Table 1 Session themes of society meetings related to venous thromboembolism

Japanese Society for Vascular Surgery								
	1999	2000	2001	2002	2003	2004	2005	2006
Symposium	–	–	Etiology, treatment, and long-term result of deep vein thrombosis	–	–	–	–	–
Pannel Discussion	–	–	–	–	–	Deep vein thrombosis: Thorough discussion of diagnosis and treatment	–	–
Workshop	–	–	–	–	–	–	–	–
Consensus Meetng	–	–	–	–	–	–	–	–
President Demand	–	–	–	–	Treatment of pulmonary thromboembolism 1	–	–	Inspection and problems of the guideline for venous thromboembolism
President Demand	–	–	–	–	Treatment of pulmonary thromboembolism 2	–	–	–

Japanese Society for Cardiovascular Surgery								
	1999	2000	2001	2002	2003	2004	2005	2006
Symposium	–	–	–	–	–	–	–	–
Pannel Discussion	–	–	–	–	–	–	–	–
Workshop	–	–	–	–	–	–	–	–
Forum	–	–	–	–	–	–	–	–
President Demand	–	–	–	–	–	–	Guidelines for treatment of venous thromboembolism	–

Japanese Association for Thoracic Surgery								
	1999	2000	2001	2002	2003	2004	2005	2006
Symposium	–	–	–	–	–	–	–	–
Pannel Discussion	–	–	–	–	–	–	–	–
Workshop	–	–	–	–	–	–	–	–
Forum	–	–	–	–	–	–	–	–

体制の整った性格の医療機関であると患者から期待されて(患者と病院の)診療契約が締結された」と認定する．そして，それらを前提としたうえで，学会発表や追試報告がなされた時期まで遡り「医療水準」の確定時期とする．有名な平成7年最高裁判決(未熟児網膜症に対する光凝固法に関する事例)は，このような判断基準によって判決が下された[3]．この瞬間，医学の改善のための良心的な「努力目標」が，司法により「努力義務」に変換させられてしまったのである．だが司法は鑑定を通して医療側に「医療水準」の明確化を求め続けてきた．さらに司法は「司法判断に利用されることを意識した医学報告」を求めている[4]．医療側のメッセージが司法に届かないのは，医師が誤ったメッセージを司法に与え続けてきたからにほかならない[5,6]．だから鑑定医は，専門家(「専門医」ではない)としてできるだけ客観的データに基づき，その分野のより多くの医師共通の判断を，司法に返さなければならない．セッション・テーマの解析は，この手続きとして有用である．

3．ガイドラインと学会

2004年の日本循環器病学会ガイドラインは学会が責任をもって推奨したものではない[2]．あくまで情報提供に過ぎない．さらに，このガイドラインは，作成された当初から血栓症のスクリーニングが組み込まれていないという大きな欠陥が指摘されていた[7]．これらの点から，このガイドラインの内容がその当時の医療水準を表していないことは明らかである．

だが司法は，ガイドラインをそのようには見ていない．司法が判断の拠り所とする判例集に，診療ガイドラインとは「EBMに基づいて作成された」ものとの記述がある[3]．司法はガイドラインの定義として，財団法人日本医療機能評価機構が公表する医療情報サービス(Minds)を参考にする．Mindsは一般の人々に向けて，平成16年からネット上で公開されている(http://minds.jcqhc.or.jp)．ここでは診療ガイドラインを，「医学的な情報や専門医の助言をまとめた文書」であり，そして「臨床医や患者が，適切な判断や決断を下せるように支援する目的で体系的に作成された文書」とする(平成19年6月21日現在)．前述の「ガイドライン作成・活用ガイド」に基づけば，第3段階の完成されたガイドラインを指している．厚生労働科学研究費補助金を受けてネット上に医療情報サービスを公開するMindsは，ガイドラインについての認識がガイドラインを作成する医師や学会と全く異なる．この点について，学会が対応をとる必要があると考える．

4．セッション・テーマ解析の意義

セッション・テーマ解析は，ガイドラインの問題点を明確にする上で有用である．セッション・テーマ解析の結果から，日本胸部外科学会と日本心臓血管外科学会の両学会は静脈疾患をテーマとしておらず，その知識を会員間で共有し蓄積しているとはいえない．一方，2004年の日本循環器学会「肺血栓塞栓症および深部静脈血栓症の診断・治療・予防に関するガイドライン」の合同研究班参加学会には，日本胸部外科学会と日本心臓血管外科学会の名前があるが，日本血管外科学会の名前はない．学会は会員のコンセンサスを得て運営される．そして一般市民から見たとき，合同研究班に参加した学会は，そのガイドラインに責任をもつもの，そして，会員が遵守することを表明したものと受け止められる．そのことを承知しておかねばならない．日本血管外科学会として，関係するガイドラインの定義を今一度明確にするとともに，会員のコンセンサスを得られたガイドラインにのみ，日本血管外科学会の名前を連ねる方針を今後とも堅持することが望ましい．

最後に

セッション・テーマの変遷を検討することにより，会員が了解する当時の医療水準や，学会の時代的変遷が明らかとなる．今後セッションごとに進歩状況，問題点と工夫，中止理由などが記録されまとめられていけば，会員のコンセンサスに基づくより良い学会運営に結びつくものと信じる．

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文　献

1) 中山健夫：EBMを用いた診療ガイドライン作成・活用ガイド，東京，2004，金原出版．
2) 安藤太三(班長)：肺血栓塞栓症および深部静脈血栓症の診断・治療・予防に関するガイドライン．日本循環器学誌，**68**：979-1134，2004．
3) 宇津木伸，町野　朔，平林勝政，他：医事法判例百

選．別冊ジュリスト，No. 183：142-144，2006，有斐閣．
4) 福田剛久，高瀬浩造：医療訴訟と専門情報，東京，2004，判例タイムズ社．
5) 川崎富夫：民事訴訟における公的医療鑑定は何のために行われるのか．ジュリスト，No. 1327：2-6，2007，有斐閣．
6) 川崎富夫：医療紛争にみられる「認識の相違」はなぜ解消されないのか．Law & Technology，37：29-37，2007，民事法研究会．
7) 川崎富夫：日本の現状に即した新しい発想の肺塞栓症予防戦略―大阪大学の試み―．日産婦会誌，57：1530-1537，2005．

Standards of Medical Treatment Seen from the Standpoint of Changes in Academic Society Session Themes: Settlement of a Lawsuit Concerning Medical Treatment of Venous Thrombosis

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Key words: Accident, Medical, Cognitive impairment, Guideline, Session-theme, Message

Academic societies send various messages to their membership through society meetings. It is important what message the membership receives and how it is understood. The message to which the membership consents is documented in the activities of the society because important messages are repeatedly delivered until the membership consents. Accordingly, in regard to venous thromboembolism, we examined the main sessions of past general meetings from 1999 to 2006 of the three societies that comprise the Japanese Board of Cardiovascular Surgery and analyzed the theme (session theme) presented in each session. We applied this method specifically to officially accepted medical judgment. The lawsuit in question was an appeal hearing in which it was asked whether there was a violation of the standard of care in 1999 for prevention of pulmonary embolism by preventing deep vein thrombosis. The method of pulmonary embolism prevention in Japan has for a long time been diagnosis and treatment of deep vein thrombosis at the early stage, and I needed to clarify the situation in 1999 as an expert witness. The result of examination of session themes was that no pertinent session on prevention of pulmonary embolism was presented by the Japanese Association for Thoracic Surgery or the Japanese Society for Cardiovascular Surgery. However, since 2001, the Japanese Society for Vascular Surgery had presented five sessions. The method of preventing pulmonary embolism by prevention of deep vein thrombosis was first discussed in 2006 by the Japanese Society for Vascular Surgery. Thus, the medical treatment performed in this case did not violate the standard of care in 1999. And it has become clear that proposed session themes characterize the specialty and identity of the society. The "standard of medical treatment at the time," which the laws require to pass judgement, can be published from the specialists' standpoint because by tracing consensus, the "upper limits" of allowable medical treatment can be shown. From now on, if the consensus resulting from each session is recorded, a more detailed analysis could be made of the message to which the membership has consented.

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Inherited and *de novo* mutations of *ADAMTS13* in a patient with Upshaw-Schulman syndrome

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Upshaw-Schulman syndrome (USS) is a congenital thrombotic and hemorrhagic diathesis characterized by a deficient activity of plasma von Willebrand factor (VWF)-cleaving protease, ADAMTS13 [1–8]. Some patients with USS develop severe jaundice soon after birth as their first symptom, and some are almost asymptomatic during childhood to adolescence, unless they have precipitating factors such as infection or pregnancy. The pathological condition of USS belongs to thrombotic thrombocytopenic purpura (TTP), which is characterized by thrombocytopenia, hemolytic anemia and microvascular thrombosis. USS, therefore, is also called congenital TTP. Most cases of TTP result from acquired deficient activity of ADAMTS13 caused by the advent of autoantibodies that inhibit the ADAMTS13 activity. In contrast, patients with USS do not carry such inhibitory antibodies in their plasma, and suffer from USS because of the compound heterozygous or homozygous mutations of the *ADAMTS13* gene. More than 80 mutations of *ADAMTS13* have been identified in patients with USS. Here, we report a first case with *de novo* mutations of *ADAMTS13*.

Patient *P* (II-2 in Fig. 1A) is the second child of unrelated Japanese parents (I-1 and I-2). The first child (II-1) was spontaneously aborted from an unknown cause at 6 weeks of pregnancy, while the fourth child (II-4) was aborted by umbilical coiling at 22 weeks. The parents and the third child (II-3) are apparently healthy. The patient showed moderate hyperbilirubinemia soon after birth and received phototherapy without exchange blood transfusion. On the occasion of catching cold at 3 years of age, he developed thrombocytopenia, microangiopathic hemolytic anemia, and renal insufficiency. He was diagnosed as having TTP and treated with fresh frozen plasma (FFP) infusions. Thereafter, he repeated these episodes several times a year, and each time he soon recovered with FFP infusions. At 21 years of age, he developed a hallmark of TTP, consisting of thrombocytopenia, microangiopathic hemolytic anemia, neurological dysfunction, renal failure and fever. He started taking prophylactic infusions of FFP (4–8 ml kg^{-1} body weight) every 2 weeks.

The plasma ADAMTS13 activities of the family members, measured by the method based on VWF-multimer analysis [1,9], are shown under each symbol in Fig. 1A. The ADAMTS13 activity of the patient was less than 3% of that of the control, which was confirmed by measuring his plasma collected after an interval of more than 1 month. The values were consistent with the data obtained by two other methods, a fluorogenic assay [10] and a chromogenic assay [11] (data not shown). As the assay of the ADAMTS13-activity inhibitors [1] showed no detectable inhibitors in the patient plasma (data not shown), the etiology of his TTP symptoms was considered a genetic deficiency of ADAMTS13, that is, USS. To clarify the underlying cause of the TTP crisis, we analyzed the nucleotide sequences of the family's *ADAMTS13* genes.

DNA experiments were carried out with the permission of the ethics committees of the National Cardiovascular Center after obtaining informed consent from the study subjects. The nucleotide sequences of all 29 exons of *ADAMTS13*, including the intron-exon boundaries, were determined by direct sequencing of polymerase chain reaction (PCR) products as described previously [12,13].

The patient was heterozygous for five nucleotide mutations, c.964T > G, c.968C > G, c.969C > A, c.970T > C and c.2723G > A. Of them, c.2723G > A was also detected in the father. The mother and sister were heterozygous for c.2708C > T, which was not found in the patient or his father (Fig. 1B).

The c.2723G > A mutation on exon 21 causing C908Y, heterozygously found in the patient and father, was previously reported by us as a causative mutation in another USS family [14]. This mutation causes the impaired secretion of ADAMTS13 [14]. The moderately decreased ADAMTS13 activity of the father in the present case could be explained by this single mutation. The c.2708C > T mutation on exon 21 causing S903L was heterozygously found in the mother and sister, whose plasma ADAMTS13 activities were normal. This suggested that S903L should not affect the ADAMTS13 activity. In fact, the allele frequency of S903L is 6.0% in the Japanese general population (our unpublished data), making it a common polymorphism.

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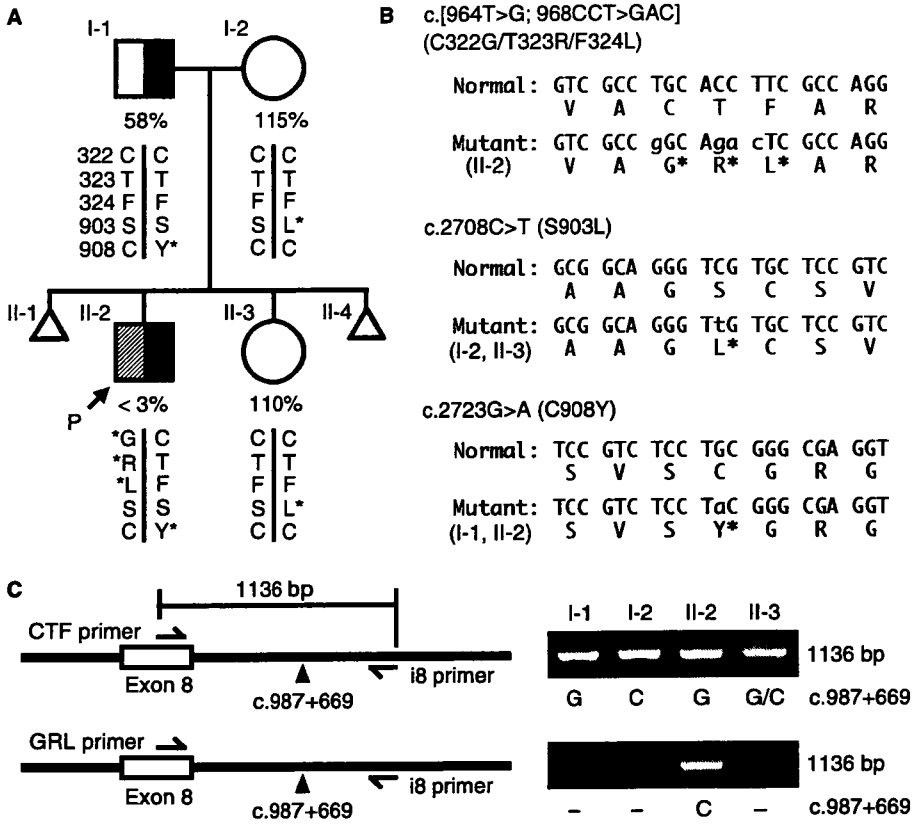

Fig. 1. *ADAMTS13* mutations in the USS patient *P* family. (A) Pedigree of the patient family. Plasma ADAMTS13 activities are shown under each symbol. The haplotype patterns of the five amino-acid residues deduced from each *ADAMTS13* gene are indicated under the ADAMTS13 activities. The arrow indicates the proband. (B) Missense mutations of *ADAMTS13* identified in this family. S903L is a common polymorphism. Asterisks indicate the mutated amino-acid residues. (C) PCR analysis of normal and mutant alleles. The 1,136-bp region was amplified by PCR using either the normal allele-specific CTF primer (5′-CCAAGGCTGTCGCCTGCACCT-3′) or the mutant allele-specific GRL primer (5′-CCAAGGCTGTCGCCGGCAGAC-3′) on exon 8 with the common reverse i8 primer (5′-TGAAGCCAGGAGTCCTAGACA-3′) on intron 8. This region contained a single nucleotide G/C polymorphism at the site of c.987 + 669. Subjects I-1 (father) and I-2 (mother) were homozygotes for G and C, respectively. The normal and mutant alleles of II-2 (patient) carried G and C, respectively.

The four mutations on exon 8, c.964T > G, c.968C > G, c.969C > A and c.970T > C, were detected only in the patient, suggesting that they should be *de novo* mutations in the patient's *ADAMTS13*. All the four mutations were excluded as common polymorphisms by the screening of 346 individuals in the Japanese general population. Cloning and sequencing of the genomic PCR products including exon 8 revealed that all the mutations were located on a single allele. Therefore, they could be described as c.[964T > G; 968CCT > GAC], resulting in three contiguous missense changes C322G/T323R/F324L within the disintegrin-like domain of ADAMTS13. The C322G mutation may disrupt a tertiary structure of the protein because of the defect in disulfide bond formation.

To determine the origin of the freshly mutated allele of the patient, a longer region including exon 8 was amplified by PCR using a combination of either the normal allele-specific CTF primer or the mutant allele-specific GRL primer and the common reverse i8 primer (Fig. 1C). The combinatorial use of CTF and i8 primers produced a 1,136-bp fragment from genomic DNAs of all the family members, whereas the use of GRL and i8 primers produced a 1,136-bp fragment only from the patient, as expected. The region contained a single nucleotide G/C polymorphism at the site of c.987 + 669. Sequencing of the fragments suggested that the father and mother were homozygotes for G and C, respectively, and that the sister was heterozygous. The normal and mutant alleles of the patient carried G and C, respectively. These results suggested that the mutant allele of the patient was derived from one of the maternal alleles. Based on all of the data, we concluded that the patient was a compound heterozygote of paternally transmitted C908Y and freshly mutated C322G/T323R/F324L on the maternal allele.

In conclusion, this is the first report of a case of compound heterozygosity of inherited and *de novo ADAMTS13* mutations resulting in USS.

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Disclosure of Conflict of Interests

The authors have no conflict of interest.

References

1 Kinoshita S, Yoshioka A, Park YD, Ishizashi H, Konno M, Funato M, Matsui T, Titani K, Yagi H, Matsumoto M, Fujimura Y. Upshaw-Schulman syndrome revisited: a concept of congenital thrombotic thrombocytopenic purpura. *Int J Hematol* 2001; **74**: 101–8.

2 Levy GG, Nichols WC, Lian EC, Foroud T, McClintick JN, McGee BM, Yang AY, Siemieniak DR, Stark KR, Gruppo R, Sarode R, Shurin SB, Chandrasekaran V, Stabler SP, Sabio H, Bouhassira EE, Upshaw JD Jr, Ginsburg D, Tsai HM. Mutations in a member of the *ADAMTS* gene family cause thrombotic thrombocytopenic purpura. *Nature* 2001; **413**: 488–94.

3 Fujimura Y, Matsumoto M, Yagi H, Yoshioka A, Matsui T, Titani K. Von Willebrand factor-cleaving protease and Upshaw-Schulman syndrome. *Int J Hematol* 2002; **75**: 25–34.

4 Kremer Hovinga JA, Studt JD, Lämmle B. The von Willebrand factor-cleaving protease (ADAMTS-13) and the diagnosis of thrombotic thrombocytopenic purpura (TTP). *Pathophysiol Haemost Thromb* 2003; **33**: 417–21.

5 Matsumoto M, Yagi H, Ishizashi H, Wada H, Fujimura Y. The Japanese experience with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. *Semin Hematol* 2004; **41**: 68–74.

6 Sadler JE, Moake JL, Miyata T, George JN. Recent advances in thrombotic thrombocytopenic purpura. *Hematology Am Soc Hematol Educ Program* 2004; 407–23.

7 Lämmle B, Kremer Hovinga JA, Alberio L. Thrombotic thrombocytopenic purpura. *J Thromb Haemost* 2005; **3**: 1663–75.

8 Miyata T, Kokame K, Banno F, Shin Y, Akiyama M. ADAMTS13 assays and ADAMTS13-deficient mice. *Curr Opin Hematol* 2007; **14**: 277–83.

9 Furlan M, Robles R, Lämmle B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood* 1996; **87**: 4223–34.

10 Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *Br J Haematol* 2005; **129**: 93–100.

11 Kato S, Matsumoto M, Matsuyama T, Isonishi A, Hiura H, Fujimura Y. Novel monoclonal antibody-based enzyme immunoassay for determining plasma levels of ADAMTS13 activity. *Transfusion* 2006; **46**: 1444–52.

12 Kokame K, Matsumoto M, Soejima K, Yagi H, Ishizashi H, Funato M, Tamai H, Konno M, Kamide K, Kawano Y, Miyata T, Fujimura Y. Mutations and common polymorphisms in *ADAMTS13* gene responsible for von Willebrand factor-cleaving protease activity. *Proc Natl Acad Sci U S A* 2002; **99**: 11902–7.

13 Kokame K, Miyata T. Genetic defects leading to hereditary thrombotic thrombocytopenic purpura. *Semin Hematol* 2004; **41**: 34–40.

14 Matsumoto M, Kokame K, Soejima K, Miura M, Hayashi S, Fujii Y, Iwai A, Ito E, Tsuji Y, Takeda-Shitaka M, Iwadate M, Umeyama H, Yagi H, Ishizashi H, Banno F, Nakagaki T, Miyata T, Fujimura Y. Molecular characterization of *ADAMTS13* gene mutations in Japanese patients with Upshaw-Schulman syndrome. *Blood* 2004; **103**: 1305–10.

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CLINICAL RESEARCH

Determinants of thrombin generation, fibrinolytic activity, and endothelial dysfunction in dual-antiplatelet therapy: involvement of factors other than platelet aggregability in Virchow's triad

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Aims The aim of the study was to assess mechanisms and clinical backgrounds in order to determine residual platelet aggregability in dual-antiplatelet therapy and to ascertain whether platelet aggregability is involved in systemic thrombogenicity.

Methods and results A cross-sectional study was conducted in 85 consecutive patients who underwent dual-antiplatelet therapy (aspirin and thienopyridine/cilostazol) after percutaneous coronary intervention (PCI). Although serum thromboxane B_2 and dephosphorylation of vasodilator-stimulated phosphoprotein were significantly abolished, the platelet aggregation tests showed inter-individual differences that could be partly explained by plasma glucose levels. Platelet aggregability was not related to other factors involved in thrombogenicity. Thrombin generation assessed by soluble fibrin was independently associated with total cholesterol ($\beta = 0.349$, $P < 0.001$), brain natriuretic peptide ($\beta = 0.222$, $P = 0.018$), and ankle-brachial index ($\beta = -0.330$, $P = 0.001$). Plasminogen activator inhibitor-1 was associated with the apnea–hypopnea index ($\beta = 0.300$, $P = 0.006$). E-selectin was correlated with diabetes mellitus ($\beta = 0.279$, $P = 0.008$) and body mass index ($\beta = 0.323$, $P = 0.002$).

Conclusion Although dual-antiplatelet therapy effectively inhibited its pharmacological targets, thrombin generation, inhibition of fibrinolytic activity, and endothelial dysfunction were determined by other clinical backgrounds. Our data suggested that some patients remain at risk of thrombotic complications after PCI and that these may benefit from anticoagulant treatment despite adequate dual-antiplatelet therapy.

Keywords Percutaneous coronary intervention • Aspirin • Thienopyridine • Antiplatelet drug resistance • Thrombin generation

Introduction

Platelet aggregation plays a central role in the development of thrombotic complications after percutaneous coronary intervention (PCI).[1–3] The role of aspirin in secondary prevention of ischaemic cardiovascular diseases is universally accepted. Furthermore, dual-antiplatelet therapy of aspirin combined with thienopyridine (and/or cilostazol) including clopidogrel or ticlopidine is

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the gold standard for preventing major cardiovascular events in patients undergoing PCI, especially since the beginning of the balloon-expandable stent era.[4–6] In contrast, nearly 20% of patients continue to have further cardiovascular events after PCI, despite the superior protection conferred by dual-antiplatelet therapy, as shown in a number of clinical trials.[7]

The mechanism by which antiplatelet therapy fails in certain patients after PCI, in part, thought to be attributed to the fact that some individuals have impaired antiplatelet responses, is referred to as 'aspirin resistance' or 'clopidogrel resistance'.[8–10] There is evidence that not all patients respond comparably to antiplatelet drugs, as evaluated by non-specific laboratory test such as aggregometry, and hence the concept of drug 'resistance' has arisen.[11–14] However, recent evidence suggest that when the definition of resistance is limited to situations in which the drugs fail to hit their pharmacological targets, resistance against antiplatelet drug appears to be rare.[15–18] Many published studies of antiplatelet resistance have been carried out using nonspecific platelet aggregation tests, which merely identify patients on antiplatelet therapy with high residual platelet activation.[7,18] Despite this drawback, identification of patients with high residual platelet reactivity may be useful for predicting individuals risks of atherothrombotic events.[7–10,13,17]

The results of clinical trials on the use of anticoagulant agents and the involvement of fibrin fibrils and inflammatory cells in the formation of occlusive thrombi suggest that not only platelets but also the coagulation cascade, fibrinolytic system, inflammation, and endothelial dysfunction may orchestrate *in vivo* thrombus formation, thereby leading to clinical treatment failure under dual-antiplatelet therapy.[19–21] Indeed, the clinical outcomes of patients undergoing PCI were reported to be associated with the levels of D-dimer, plasminogen activator inhibitor-1 (PAI-1), E-selectin, and markers for thrombin generation.[22–25] However, there is no sufficient data that correlate heightened platelet reactivity during dual-antiplatelet therapy with other markers for coagulation, fibrinolysis, and endothelial dysfunction. The aims of the present study were to assess the various clinical backgrounds associated with high residual platelet aggregability under dual-antiplatelet therapy and to clarify any association with thrombin generation, fibrinolytic activity, and endothelial dysfunction that might lead to clinical failure against antiplatelet therapy.

Methods

Patients and study protocol

The institutional review board at the Jichi Medical University approved the study protocols, and written informed consent was obtained from all participants. We enrolled consecutive hospitalized patients from July 2006 to April 2007 who were treated by PCI because of symptomatic coronary artery disease, including unstable angina, and non-ST-elevation or ST-elevation myocardial infarction. We estimated the sample size required using a general formula for the correlation coefficient.[26] We set $\alpha = 0.05$, $\beta = 0.20$, and expected a correlation coefficient, $r = 0.30–0.35$. Using the formula, at least 62–85 participants would be required for the study. All patients had taken dual-antiplatelet therapy, consisting of 100 mg/day of aspirin and 200 mg/day of ticlopidine, 75 mg/day of clopidogrel, or 200 mg/day of cilostazol (*Table 1*). The exclusion criteria were as follows: acute coronary syndrome within 10 days; New York Heart Association Class III or IV heart failure; ingestion of other drugs affecting platelet function or coagulation; platelet counts of $<10\times10^7$ or $>40\times10^7$ ml^{-1}; myeloproliferative disorders; autoimmune diseases; malignant diseases; and atrial fibrillation. Compliance with antiplatelet drugs was determined by nursing staff during hospitalization. After normalization of cardiac enzymes (just before discharge), patients underwent blood sampling, ambulatory blood pressure monitoring (ABPM; TM-2425; A&D Co., Inc., Tokyo, Japan), ankle-brachial index (ABI) monitoring (FORM/ABI; Colin Co. Ltd., Ehime, Japan), and cardiorespiratory monitoring (Somte; Compumedics, Melbourne, Australia).

Table 1 Characteristics of the study population

Variables	Total subjects ($n = 85$)
Age (years)	60.0 ± 13.1
Men, *n* (%)	70 (82)
Body mass index (kg/m^2)	24.3 ± 3.3
Current smoker, *n* (%)	50 (59)
Family history of coronary artery disease, *n* (%)	26 (31)
Hypertension, *n* (%)	59 (69)
Diabetes mellitus, *n* (%)	36 (42)
Dyslipidemia, *n* (%)	75 (88)
Prior myocardial infarction, *n* (%)	10 (12)
Presenting symptoms, *n* (%)	
Unstable angina	22 (26)
Myocardial infarction	63 (74)
Coronary artery disease, *n* (%)	
One-vessel disease	41 (48)
Two-vessel disease	26 (31)
Three-vessel disease	18 (21)
Concomitant medications	
Antiplatelet agents, *n* (%)	
Aspirin	85 (100)
Ticlopidine	72 (85)
Clopidogrel	3 (4)
Cilostazol	10 (12)
Antihypertensive medication, *n* (%)	
Beta blocker	51 (60)
Angiotensin-converting enzyme inhibitor	39 (46)
Angiotensin II receptor blocker	32 (38)
Calcium channel blocker	17 (20)
Diuretic	14 (16)
Nitrate, *n* (%)	5 (6)
Statin, *n* (%)	66 (78)
Proton pump inhibitor, *n* (%)	1 (1)
Non-steroidal anti-inflammatory drug, *n* (%)	0 (0)

Data for continuous variables are expressed as the mean ± SD.

To assess the effects of antiplatelet therapy, 20 healthy individuals who were not taking any antiplatelet drugs were enrolled as controls.

Platelet aggregation

A fasting venous sample was carefully collected via a 21-gauge needle into a syringe containing 1/10 volume of sodium citrate between 07:30 and 08:00 h. Platelet-rich plasma (PRP) was obtained by centrifuging whole blood at 200 *g* for 12 min. The time from blood collection to measurement was standardized to 1 h. The aggregation response was measured based on the light scattering intensities obtained with a PA-200 Platelet Aggregation Analyzer (Kowa Co. Ltd., Tokyo, Japan).[27] This device is particularly sensitive for detecting the sizes of small platelet aggregates.[27,28] Platelet aggregation was performed without any agonists, or with collagen (Hormon-Chemie, Munich, Germany), adenosine diphosphate (ADP) (MC Medical Co., Tokyo, Japan), and thrombin receptor-activating peptide (TRAP; Invitrogen Co., Carlsbad, CA, USA), a specific agonist for protease-activating receptor-1. Spontaneous small platelet aggregation was defined by small aggregate formation by stirring without agonist.

Phosphorylation of vasodilator-stimulated phosphoprotein in platelets

Phosphorylation of vasodilator-stimulated phosphoprotein (VASP) is regulated by the cAMP level, which is thus believed to be a marker of $P2Y_{12}$ receptor reactivity.[29] To determine the VASP phosphorylation state of whole blood, we used a standardized flow cytometric assay (PLT VASP/P2Y12; Biocytex, Marseille, France) with some modifications. We found that the commercially available VASP phosphorylation assay appeared to contain an extremely high concentration of ADP. In our protocol, cAMP elevation by 1 μM PGI_2 increased the VASP phosphorylation level by stimulation of adenylate cyclase. When simultaneously stimulated with 2 μM ADP, the signaling from G_i activation mediated via $P2Y_{12}$ reduced the phosphorylation of VASP induced by PGI_2. However, when the $P2Y_{12}$ receptor was successfully inhibited by active metabolites of thienopyridines, or phosphodiesterase that was inhibited by cilostazol, ADP was unable to reduce PGI_2-induced VASP phosphorylation. The phosphorylation of VASP was quantified by flow cytometry according to the manufacturer's instructions. The reduction of VASP phosphorylation induced by ADP was expressed as the % of PGI_2; the mean fluorescence intensity of PGI_2 plus ADP was devided by that of PGI_2.

Laboratory testing, ambulatory blood pressure monitoring, ankle-brachial index, and cardiorespiratory monitoring

Methods are described in detail in the supplementary materials. The intraassay and interassay coefficients of laboratory tests were all <10%. The data obtained from patients are shown in *Table 2*.

Statistical analysis

All statistical analyses were performed with SPSS version 11 software (SPSS, Inc., Chicago, IL, USA). The Mann–Whitney *U*-test was used to compare measurements of platelet activation between patients and healthy volunteers. The associations between the individual parameters were calculated using Spearman's correlation method. To identify independent factors, we used a step-wise multivariable linear regression analysis in which a *P*-value of 0.05 or less in a simple regression analysis was used as the criterion for entry into the model. We validated independent explanatory variables by Mann–Whitney *U*-test after categorization into two groups. All reported *P*-values are two-sided; a *P*-value of less than 0.05 was considered to be statistically significant.

Table 2 Physiological and biochemical characteristics of the study population

Biochemical markers	
White blood cells ($\times 1000\ mm^{-3}$)	7.1 ± 1.8
Haemoglobin (g/dL)	13.7 ± 1.8
Platelets ($\times 1000\ mm^{-3}$)	306.7 ± 84.0
Fasting glucose (mg/dL)	116.2 ± 46.7
Total cholesterol (mg/dL)	167.9 ± 35.6
Triglycerides (mg/dL)	130.9 ± 52.6
High-density lipoprotein cholesterol (mg/dL)	41.3 ± 11.8
Low-density lipoprotein cholesterol (mg/dL)	100.3 ± 29.5
Adrenalin (pg/mL)	31.1 ± 21.9
hsCRP (mg/L)	5.69 ± 7.60
Brain natriuretic peptide (pg/mL)	145.7 ± 174.7
PAI-1 (ng/mL)	56.6 ± 20.2
E-selectin (ng/mL)	20.4 ± 10.1
D-dimer (μg/mL)	1.8 ± 2.5
Soluble fibrin (μg/mL)	4.3 ± 7.5
Physiological markers	
24-h SBP (mmHg)	120.0 ± 14.5
24-h DBP (mmHg)	72.6 ± 9.1
24-h HR (b.p.m.)	68.2 ± 11.1
AHI ≥ 5/h, *n* (%)	75 (88)
AHI ≥ 15/h, *n* (%)	50 (59)
ABI	1.08 ± 0.123

Data for continuous variables are expressed as the mean ± SD.
hsCRP, high-sensitivity C-reactive protein; PAI-1, plasminogen activator inhibitor-1; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; AHI, apnea–hypopnea index; ABI, ankle-brachial index.

Results

Patients

Of the 94 patients recruited, two were not included because of advanced gastric cancer or spastic angina, and three did not take dual-antiplatelet drugs at the time blood was collected. An additional four patients were excluded from the analysis because of incomplete blood collection or failure of polysomnography or ABPM. Thus, 85 patients were finally included in the analysis (*Table 1*).

Dual-antiplatelet therapy effectively inhibits its pharmacological targets

To precisely assess the effects of aspirin, we measured serum thromboxane B_2 (TxB_2) concentration, which reflects platelet-cyclooxygenase (COX)-dependent TxA_2 production. As has been described,[15,17] the serum TxB_2 concentration was uniformly abolished in all patients compared with control patients (*Figure 1A*). We also simultaneously evaluated VASP dephosphorylation after ADP stimulation, which reflects Gi-dependent cAMP reduction. As shown in *Figure 1B*, cAMP reduction by ADP was effectively

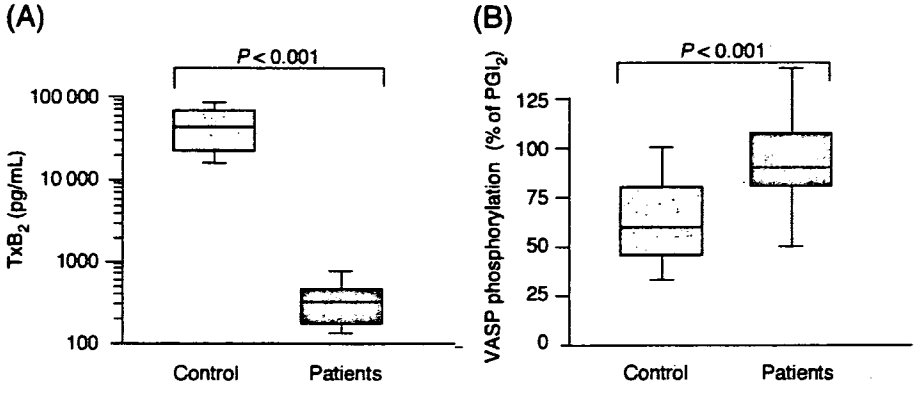

Figure 1 Serum thromboxane B_2 (TxB_2) concentration and vasodilator-stimulated phosphoprotein index in patients taking dual-antiplatelet therapy. (*A*) The serum concentration of TxB_2 was measured by EIA. (*B*) The vasodilator-stimulated phosphoprotein phosphorylation was assessed by flow cytometry. ADP-induced vasodilator-stimulated phosphoprotein dephosphorylation was expressed as % of PGI_2. Data are expressed as box-and-whisker plots.

inhibited by dual-antiplatelet therapy. These data suggested that dual-antiplatelet therapy efficiently inhibits its pharmacological targets in patients undergoing PCI.

Inter-individual differences in platelet reactivity under dual-antiplatelet therapy

Next, we examined the effects of dual-antiplatelet therapy on platelet aggregation patterns using an aggregometry method that simultaneously measures both light transmission and light scattering. Although platelet aggregation assessed by light transmission was significantly decreased in the patients, the results of platelet aggregation tests induced by different agonists showed some inter-individual differences compared with serum TxB_2 and VASP phosphorylation (*Figure 2A*). We compared the changes of VASP phosphorylation and all platelet aggregations in the cilostazol group ($n = 10$) with those in the thienopyridine group ($n = 75$). We did not find any significant differences in platelet activation status, suggesting that drug differences could not explain the heterogeneity of platelet aggregation. Use of a laser-light scattering method to quantitatively evaluate the aggregate sizes and numbers revealed that the number of small aggregates increased after stimulation with all agonists, except for the lower concentration of ADP (*Figure 2B*). The inhibition of medium and large aggregates was clearer for low-dose agonist stimulation (data not shown), indicating that the platelet reactivity generating large platelet aggregates from small aggregates after agonist stimulation was highly concentration-dependent. Furthermore, the degrees of platelet aggregation induced by different agonists within a given subject significantly correlated with each other (*Table 3*). The number of small platelet aggregates spontaneously formed without agonist stimulation was significantly correlated with the collagen-induced platelet aggregation assessed by light transmission ($R = 0.398$, $P < 0.001$). We also found that small aggregate formation induced by a lower dose of agonist (1 μg/mL of collagen or 2 μM ADP) strongly correlated with light transmission induced by all higher concentrations of agonist ($R = 0.563$–0.815, $P < 0.001$). These data suggested that platelet aggregability under dual-antiplatelet therapy may be determined by differences in the thresholds of each patient's platelets, rather than by differences in antiplatelet drug efficacies.

As activated platelets offer the scaffold of a coagulation cascade in arterial thrombus formation, we supposed that residual platelet activation under dual-antiplatelet therapy may be involved in a systemic thrombin generation. To determine whether *in vitro* platelet aggregation is related to blood thrombogenicity, we compared the results of platelet aggregation tests with the plasma levels of SF (a marker for thrombin generation), D-dimer (a marker for fibrinolysis), PAI-1 (an inhibitor of fibrinolysis), and E-selectin (a marker for endothelial dysfunction). None of these variables was associated with the results of platelet aggregation (*Table 3*). Next, we attempted to determine factors influencing platelet aggregability by comparing the clinical backgrounds and other laboratory tests. Interestingly, we found that only the fasting glucose level was significantly correlated with the number of spontaneously formed small platelet aggregates and collagen-induced platelet aggregates ($R = 0.498$, $P < 0.001$ and $R = 0.243$, $P = 0.025$, respectively), regardless of the presence of diabetes mellitus (*Table 4*). Although many drugs including angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and statin can influence platelet activation and blood coagulation, the use of these drugs did not affect the results of platelet aggregation tests, or the levels of PAI-1, D-dimer, SF, or E-selectin (data not shown).

Determinants of thrombin generation, fibrinolytic activity, and endothelial dysfunction

Finally, we examined the clinical characteristics that determine thrombin generation, fibrinolytic activity, and endothelial dysfunction. SF was significantly correlated with total cholesterol, BNP, ABI, and the number of coronary vessels affected *Table 4*. By multivariable regression analysis including these significant covariates,

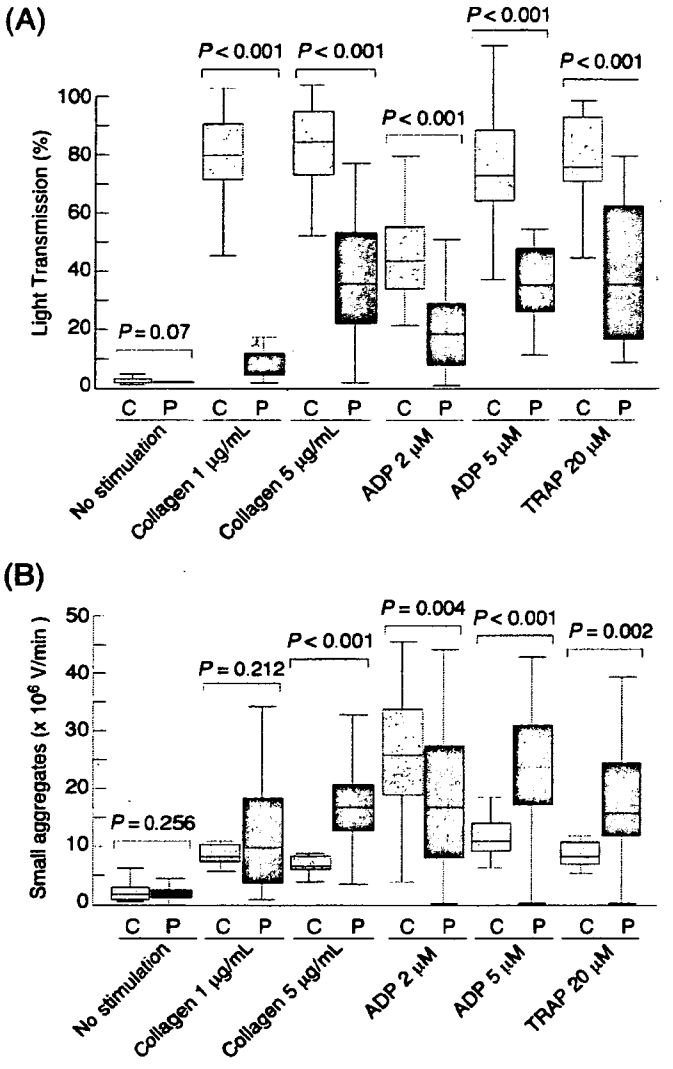

Figure 2 Platelet aggregation patterns in patients taking dual-antiplatelet therapy. Platelets in platelet-rich plasma obtained from control subjects (*C*) or patients taking dual-antiplatelet therapy (P) were stimulated with the indicated agonists for 5 min. (*A*) Changes in the maximum light transmission were monitored using conventional methods. (*B*) Light scattering intensities that represent small aggregate formation were measured simultaneously. Data are expressed as box-and-whisker plots.

total cholesterol, BNP, and ABI remained independently correlated with the SF level (*Table 5*). BNP was also an independent predictor of the D-dimer level in a multivariable regression analysis (*Table 5*). On the other hand, PAI-1 was significantly correlated with body mass index (BMI) and AHI *Table 4*. By multivariable analysis, only AHI remained independently correlated with the PAI-1 level (*Table 5*). E-selectin was significantly associated with age, BMI, diabetes mellitus, 24 h DBP, and AHI (*Table 4*). By multivariable regression analysis, BMI and diabetes mellitus remained independently correlated with the E-selectin level (*Table 5*). The significance of these explanatory variables was confirmed by Mann–Whitney *U*-test after categorization into two groups (see Supplementary material online, Figure S1). These results suggested that total thrombogenicity under antiplatelet therapy may be orchestrated by a variety of patient backgrounds that affect platelet reactivity, thrombin generation, fibrinolysis, and endothelial dysfunction.

Discussion

Activated platelets are critically involved in thrombotic complications after PCI and in acute coronary syndrome.[8–10] The issue of resistance to antiplatelet agents has been emphasized in the literature, leading to growing concern about the efficacy of antiplatelet therapy and about possible unfavorable clinical outcomes.[10–12] However, the term 'resistance' is frequently misleading when it refers to individuals who develop cardiovascular events despite antiplatelet therapy.[10–12] More accurately, we should properly

Table 3 Spearman's correlation coefficients among platelet aggregation (light transmission), PAI-1, D-dimer, SF, and E-selectin

	Collagen (1 μg/mL)	Collagen (5 μg/mL)	ADP (2 μg/ml)	ADP (5 μg/mL)	TRAP	PAI-1	D-dimer	Soluble fibrin
Collagen (1 μg/mL)	–	–	–	–	–	–	–	–
Collagen (5 μg/mL)	0.788 ($P < 0.001$)	–	–	–	–	–	–	–
ADP (2 μg/mL)	0.635 ($P < 0.001$)	0.853 ($P < 0.001$)	–	–	–	–	–	–
ADP (5 μg/mL)	0.608 ($P < 0.001$)	0.868 ($P < 0.001$)	0.931 ($P < 0.001$)	–	–	–	–	–
TRAP	0.417 ($P < 0.001$)	0.716 ($P < 0.001$)	0.646 ($P < 0.001$)	0.688 ($P < 0.001$)	–	–	–	–
PAI-1	−0.142 ($P = 0.194$)	−0.121 ($P = 0.271$)	−0.163 ($P = 0.137$)	−0.177 ($P = 0.105$)	−0.016 ($P = 0.884$)	–	–	–
D-dimer	0.050 ($P = 0.648$)	−0.007 ($P = 0.947$)	−0.007 ($P = 0.948$)	−0.047 ($P = 0.669$)	−0.177 ($P = 0.104$)	−0.169 ($P = 0.122$)	–	–
Soluble fibrin	0.100 ($P = 0.364$)	−0.046 ($P = 0.675$)	−0.022 ($P = 0.840$)	−0.021 ($P = 0.852$)	−0.170 ($P = 0.121$)	−0.061 ($P = 0.580$)	0.190 ($P = 0.082$)	–
E-selectin	−0.087 ($P = 0.427$)	−0.062 ($P = 0.572$)	−0.073 ($P = 0.506$)	−0.083 ($P = 0.049$)	0.031 ($P = 0.781$)	0.232 ($P = 0.032$)	0.042 ($P = 0.701$)	−0.105 ($P = 0.340$)

TRAP, thrombin receptor-activating peptide; PAI-1, plasminogen activator inhibitor-1.

distinguish patients who develop cardiovascular events despite antiplatelet therapy as 'treatment failure'.[30] From the viewpoint of Virchow's triad, arterial thrombosis may occur through complex interactions of a variety of components, including platelet activation, coagulation/fibrinolytic activity, endothelial dysfunction, and blood flow.[31,32]

On the basis of the results of our study, true antiplatelet drug resistance as defined by a specific test appears rare. This observation is consistent with recent studies, reporting that aspirin resistance other than non-compliance appears to be exceptional.[15–18,33] Although studies that used specific tests to measure the pharmacological effects of thienopyridines showed a wide variability in the responses to these drugs,[12] VASP dephosphorylation was significantly inhibited by dual-antiplatelet therapy, and was not associated with ADP-induced platelet aggregation (data not shown). This discrepancy may be because of differences in the concentrations of ADP used; the commercially available VASP phosphorylation kit appears to use a high concentration of ADP (see Materials and Methods). As well, it is possible that pharmacokinetic differences related to race exist in the metabolism of thienopyridine antiplatelet drugs.

Although antiplatelet resistance has been defined by *in vitro* platelet function, there appears a widespread misunderstanding that *in vitro* platelet function directly represents inhibition of a drug target.[30] Here, we found that platelet aggregation elicited by different agonists were significantly correlated with each other and associated with small aggregate formation without or with lower agonist stimulation. These data suggest that the platelet aggregability under dual-antiplatelet therapy may be determined by differences in the thresholds of each patient's platelets, rather than by differences in antiplatelet drug efficacies. Our finding is supported by recent reports that a 150 mg maintenance dose of clopidogrel is associated with enhanced antiplatelet effects compared with a 75 mg dose, although suboptimal responses were still present in 60% of patients.[34] Furthermore, Michelson *et al.*[35] reported that pre-existing variability in platelet responses to ADP accounts for clopidogrel resistance assessed by aggregometory.

We previously showed that an unknown factor, other than COX-1, determines inter-individual differences in platelet aggregation in aspirin-treated patients.[17] In this study, only fasting glucose level was significantly correlated with platelet aggregability, regardless of diabetes mellitus. Acute hyperglycemia during oral glucose tolerance tests was correlated with the number of small platelet aggregates.[28] Angiolillo *et al.*[34] reported that patients with hyperglycemia exhibit increased platelet reactivity, despite dual-antiplatelet therapy, that continues to persist even after administration of a higher maintenance dose of clopidogrel. These findings indicate the importance of suppressing transient hyperglycemia by tight glucose control to prevent thrombotic complications after PCI. Indeed, elevated plasma glucose, with or without a diabetic status, was reportedly an independent predictor of outcomes in acute coronary syndrome patients.[36,37]

Treatment failure under antiplatelet drug therapy may be influenced by many factors. The coagulation cascade and its regulation are important contributors to clinical events after PCI.[19–21] Activated platelets provide phosphatidylserine exposure on their

Table 4 Spearman's correlation coefficients between patient characteristics and thrombogenetic factors in patients taking dual-antiplatelet therapy

	Platelet aggregation[a]	PAI-1	E-selectin	D-dimer	Soluble fibrin
Patient characteristics					
Age (years)	0.038 ($P = 0.729$)	−0.051 ($P = 0.645$)	−0.241 ($P = 0.026$)*	0.167 ($P = 0.127$)	0.079 ($P = 0.472$)
BMI (kg/m^2)	−0.057 ($P = 0.804$)	0.234 ($P = 0.032$)*	0.310 ($P = 0.004$)**	−0.254 ($P = 0.020$)*	−0.163 ($P = 0.139$)
Hypertension	0.042 ($P = 0.452$)	0.181 ($P = 0.132$)	−0.166 ($P = 0.128$)	−0.016 ($P = 0.396$)	0.029 ($P = 0.794$)
Diabetes mellitus	0.063 ($P = 0.570$)	−0.150 ($P = 0.172$)	0.253 ($P = 0.019$)*	−0.009 ($P = 0.943$)	0.145 ($P = 0.186$)
Prior myocardial infarction	−0.106 ($P = 0.334$)	0.058 ($P = 0.598$)	0.131 ($P = 0.131$)	−0.273 ($P = 0.011$)*	−0.046 ($P = 0.579$)
Number of vessel diseases	0.089 ($P = 0.420$)	−0.006 ($P = 0.953$)	0.011 ($P = 0.918$)	−0.185 ($P = 0.073$)	0.248 ($P = 0.022$)*
Biochemical markers					
Fasting glucose	0.243 ($P = 0.025$)*	−0.119 ($P = 0.277$)	0.205 ($P = 0.059$)	0.090 ($P = 0.411$)	−0.097 ($P = 0.378$)
Total cholesterol	−0.010 ($P = 0.928$)	−0.125 ($P = 0.256$)	−0.154 ($P = 0.158$)	0.095 ($P = 0.387$)	0.426 ($P < 0.001$)***
BNP	0.146 ($P = 0.191$)	−0.071 ($P = 0.527$)	−0.177 ($P = 0.111$)	0.411 ($P < 0.001$)***	0.296 ($P = 0.005$)**
Adrenalin	0.099 ($P = 0.367$)	0.169 ($P = 0.122$)	0.022 ($P = 0.843$)	0.002 ($P = 0.969$)	0.148 ($P = 0.177$)
hsCRP	0.009 ($P = 0.932$)	0.115 ($P = 0.295$)	0.132 ($P = 0.230$)	0.064 ($P = 0.581$)	0.049 ($P = 0.655$)
Physiological markers					
AHI	0.048 ($P = 0.671$)	0.304 ($P = 0.005$)**	0.269 ($P = 0.015$)*	0.082 ($P = 0.465$)	0.111 ($P = 0.320$)
ABI	−0.078 ($P = 0.480$)	−0.010 ($P = 0.920$)	−0.111 ($P = 0.836$)	−0.009 ($P = 0.933$)	−0.452 ($P < 0.001$)***
24-h SBP	−0.128 ($P = 0.494$)	0.109 ($P = 0.333$)	0.135 ($P = 0.231$)	−0.056 ($P = 0.620$)	0.032 ($P = 0.779$)
24-h DBP	−0.188 ($P = 0.255$)	0.148 ($P = 0.186$)	0.292 ($P = 0.008$)**	−0.042 ($P = 0.707$)	−0.120 ($P = 0.285$)

[a]Light transmission assessed by 1 μg/mL of collagen.

BMI, body mass index; hsCRP, high-sensitivity C-reactive protein; BNP, brain natriuretic peptide; PAI-1, plasminogen activator inhibitor-1; SBP, systolic blood pressure; DBP, diastolic blood pressure; AHI, apnea–hypopnea index; ABI, ankle-brachial index.

*$P < 0.05$.

**$P < 0.01$.

***$P < 0.001$.

Table 5 Multivariate analyses for determination of thrombogenetic factors in patients taking dual-antiplatelet therapy

	(R^2; P)	Variables	β*	β (95%CI)	P
PAI-I	(0.09, 0.006)	BMI	0.167	–	0.147
		AHI	0.300	0.402 (0.116–0.687)	0.006
E-selectin	(0.203, <0.001)	Age	−0.127	–	0.236
		BMI	0.323	0.983 (0.365–1.601)	0.002
		Diabetes mellitus	0.279	5.736 (1.566–9.906)	0.008
		AHI	0.126	–	0.253
		24-h DBP	0.169	–	0.136
D-dimer	(0.126, 0.001)	BMI	−0.064	–	0.564
		Prior MI	−0.075	–	0.484
		BNP	0.356	1.928 (0.793–3.063)	0.001
Soluble fibrin	(0.366, <0.001)	Number of VD	0.085	–	0.372
		Total cholesterol	0.349	0.075 (0.035–0.113)	<0.001
		BNP	0.222	3.681 (0.651–6.711)	0.018
		ABI	−0.330	−17.953 (−28.203–7.704)	0.001

β*, standardized coefficient; CI, confidence interval; PAI-1, plasminogen activator inhibitor-1; AHI, apnea–hypopnea index; BMI, body mass index; BNP, brain natriuretic peptide; DBP, diastolic blood pressure; ABI, ankle-brachial index; VD, vessel diseases.

surface that provokes the coagulation cascade, thereby amplifying thrombin generation.[38,39] However, residual platelet activation was not correlated with systemic thrombin generation assessed by plasma SF and resultant fibrinolytic activation assessed by the D-dimer level. The major determinant of thrombin generation was found to be independently associated with total cholesterol, BNP, and ABI, suggesting that thrombin generation in PCI subjects under dual-antiplatelet therapy is mainly determined by the degree of impaired cardiac function and/or arteriosclerosis. Plasma PAI-1 was also associated with the presence of sleep apnea syndrome. Although circulating platelets account for increases in plasma PAI-1 and release it following activation,[40] platelet aggregability was not associated with PAI-1. Taken together, these data suggested that many factors may be involved in systemic thrombogenicity, independent of platelet aggregability.

Our data suggested that some patients may benefit from the addition of anticoagulant treatment after PCI. The American College of Cardiology/American Heart Association guidelines recommend anticoagulant therapy in patients with an acute ST-elevation myocardial infarction with extensive regional wall motion abnormalities. However, the routine use of anticoagulant drugs without thienopyridine should be avoided in patients who have undergone PCI because treatment with aspirin and ticlopidine results in a lower rate of stent thrombosis as compared with a combination of aspirin plus warfarin.[41] No trial has closely evaluated the safety and efficacy of anticoagulant therapy in combination with dual-antiplatelet therapy in patients undergoing PCI. Large-scale trials are thus needed to confirm any recommendations. Our study should be interpreted in light of its limitations; for ethical reasons we could not obtain proper control patients who had not taken any antiplatelet drug after PCI. This was because dual-antiplatelet therapy is the gold standard to reduce clinical events in patients who have undergone PCI.

In conclusion, the current study has demonstrated that dual-antiplatelet therapy effectively inhibited its pharmacological targets, although we found inter-individual variability in platelet aggregation, which was at least partly explained by hyperglycemia. On the other hand, thrombin generation, inhibition of fibrinolytic activity, and endothelial dysfunction were not determined by platelet aggregability, but by other aspects of the patients' backgrounds, such as obesity, sleep apnea, diabetes mellitus, cardiac dysfunction, and/or atherosclerotic burden. Our findings indicated that some patients remain at risk of subsequent thrombotic complications after PCI despite adequate dual-antiplatelet therapy. Large-scale prospective studies are required to determine which markers are associated with the risk of further cardiovascular events after PCI and to examine interventions such as tight plasma glucose control, anticoagulation, and continuous positive air way pressure therapy.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: none declared.

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References

1. Mehta SR, Yusuf S, Peters RJ, Bertrand ME, Lewis BS, Natarajan MK, Malmberg K, Rupprecht H, Zhao F, Chrolavicius S, Copland I, Fox KA. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. *Lancet* 2001;**358**:527–533.
2. Schomig A, Neumann FJ, Kastrati A, Schuhlen H, Blasini R, Hadamitzky M, Walter H, Zitzmann-Roth EM, Richardt G, Alt E, Schmitt C, Ulm K. A randomized comparison of antiplatelet and anticoagulant therapy after the placement of coronary-artery stents. *N Engl J Med* 1996;**334**:1084–1089.
3. Grines CL, Bonow RO, Casey DE Jr, Gardner TJ, Lockhart PB, Moliterno DJ, O'Gara P, Whitlow P. Prevention of premature discontinuation of dual antiplatelet therapy in patients with coronary artery stents: a science advisory from the American Heart Association, American College of Cardiology, Society for Cardiovascular Angiography and Interventions, American College of Surgeons, and American Dental Association, with representation from the American College of Physicians. *Circulation* 2007;**115**:813–818.
4. Steinhubl SR, Berger PB, Mann JT III, Fry ET, DeLago A, Wilmer C, Topol EJ. Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. *J Am Med Assoc* 2002;**288**:2411–2420.
5. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N Engl J Med* 2001;**345**:494–502.
6. Chen ZM, Jiang LX, Chen YP, Xie JX, Pan HC, Peto R, Collins R, Liu LS. Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. *Lancet* 2005;**366**:1607–1621.
7. Gurbel PA, Bliden KP, Guyer K, Cho PW, Zaman KA, Kreutz RP, Bassi AK, Tantry US. Platelet reactivity in patients and recurrent events post-stenting: results of the PREPARE POST-STENTING Study. *J Am Coll Cardiol* 2005;**46**:1820–1826.
8. Matetzky S, Shenkman B, Guetta V, Shechter M, Bienart R, Goldenberg I, Novikov I, Pres H, Savion N, Varon D, Hod H. Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. *Circulation* 2004;**109**:3171–3175.
9. Bliden KP, DiChiara J, Tantry US, Bassi AK, Chaganti SK, Gurbel PA. Increased risk in patients with high platelet aggregation receiving chronic clopidogrel therapy undergoing percutaneous coronary intervention: is the current antiplatelet therapy adequate? *J Am Coll Cardiol* 2007;**49**:657–666.
10. Cuisset T, Frere C, Quilici J, Barbou F, Morange PE, Hovasse T, Bonnet JL, Alessi MC. High post-treatment platelet reactivity identified low-responders to dual antiplatelet therapy at increased risk of recurrent cardiovascular events after stenting for acute coronary syndrome. *J Thromb Haemost* 2006;**4**:542–549.
11. Maree AO, Fitzgerald DJ. Variable platelet response to aspirin and clopidogrel in atherothrombotic disease. *Circulation* 2007;**115**:2196–2207.
12. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Alfonso F, Macaya C, Bass TA, Costa MA. Variability in individual responsiveness to clopidogrel: clinical implications, management, and future perspectives. *J Am Coll Cardiol* 2007;**49**:1505–1516.
13. Gum PA, Kottke-Marchant K, Welsh PA, White J, Topol EJ. A prospective, blinded determination of the natural history of aspirin resistance among stable patients with cardiovascular disease. *J Am Coll Cardiol* 2003;**41**:961–965.
14. Eikelboom JW, Hirsh J, Weitz JI, Johnston M, Yi Q, Yusuf S. Aspirin-resistant thromboxane biosynthesis and the risk of myocardial infarction, stroke, or cardiovascular death in patients at high risk for cardiovascular events. *Circulation* 2002;**105**:1650–1655.
15. Frelinger AL III, Furman MI, Linden MD, Li Y, Fox ML, Barnard MR, Michelson AD. Residual arachidonic acid-induced platelet activation via an adenosine diphosphate-dependent but cyclooxygenase-1- and cyclooxygenase-2-independent pathway: a 700-patient study of aspirin resistance. *Circulation* 2006;**113**:2888–2896.
16. Gurbel PA, Bliden KP, DiChiara J, Newcomer J, Weng W, Neerchal NK, Gesheff T, Chaganti SK, Etherington A, Tantry US. Evaluation of dose-related effects of aspirin on platelet function: results from the Aspirin-Induced Platelet Effect (ASPECT) study. *Circulation* 2007;**115**:3156–3164.
17. Ohmori T, Yatomi Y, Nonaka T, Kobayashi Y, Madoiwa S, Mimuro J, Ozaki Y, Sakata Y. Aspirin resistance detected with aggregometry cannot be explained by cyclooxygenase activity: involvement of other signaling pathway(s) in cardiovascular events of aspirin-treated patients. *J Thromb Haemost* 2006;**4**:1271–1278.
18. Tantry US, Bliden KP, Gurbel PA. Overestimation of platelet aspirin resistance detection by thrombelastograph platelet mapping and validation by conventional aggregometry using arachidonic acid stimulation. *J Am Coll Cardiol* 2005;**46**:1705–1709.
19. Hurlen M, Abdelnoor M, Smith P, Erikssen J, Arnesen H. Warfarin, aspirin, or both after myocardial infarction. *N Engl J Med* 2002;**347**:969–974.
20. ten Berg JM, Hutten BA, Kelder JC, Verheugt FW, Plokker HW. Oral anticoagulant therapy during and after coronary angioplasty: the intensity and duration of anticoagulation are essential to reduce thrombotic complications. *Circulation* 2001;**103**:2042–2047.
21. Iakovou I, Schmidt T, Bonizzoni E, Ge L, Sangiorgi GM, Stankovic G, Airoldi F, Chieffo A, Montorfano M, Carlino M, Michev I, Corvaja N, Briguori C, Gerckens U, Grube E, Colombo A. Incidence, predictors, and outcome of thrombosis after successful implantation of drug-eluting stents. *J Am Med Assoc* 2005;**293**:2126–2130.
22. Wiman B, Andersson T, Hallqvist J, Reuterwall C, Ahlbom A, deFaire U. Plasma levels of tissue plasminogen activator/plasminogen activator inhibitor-1 complex and von Willebrand factor are significant risk markers for recurrent myocardial infarction in the Stockholm Heart Epidemiology Program (SHEEP) study. *Arterioscler Thromb Vasc Biol* 2000;**20**:2019–2023.
23. Moss AJ, Goldstein RE, Marder VJ, Sparks CE, Oakes D, Greenberg H, Weiss HJ, Zareba W, Brown MW, Liang CS, Lichstein E, Little WC, Gillespie JA, Van Voorhees L, Krone RJ, Bodenheimer MM, Hochman J, Dwyer EM Jr, Arora R, Marcus FI, Watelet LF, Case RB. Thrombogenic factors and recurrent coronary events. *Circulation* 1999;**99**:2517–2522.
24. Kontny F, Dempfle CE, Abildgaard U. Fibrin monomer antigen: a novel marker of mortality in acute myocardial infarction. *Eur Heart J* 1999;**20**:808–812.
25. Kilickap M, Tutar E, Aydintug O, Pamir G, Erol C, Tutkak H, Oral D. Increase in soluble E-selectin level after PTCA and stent implantation: a potential marker of restenosis. *Int J Cardiol* 2004;**93**:13–18.

26. Hulley SB, Cummings SR, Browner WS, Grady DG, Newman TB, eds. *Designing Clinical Research*. 3rd ed. Lippincott Williams & Wilkins; 2006. p85.
27. Ozaki Y, Satoh K, Yatomi Y, Yamamoto T, Shirasawa Y, Kume S. Detection of platelet aggregates with a particle counting method using light scattering. *Anal Biochem* 1994;**218**:284–294.
28. Sakamoto T, Ogawa H, Kawano H, Hirai N, Miyamoto S, Takazoe K, Soejima H, Kugiyama K, Yoshimura M, Yasue H. Rapid change of platelet aggregability in acute hyperglycemia: detection by a novel laser-light scattering method. *Thromb Haemost* 2000;**83**:475–479.
29. Schwarz UR, Geiger J, Walter U, Eigenthaler M. Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of activating and inhibitory signal transduction pathways in human platelets—definition and detection of ticlopidine/clopidogrel effects. *Thromb Haemost* 1999;**82**:1145–1152.
30. Cattaneo M. Resistance to antiplatelet drugs: molecular mechanisms and laboratory detection. *J Thromb Haemost* 2007;**5**(Suppl. 1):230–237.
31. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes 1. *N Engl J Med* 1992;**326**:242–250.
32. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes 2. *N Engl J Med* 1992;**326**:310–318.
33. Meadows TA, Bhatt DL. Clinical aspects of platelet inhibitors and thrombus formation. *Circ Res* 2007;**100**:1261–1275.
34. Angiolillo DJ, Shoemaker SB, Desai B, Yuan H, Charlton RK, Bernardo E, Zenni MM, Guzman LA, Bass TA, Costa MA. Randomized comparison of a high clopidogrel maintenance dose in patients with diabetes mellitus and coronary artery disease: results of the Optimizing Antiplatelet Therapy in Diabetes Mellitus (OPTIMUS) study. *Circulation* 2007;**115**:708–716.
35. Michelson AD, Linden MD, Furman MI, Li Y, Barnard MR, Fox ML, Lau WC, McLaughlin TJ, Frelinger AL. Evidence that pre-existent variability in platelet response to ADP accounts for 'clopidogrel resistance'. *J Thromb Haemost* 2007;**5**:75–81.
36. Stranders I, Diamant M, van Gelder RE, Spruijt HJ, Twisk JW, Heine RJ, Visser FC. Admission blood glucose level as risk indicator of death after myocardial infarction in patients with and without diabetes mellitus. *Arch Intern Med* 2004;**164**:982–988.
37. Abizaid A, Kornowski R, Mintz GS, Hong MK, Abizaid AS, Mehran R, Pichard AD, Kent KM, Satler LF, Wu H, Popma JJ, Leon MB. The influence of diabetes mellitus on acute and late clinical outcomes following coronary stent implantation. *J Am Coll Cardiol* 1998;**32**:584–589.
38. Ruggeri ZM. Platelets in atherothrombosis. *Nat Med* 2002;**8**: 1227–1234.
39. Rauch U, Osende JI, Fuster V, Badimon JJ, Fayad Z, Chesebro JH. Thrombus formation on atherosclerotic plaques: pathogenesis and clinical consequences. *Ann Intern Med* 2001;**134**:224–238.
40. Brogren H, Karlsson L, Andersson M, Wang L, Erlinge D, Jern S. Platelets synthesize large amounts of active plasminogen activator inhibitor 1. *Blood* 2004;**104**:3943–3948.
41. Leon MB, Baim DS, Popma JJ, Gordon PC, Cutlip DE, Ho KK, Giambartolomei A, Diver DJ, Lasorda DM, Williams DO, Pocock SJ, Kuntz RE. A clinical trial comparing three antithrombotic-drug regimens after coronary-artery stenting. Stent Anticoagulation Restenosis Study Investigators. *N Engl J Med* 1998;**339**:1665–1671.

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Successful Treatment of Primitive Neuroectodermal Tumor-associated Microangiopathy with Multiple Bone Metastases

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We report here a 16-year-old male with primitive neuroectodermal tumor (PNET)-associated probable microangiopathy with multiple bone metastases. Laboratory findings excluded the possibility of amegakaryocytic or immune thrombocytopenia and/or disseminated intravascular coagulation. He was first treated with plasma-exchange (PE), followed by platelet transfusions, steroid pulse therapy and combined chemotherapy. PE and steroid pulse therapy reduced his plasma CRP level. Combined chemotherapy drastically increased his platelet count until it had almost normalized without further transfusion. The plasma level of von Willebrand factor-cleaving protease (ADAMTS13) activity measured before PE was not severely deficient (48% of normal) and an unusually large von Willebrand factor multimer (UL-VWFM) was detected. We consider that this therapeutic strategy has the following benefits: (1) reduction of plasma levels of factors that are harmful to both platelet activation and endothelial cell injury; and (2) the safe transfusion of platelet concentrate in thrombotic microangiopathy. This strategy should be confirmed in further cases.

Key words: PNET – microangiopathy – chemotherapy – ADAMTS13 – UL-VWFM

INTRODUCTION

Malignancy-associated thrombotic microangiopathy (TMA), characterized by thrombocytopenia and microangiopathic hemolytic anemia, is a rare but life-threatening complication of sarcoma and its treatment remains controversial. Recent studies, however, have indicated that such patients usually have normal plasma von Willebrand factor-cleaving protease (ADAMTS13) activity (1,2), and that platelet transfusions are generally contra-indicated in these patients because transfusions have been associated with disease exacerbation (3,4). We report here a case of PNET-associated probable TMA that was successfully treated by platelet transfusion after extensive plasmapheresis (PE) followed by chemotherapy.

CASE REPORT

The patient was a 16-year-old male (body weight, 60 kg) who had complained of a high fever and fatigue beginning in June 2002. He was admitted to a nearby hospital on 14 June and received penicillin injections for three days. Suspicion of meningitis, sepsis, viral infection and immunologic diseases was excluded by negative results of leucocytosis in the cerebrospinal fluid, culture of blood and cerebrospinal fluid and antibodies against certain viruses and nucleus. A lytic area in the right eighth rib was then noted on radiography. Bone scintigraphy showed multiple hot lesions on 18 June. Bone marrow examination performed on 19 June was normal without invasion of malignant cells. Pathological examination using biopsy specimens together with the demonstration of EWS-FLI1 translocation confirmed a diagnosis of a PNET (Fig. 1). On 25 June, he developed slight bilirubinemia (1.2 mg/dl) and thrombocytopenia (94×10^9/l), which then rapidly progressed together with hemolytic signs consisting of rouleaux formation and poikilocytosis of erythrocytes in the peripheral blood, and microscopic hematuria. Normoblasts and immature myeloid cells in the peripheral blood were also found as leucoerythroblastic features. Because of this complex clinical picture, he was transferred to our hospital on 8 July. On admission, he had anemia (Hb 105 g/l) (normal range: 135–176), thrombocytopenia (26×10^9/l), and high serum levels of CRP

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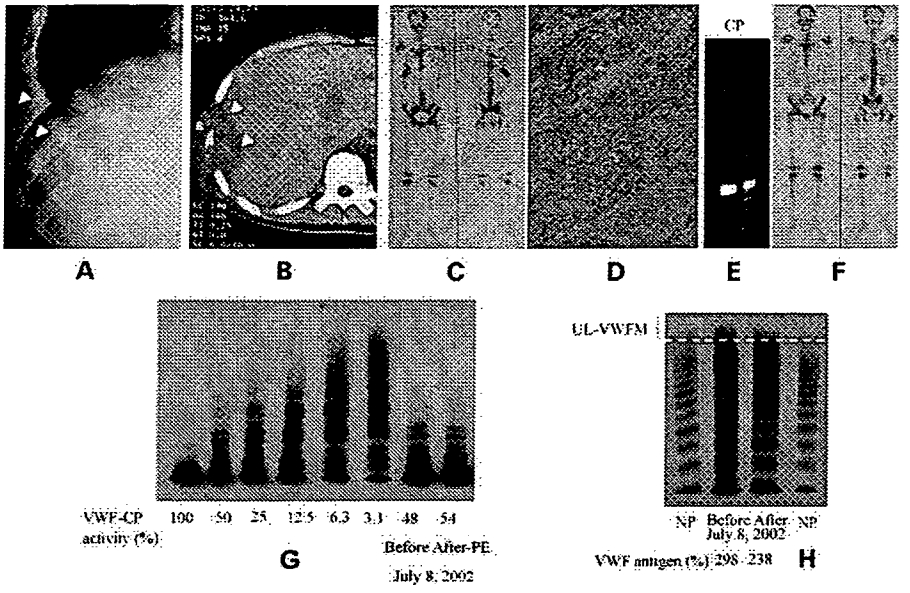

Figure 1. Results of clinical and diagnostic images. Plain radiograph (A) and CT (B) showing a lytic lesion and large expansive tumor in the right eighth rib. Bone scintigram (C) showing multiple areas of abnormal uptake in the skull, spine, pelvis, ribs, shoulders and knees. Histology of the biopsy specimen (D) was compatible with primitive neuroectodermal tumor. Demonstration of EWS-FLI1 translocation (E) by reverse transcription of polymerase chain reaction; c, positive control; p, patient. Bone scintigram, 10 months after diagnosis (F), did not show any abnormal uptake. Assay of plasma VWF-cp activity before and after plasma exchange (PE) on 8 July 2002 (G). Detection of unusually large von Willebrand factor multimers (UL-VWF) and VWF antigen level in the patient plasma before and after PE on 8 July 2002 (H). Note that the plasma VWF antigen level decreased after PE.

(251 mg/l), LDH (1787 U/l) (normal range: 106–211 U/l), GOT (49 U/l) (normal range: 12–32 U/L), and ALP (2,100 U/l) (normal range: 200–760 U/l). Other laboratory findings were as follows: BUN 6.78 μmol/l (normal range: 2.85–7.12 μmol/l), creatinine 53.04 μmol/l (normal range: 26.52–79.56 μmol/l), and total bilirubin 18.8 μmol/l (normal range: 5.1–18.8 μmol/l). His blood type was O-Rho (D) positive and both direct and indirect Coombs tests were negative. Antiplatelet antibody determined by mixed passive hemaglutination assay was negative. He had never previously undergone chemotherapy or blood transfusion. Coagulation screening tests including the levels of antithrombin (86%) and fibrinogen (5.91 g/l) were within normal ranges; however, his serum FDP level had increased slightly to 43.7 μg/ml. Plasma ADAMTS13 activity determined by the multimer assay was not immediately available. Based on these clinical and laboratory findings, we suspected that the patient had PNET-associated TMA rather than immune thrombocytopenia or disseminated intravascular coagulation (DIC). Because of his extremely poor general condition, surgical and/or chemotherapeutic approaches were thought to be inadvisable.

Thus, we prepared a protocol consisting an initial plasma exchange (PE) followed by transfusion of a single-donor platelet concentrate (PC) supplied by the Japan Red Cross Blood Center. This regimen was repeated for five consecutive days, together with steroid pulse therapy (methyl prednisolone 1 g/d for 3 days). Using this approach, PC was transfused without any appreciable adverse reactions. The expected rise in platelet count was identified 1 h after each infusion. It then decreased to the pre-infusion level ($23–33 \times 10^9$/l) over the next few days. Bone marrow examination on the fourth hospital day (11 July) demonstrated a normal nuclear cell count (137×10^9/l), of which malignant cells accounted for 29.5%. Meanwhile, the megakaryocyte count was normal or had increased slightly (200/μl), supporting the concept of enhanced consumption of newly-produced platelets. After sequential PE, a marked decrease in the CRP level was observed, and the general condition of the patient appeared to improve. However, the LDH level remained elevated and even increased slightly while the anemia worsened, indicating invasive expansion of tumor cells. Thus, on 13 July, we started combined chemotherapy, consisting of vincristine (VCR), adriamycin (ADR), and cyclophosphamide (CPA), that resulted in a dramatic increase in the platelet count with a concomitant decrease in LDH. Furthermore, the anemia ceased to progress, with no red blood cell transfusion required throughout this clinical course. Partial response was confirmed by resection of the right eighth rib after chemotherapy. Total body and local irradiation was performed after the Hi-MEC regimen, resulting in an absence of abnormal accumulation on bone scintigraphy 10 months after diagnosis. However, 13 months after diagnosis, recurrences in the right hip joint and orbit were detected and the patient died of disease. Survival after diagnosis was 23 months.

Before PE, his plasma VWF antigen level was elevated (298%) and an unusually-large VWF multimer (UL-VWFM) was present (Fig. 2).

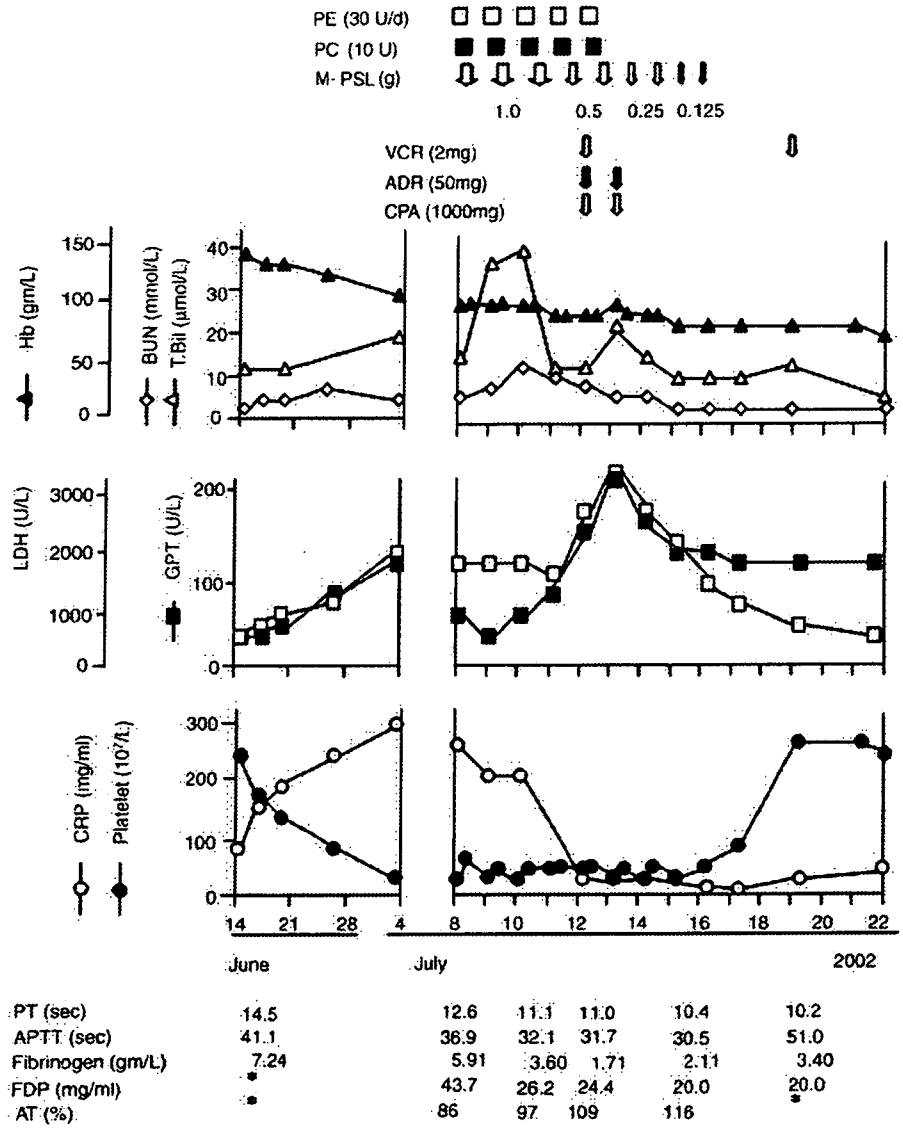

Figure 2. Time course of laboratory parameters and therapeutic regimen. PE, plasma exchange; PC, platelet concentration; m-psl, methyl prednisolone; VCR, vincristine; ADR, adriamycin; CPA, cyclophosphamide; Hb, hemoglobin; BUN, blood urea nitrogen; T.Bil, total bilirubin; LDH, lactate dehydrogenase; GPT, glutamic-pyruvic transaminase; CRP, C-reactive protein; PT, prothrombin time; APTT, activated partial thromboplastin time, FDP; fibrinogen degradation products; AT, antithrombin.

DISCUSSION

The diagnosis of TMA was based on schistocytosis and evidence of hemolysis. In our case, an elevated level of LDH was evident; however, schistocytosis was not tested in our hospital.

Thrombocytopenia occurred as a result of low platelet production and/or increased breakdown. In our case, a normal or slight increase in the production of platelets in the bone marrow was confirmed during hospitalization. Therefore, the increased breakdown of platelets was assumed. Spherocytes in the peripheral blood, which are characteristic in immune and hereditary hemolysis, were not found before transition. Considering these data along with the negative results on direct and indirect Coombs tests, the possibility of immune hemolysis was considered highly improbable and DIC was also excluded by the absence of signs indicating decreased ATIII.

Though a high CRP value persisted, severe infection, including meningitis, sepsis and viral infection were excluded by intensive examination. High fever and leucocytosis were thus considered characteristic symptoms of PNET and not owing to infection. Splenomegaly as a sign of increased breakdown of platelets was not confirmed by CT. There was no history of blood transfusion.

Invasion of malignant cells as confirmed by bone marrow examination occurred between 11 June and 19 July, and thrombocytopenia with hemolytic anemia occurred concomitant with this invasion, though multiple bone metastases had

already been confirmed by bone scintigraphy. Therefore the formation of bone metastases is insufficient to explain thrombocytopenia in this case. We consider that thrombocytopenia was probably as a result of malignant tumor-associated TMA.

Detection of UL-VWFM, released from endothelial cells and cleaved by ADAMTS13, and subnormal activity of ADAMTS13, reported as a marker to differentiate between TTP and HUS (3), were also confirmed later. Detection of UL-VWFM suggests injury of the endothelial cells or obstruction of cleavage by ADAMTS13. In our case, ADAMTS13 activity was subnormal, which agreed with the findings in the majority of TMA reported (1). Therefore, detection of UL-VWFM suggested injury of endothelial cells (2). Invasion of malignant cells, synchronously occurring, may have caused endothelial cell injury. Histological examination may help clarify the mechanism.

Cytokines have recently been reported to mediate UL-VWFM release from vascular endothelial cells (5). Furthermore, it was proposed that cytokines that injure vascular endothelial cells may interfere with the efficient supply of ADAMTS13 (5). Thus, cytokines may be another cause of TMA.

Systemic chemotherapy is usually indicated except in cases of chemotherapy-induced TMA. However, low platelet count made the initiation of this therapy inadvisable. Thus, transfusion of platelets was performed after PE to prevent adverse reaction. It has been proposed that PC transfusion is contra-indicated in TMA because uncleaved UL-VWFM induces platelet aggregation under high shear stress and exacerbates thrombosis (3). However, after removal of factors including UL-VWFM and cytokines from the circulation by PE, PC transfusion was performed safely and quickly resulted in raising the platelet count over the short time. However, the basic conditions, for example, expansion of tumor cells, may gradually lower the platelet count again. Thus, treatment of the tumor itself is necessary. Combined chemotherapy after PE dramatically improved TMA in our case. This also supports the hypothesis that malignant cells were related to injury of the endothelial cells. Prognosis of TMA depends on the chemosensitivity of the tumor itself. Further experience is necessary to confirm this regimen.

The present findings may improve our understanding the reason why malignancy-associated TMA responds poorly to PE therapy alone, as has been commonly accepted.

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Conflict of interest statement

None declared.

References

1. Fontana S, Gerritsen E, Hovinga KJ, Furlan M, Lammle B. Microangiopathic hemolytic anemia in metastatizing malignant tumor is not associated with a severe deficiency of the von Willebrand factor-cleaving protease. *Br J Haematol* 2001;113:100–2.
2. Blot E, Decaudin D, Veyardier A, Bardier A, Zagame OL, Pouillart P. Cancer-related thrombotic microangiopathy secondary to Von Willebrand factor-cleaving protease deficiency. *Thromb Res* 2002;106:127–30.
3. Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, et al. Von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med* 1998;339:1578–84.
4. Allford SL, Hunt BJ, Rose P, Machin SJ. Guidelines on the diagnosis and management of the thrombotic microangiopathic haemolytic anaemia. *Br J Hematol* 2003;120:556–73.
5. Bernardo A, Ball C, Nolasco L, Moake JF, Dong J-F. Effects of inflammatory cytokines on the release and cleavage of the endothelial cell-derived ultralarge von Willebrand factor multimers under flow. *Blood* 2004;104:100–6.