodialysis patients: a seroepidemiological survey. Nephrol Dial Transplant 1997;12:133–136.
19 Arankalle VA, Chobe LP: Hepatitis E virus: can it be transmitted parenterally? J Viral Hepat 1999;6:161–164.
20 Mitsui T, Tsukamoto Y, Yamazaki C, Masuko K, Tsuda F, Takahashi M, Nishizawa T, Okamoto H: Prevalence of hepatitis E virus infection among hemodialysis patients in Japan: evidence for infection with a genotype 3 HEV by blood transfusion. J Med Virol 2004;74:563–572.
21 Inagaki M, Miura T, Taki M, Saito N, Yamada K, Unuma T, Tsuda F, Miyakawa Y, Mayumi M: Infection with hepatitis delta and human immunodeficiency viruses among hemophiliacs in Japan. Transfusion 1988;28:18–20.
22 Kashiwagi S, Hayashi J, Ikematsu H, Noguchi A, Ikeda K, Kishida K, Shirakawa M, Takenaka A, Mori R: Prevalence of human immunodeficiency virus (HIV) infection among hemophiliacs in Fukuoka, Japan. Microbiol Immunol 1988;32:851–855.
23 Isobe K, Imoto M, Fukuda Y, Koyama Y, Nakano I, Hayakawa T, Takamatsu J: Hepatitis C virus infection and genotypes in Japanese hemophiliacs. Liver 1995;15:131–134.
24 Makris M, Preston FE, Rosendaal FR, Underwood JCE, Rice KM, Triger DR: The natural history of chronic hepatitis C in haemophiliacs. Br J Haematol 1996;94:746–752.
25 Darby SC, Ewart DW, Giangrande PLF, Spooner RJD, Rizza CR, Dusheiko GM, Lee CA, Ludlam CA, Preston FE: Mortality from liver cancer and liver disease in haemophilic men and boys in UK given blood products contaminated with hepatitis C. UK Haemophilia Centre Directors' Organisation. Lancet 1997;350:1425–1431.
26 Hayashi K, Fukuda Y, Nakano I, Katano Y, Nagano K, Yokozaki S, Hayakawa T, Toyoda H, Takamatsu J: Infection of hepatitis A virus in Japanese haemophiliacs. J Infect 2001;42:1–4.
27 Toyoda H, Fukuda Y, Hayakawa T, Takamatsu J, Saito H, Okamoto H: GB virus C/hepatitis G virus isolates in Japanese haemophiliacs and their origins. Thromb Haemost 1998;80:242–245.
28 Li TC, Zhang J, Shinzawa H, Ishibashi M, Sata M, Mast EE, Kim K, Miyamura T, Takeda N: Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. J Med Virol 2000;62:327–333.
29 Fukuda S, Sunaga J, Saito N, Fujimura K, Itoh Y, Sasaki M, Tsuda F, Takahashi M, Nishizawa T, Okamoto H: Prevalence of antibodies to hepatitis E virus among Japanese blood donors: identification of three blood donors infected with a genotype 3 hepatitis E virus. J Med Virol 2004;73:554–561.
30 Mannhalter JW, Ahmad R, Leibl H, Göttlicher J, Wolf HM, Eibl MM: Comparable modulation of human monocyte functions by commercial factor VIII concentrates of varying purity. Blood 1988;71:1662–1668.
31 Wadsworth C, Hanson LA, Kjellman H, Söderström T, Blombäck M: Some characteristics of aggregates of IgG and plasma proteins in heat-treated factor VIII concentrates. Blut 1989;58:133–141.
32 Meng XJ, Purcell RH, Halbur PG, Lehman JR, Webb DM, Tsareva TS, Haynes JS, Thacker BJ, Emerson SU: A novel virus in swine is closely related to the human hepatitis E virus. Proc Natl Acad Sci USA 1997;94:9860–9865.
33 Meng XJ, Halbur PG, Shapiro MS, Govindarajan S, Bruna JD, Mushahwar IK, Purcell RH, Emerson SU: Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J Virol 1998;72:9714–9721.
34 Erker JC, Desai SM, Schlauder GG, Dawson GJ, Mushahwar IK: A hepatitis E virus variant from the United States: molecular characterization and transmission in cynomolgus macaques. J Gen Virol 1999;80:681–690.
35 Halbur PG, Kasorndorkbua C, Gilbert C, Guenette D, Potters MB, Purcell RH, Emerson SU, Meng XJ: Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. J Clin Microbiol 2001;39:918–923.
36 Okamoto H, Takahashi M, Nishizawa T, Fukai K, Muramatsu U, Yoshikawa A: Analysis of the complete genome of indigenous swine hepatitis E virus isolated in Japan. Biochem Biophys Res Commun 2001;289:929–936.
37 Nishizawa T, Takahashi M, Mizuo H, Miyajima H, Gotanda Y, Okamoto H: Characterization of Japanese swine and human hepatitis E virus isolates of genotype IV with 99% identity over the entire genome. J Gen Virol 2003;84:1245–1251.
38 Tei S, Kitajima N, Takahashi K, Mishiro S: Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 2003;362:371–373.
39 Yazaki Y, Mizuo H, Takahashi M, Nishizawa T, Sasaki N, Gotanda Y, Okamoto H: Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. J Gen Virol 2003;84:2351–2357.
40 Matsuda H, Okada K, Takahashi K, Mishiro S: Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. J Infect Dis 2003;188:944.
41 Tamada Y, Yano K, Yatsuhashi H, Inoue O, Mawatari F, Ishibashi H: Consumption of wild boar linked to cases of hepatitis E. J Hepatol 2004;40:869–870.
42 Chauhan A, Jameel S, Dilawari JB, Chawla YK, Kaur U, Ganguly NK: Hepatitis E virus transmission to a volunteer. Lancet 1993;341:149–150.
43 Mitsui T, Tsukamoto Y, Suzuki S, Yamazaki C, Masuko K, Tsuda F, Takahashi M, Tsatsralt-Od B, Nishizawa T, Okamoto H: Serological and molecular studies on subclinical hepatitis E virus infection using periodic serum samples obtained from healthy individuals. J Med Virol 2005;76:526–533.
44 Ding X, Li TC, Hayashi S, Masaki N, Tran TTH, Hirano M, Yamaguchi M, Usui M, Takeda N, Abe K: Present state of hepatitis E virus epidemiology in Tokyo, Japan. Hepatol Res 2003;27:169–173.
45 Tanaka E, Matsumoto A, Takeda N, Li TC, Umemura T, Yoshizawa K, Miyakawa Y, Miyamura T, Kiyosawa K: Age specific antibody to hepatitis E virus has remained constant during the past 20 years in Japan. J Viral Hepat 2005;12:439–442.