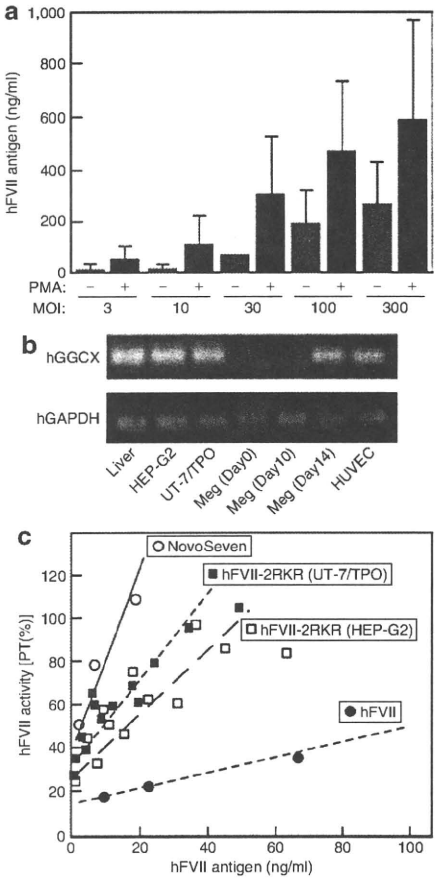

Figure 3 Production of functional hFVII-2RKR by UT-7/TPO cells transduced with simian immunodeficiency virus (SIV) vector. (**a**) UT-7/TPO cells were transduced with SIV-CMV-hFVII-2RKR at the multiplicity of infection (MOI) indicated. hFVII antigen in the supernatant from the cells treated without or with 5 μmol/l phorbol 12-myristate 13-acetate (PMA) for 24 hours were measured using enzyme-linked immunosorbent assay. Columns and error bars represent the mean ± SD (n = 3). (**b**) mRNA expression of γ-glutamyl carboxylase (GGCX) was determined using reverse transcriptase-PCR (RT-PCR). As a control, RT-PCR analysis for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was performed simultaneously. (**c**) Comparison between activity and antigen levels of hFVII-2RKR produced by UT-7/TPO and HEP-G2 cells, NovoSeven, and plasma-derived full-length hFVII. CMV, cytomegalovirus; hFVII, human factor VII; HUVEC, human umbilical vein endothelial cells; Meg, CD34$^+$-derived megakaryocytes at days 0, 10, and 14 after the start of differentiation with 10 ng/ml of interleukin-3 and 50 ng/ml of thrombopoietin.

factor, was confirmed in megakaryocytes (**Figure 3b**). These data suggest that platelet precursor megakaryocytes efficiently produce functional FVIIa after transduction.

We next examined whether transduction of HSCs enabled FVIIa expression in platelets. For this purpose, hFVII was used to discriminate transduced FVII from endogenous mFVII. An SIV vector equipped with *hFVII-2RKR* driven by *GPIbα* promoter was created (**Figure 2b**) and used for infecting unfractionated bone marrow cells. After transplantation of the transduced cells into lethally irradiated FVIII-deficient mice, hFVII antigen levels were detected in platelet lysates for at least 90 days (**Figure 4a**). Importantly, hFVII antigen was not detected in the plasma even after stimulation of the

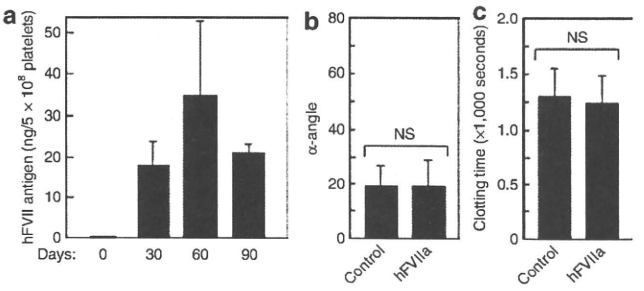

Figure 4 Human factor VII (hFVII) expression in platelets after transplantation of transduced stem cells. FVIII-deficient mice were given transplants of unfractionated bone marrow cells transduced without (control) or with SIV-GPIbα-hFVII-2RKR (hFVIIa). (**a**) Peripheral blood was drawn from the transplant-recipient mice at the indicated times, and the hFVII antigen levels in platelet lysates were measured using enzyme-linked immunosorbent assay. Columns and error bars represent the mean ± SD (n = 4 per group). (**b** and **c**) Whole-blood coagulation was assessed using thromboelastography. Quantitative data of panel **b** α-angle and panel **c** clotting time are shown (n = 7 for control; n = 8 for hFVIIa). Columns and error bars represent the mean ± SEM. Differences between the two groups were analyzed statistically using Student's t-test. NS, not significant. SIV, simian immunodeficiency virus.

platelets with collagen and phorbol 12-myristate 13-acetate (data not shown); therefore, we did not assay for FVII activity in plasma.

We next examined the improvement of whole-blood coagulation in the transplant-recipient mice. The conventional activated partial thromboplastin time, a useful marker for gene therapy in mouse models of hemophilia, did not seem to reflect the phenotypic correction directly, as seen from the fact that platelet-derived hFVII antigen could not be detected in plasma. Therefore we employed thromboelastography (TEG) to record the continuous profile of whole-blood coagulation.[10] TEG can be used for evaluating the effects of hemostatic agents such as rhFVIIa,[11] and to assess the effects of different pharmacological interventions on various factors (coagulation, platelet activation, and platelet–fibrin interaction) involved in clot formation. As shown in **Figure 4b** and **c**, whole-blood coagulation in FVIII-deficient mice, as assessed by TEG, did not improve after transplantation. The mortality rate after tail clipping was not altered by transplantation (data not shown).

In order to investigate why phenotypic correction was not observed after transplantation, we validated the expression and localization of hFVII by immunogold electron microscopy. We confirmed that hFVII antigen was abundant in cytoplasm of platelets obtained from transplant-recipient mice (**Figure 5a**). When platelets were stimulated with phorbol 12-myristate 13-acetate, most of the hFVII antigen was redistributed to the sub-membrane zone, and was partly expressed on the surface (**Figure 5b**). These data confirmed that hFVII is efficiently stored in platelets after transplantation of transduced bone marrow cells, and is expressed on the cell surface after platelet activation.

Phenotype correction of FVIII-deficient mice by expression of mFVII-2RKR in platelets

We hypothesized that the failure of hFVII-2RKR expression in platelets may be because of inefficient interaction of hFVII-2RKR with murine TF.[12,13] Indeed, higher concentrations of rhFVIIa (NovoSeven; ≥20 μg/ml) were required to restore coagulation in

FVIII-deficient mice (data not shown). We therefore generated an mFVII construct in an analogous fashion (mFVII-2RKR) to further prove its efficacy in FVIII-deficient mice (**Figure 2b**). Because we could not create an enzyme-linked immunosorbent assay for mFVII antigen, an *eGFP* gene driven by the internal ribosomal entry site was inserted just after the *mFVII-2RKR* gene to give indirect confirmation of mFVII-2RKR expression in platelets (**Figure 2b**). Although eGFP expression in platelets after transplantation was limited to 3–12% of the platelets, the pattern of eGFP expression under the control of the internal ribosomal entry site sequence was much weaker than that driven directly by the upstream promoter (data not shown). It appears, therefore, that the levels of ectopically expressed mFVII-2RKR in platelets are much higher than would be expected from the results of eGFP expression. This reasoning was supported by the results of proviral integration into the genome (1.15 ± 2.42; $n = 13$), which were similar to those obtained from mice expressing eGFP in 28.6–74.3% of platelets by transduction with SIV-GPIbα-eGFP (see **Figure 1c**).

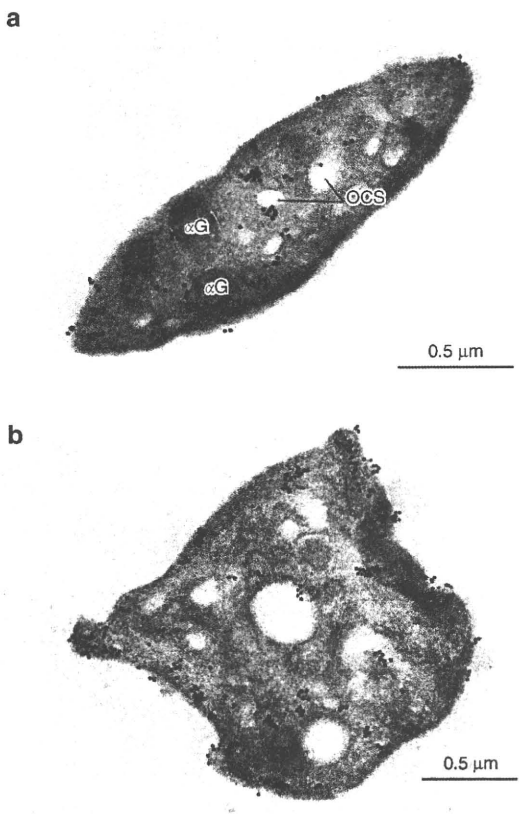

Figure 5 Immunoelectron microscopic localization of ectopically expressed hFVII-2RKR in platelets. FVIII-deficient mice were given transplants of unfractionated bone marrow cells transduced with SIV-GPIbα-hFVII-2RKR. Isolated platelets from the transplant-recipient mice were stimulated (**a**) without or (**b**) with 100 nmol/l phorbol 12-myristate 13-acetate for 15 minutes. Cells were incubated with a biotin-labeled rabbit anti-hFVII antibody. Bound antibodies were detected using a colloidal gold–conjugated goat anti-biotin secondary antibody. hFVII expression in platelets was examined by electron microscopy. αG, α-granule; hFVII, human factor VII; OCS, open canalicular system; SIV, simian immunodeficiency virus.

In comparison with the results obtained from hFVII-2RKR, whole-blood coagulation, as assessed by TEG, was significantly improved in mice that had received the transplant (**Figure 6a**). The α-angle, which represents the rate of clot formation, was enhanced (**Figure 6b**), and the clotting time was significantly shortened

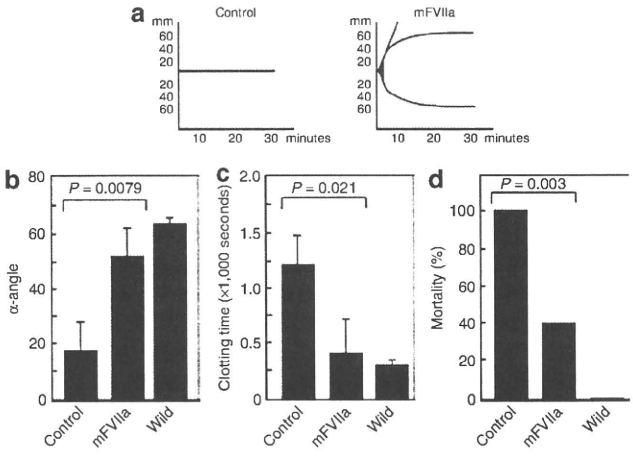

Figure 6 Phenotype correction of factor VIII (FVIII)-deficient mice by ectopic expression of mFVII-2RKR in platelets. FVIII-deficient mice were given transplants of unfractionated bone marrow cells transduced without (control) or with SIV-GPIbα-mFVII-2RKR (mFVIIa). (**a**) Representative thromboelastography data obtained from control and mFVII-2RKR-transfected mice. (**b** and **c**) Quantitative data of panel **b** α-angle and panel **c** clotting time are shown ($n = 8$ for control; $n = 8$ for mFVIIa). The data obtained from wild-type mice are also shown ($n = 6$). Columns and error bars represent the mean ± SEM. Differences between the two groups were analyzed statistically using Student's *t*-test. (**d**) Mortality rates within 24 hours of tail clipping in wild-type mice ($n = 6$) or FVIII-deficient mice given transplants of control or SIV-GPIbα-mFVII-2RKR-transduced bone marrow cells ($n = 10$ for control; $n = 10$ for mFVIIa). The mortality rate was analyzed statistically using the χ^2-test. mFVIIa, murine activated factor VII; SIV, simian immunodeficiency virus.

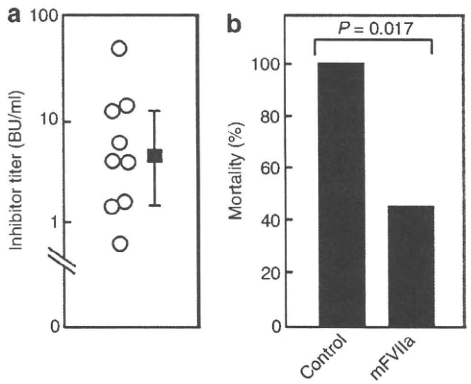

Figure 7 Effects of mFVII-2RKR expression in platelets on phenotypic correction in factor VIII (FVIII)-deficient mice in the presence of anti-FVIII inhibitors. (**a**) Circulating inhibitors were detected in 9 of the 13 FVIII-deficient mice after peritoneal injection of recombinant hFVIII. (**b**) In the presence of circulating inhibitors, mortality ratio at 24 hours after tail clipping was examined in FVIII-deficient mice given transplants of control or SIV-GPbα-mFVII-2RKR-transduced bone marrow cells [$n = 10$ for control; $n = 9$ for mFVII-2RKR (mFVIIa)]. The mortality rate was analyzed statistically using the χ^2-test. mFVIIa, murine activated factor VII; SIV, simian immunodeficiency virus.

(**Figure 6c**). The mortality rate after tail clipping was significantly reduced in mice with transplants (**Figure 6d**). In addition, five of the nine FVIII-deficient mice that received bone marrow cells transduced with SIV-GPIbα-mFVII-2RKR survived after tail clipping despite the presence of circulating inhibitors against hFVIII (**Figure 7a** and **b**). Blood coagulation, as assessed by TEG, was similarly corrected in FVIII-deficient mice having FVIII-neutralizing inhibitors, by treating them with SIV-GPIbα-mFVII-2RKR (data not shown). Taking these results together, platelet-specific mFVII-2RKR expression results in efficient bypass therapy to activate factor X activation, thereby resulting in thrombin generation on platelet surfaces in FVIII-deficient mice.

DISCUSSION

Hemophilia is considered to be a suitable condition for gene therapy because it is caused by a single gene abnormality, and therapeutic coagulation factor levels may vary across a broad range (5–100%).[14] Although sustained therapeutic expression of FVIII has been achieved in preclinical studies using a wide range of gene transfer technologies targeted at different tissues, the emergence of neutralizing antibodies often limits the clinical applications.[15] Blood platelets have been receiving much attention as novel target cells for hemophilia gene therapy, because platelet-specific expression of FVIII abolishes the emergence of neutralizing antibodies, and platelet-derived FVIII supports hemostasis in the presence of high titers of FVIII-neutralizing antibodies.[2–5] Here, we extended the application of platelet-directed gene therapy to demonstrate that FVII-2RKR expression in platelets improved the bleeding phenotype of FVIII-deficient mice, even in the presence of FVIII-neutralizing antibodies. Given that rhFVIIa has proven efficacy in the treatment of hemophilia patients with the inhibitors,[8] platelets expressing FVII-2RKR may be a potential alternative to bolus administration of rhFVIIa in such patients.

It is possible that platelets store ectopically expressed FVII-2RKR in the cytoplasm, and that this is specifically expressed on the cell surface after activation. Although hFVII antigen levels achieved here seemed to be much lower than the therapeutic level, whole-blood clotting, as assessed by TEG, and mortality rate after tail clipping were significantly improved when mFVII-2RKR was expressed. These data suggest that FVII-2RKR in platelets can locally generate a thrombin burst at the site of vascular injury. Although an important drawback of rhFVIIa administration is its short half-life (2.6–2.8 hours), which necessitates frequent bolus injections to stop bleeding,[7,16] platelets may be able to store stable FVII-2RKR in the circulation. The importance of coagulation factor stored in platelets is supported by the role of platelet factor V in hemostasis. Platelet-derived platelet factor V appears to support hemostasis even in patients with an acquired platelet factor V inhibitor, thereby suggesting that platelets can deliver coagulation factors and protect them from being degraded by platelet factor V inhibitors.[17] Recently, erythroid-specific factor IX expression, driven by the *β-globin* promoter, has been shown to result in phenotypic correction of hemophilia B in mice.[18] However, given the context of the specific release or expression of a target protein at the site of thrombus formation, platelet-directed gene therapy has an advantage as a therapy for inherited coagulation factor deficiencies.

Contrary to our initial expectations, the sub-localization of ectopically expressed hFVII-2RKR in platelets is quite different from that described in previous reports of FVIII expression.[3,19] Retroviral transduction of FVIII in human $CD34^+$ HSCs enables FVIII-transduced megakaryocytes to store human FVIII with von Willebrand factor, a natural carrier protein of FVIII, within α-granules.[19] In this study, hFVII-2RKR was found to be localized in the cytoplasm, but not in α-granules, suggesting that storage of this ectopically expressed protein in α-granules requires specific binding with an endogenous protein, as is the case for the FVIII–von Willebrand factor interaction.[20] Despite the failure to localize in α-granules, cytoplasmic hFVII-2RKR translocated to a sub-membrane fraction and was expressed on the cell surface after platelet activation. It is possible that activated platelets can express FVII-2RKR through a mechanism of protein secretion other than granule release. Phosphatidylserine flip-flop is one candidate that may be responsible for the surface expression of FVII-2RKR; however, we do not yet have a clear explanation for this. Recently, it has been reported that platelets supply their own TF for thrombin generation in a temporally and spatially circumscribed process.[21,22] This would suggest that TF expression in platelets is involved in the hemostatic function of ectopically expressed FVII-2RKR, even in the absence of soluble TF. The failure of hFVII-2RKR expression in platelets to correct the bleeding phenotype, in contrast to mFVII-2RKR, further demonstrates the TF-dependence of coagulation mediated by platelet-derived FVIIa; murine TF appears to be more species-specific and interacts poorly with hFVIIa.[12,13]

Before proceeding to further clinical application, we should weigh the clinical benefits and risks of FVII-2RKR expression in platelets. The most important drawback of FVIIa expression is a potential risk for thromboembolic events. In this study, the clinical effect of FVII-2RKR expression seemed to be less than that of FVIII expression in platelets. Although a correction of as little as 1% of FVIII in platelets was reportedly enough to cure FVIII-null mice,[3–5] our strategy resulted only in a partial cure in the current model. One explanation of the lower efficacy is that much higher expression of FVII-2RKR is needed to generate sufficient thrombin in FVIII-deficient mice. We will need to further improve the transduction efficiency and modify the enzymatic activity of FVIIa. We believe that megakaryocyte- and platelet-specific expression of FVIIa in targeting HSCs is important for safety, given that expression of FVIIa in neutrophils or monocytes may alter the coagulation properties of blood cells, leading to an unexpected thrombosis similar to disseminated intravascular coagulation. Once the approach involving the targeting of platelets has been optimized for greater efficacy, it is possible that the risk of unexpected thrombosis will become a major concern. Further, we must consider the risk of insertional mutagenesis of the integrating vector. While lentiviral vectors offer a means to correct genetic diseases by integration into chromosomal DNA permanently, all the integrating gene transfer vectors in current use carry a risk of insertional mutagenesis.[23] In view of the fact that the target diseases for platelet-directed gene therapy, including hemophilia, are not generally lethal disorders, we have to continue investigating the safety of platelet-directed gene therapy using integrating vectors to the maximum. Observations should be carried on for extended durations in order

to substantiate long-term *in vivo* gene expression and vector safety. Our observations were limited to 3 months.

In this study, we demonstrated that FVII-2RKR expression in platelets by SIV vectors could be an important strategy for treating hemophilia A. Given that platelets play a central role and provide the scaffolding for the coagulation cascade, this would be a reasonable approach, similar to the proven therapeutic effects of rhFVIIa infusion, for treating a number of hemorrhagic diseases, such as hemophilia, Glanzmann's thrombasthenia, and FVII deficiency. Further evaluations utilizing larger animals such as Cynomolgus monkeys or dogs will be necessary to determine efficient and safe protocols for platelet-directed gene therapy.

MATERIALS AND METHODS

The materials and methods for cell culture, reverse transcriptase-PCR, proviral integration, mouse blood preparation, enzyme-linked immunosorbent assay for hFVII antigen, determination of hFVII activity, and flow cytometry are described in the **Supplementary Materials and Methods.**

Plasmid constructs and production of SIV lentiviruses. The methods for cDNA cloning and plasmid construction are described in detail in **Supplementary Materials and Methods.** A self-inactivating SIV vector plasmid was generated as described earlier (**Figure 1b**).[24] SIV lentiviral vectors were produced as described earlier.[4] Briefly, the SIV vector and each packaging vector (*gag/pol*, *rev*, and *VSV-G*) were co-transfected into HEK293T cells using the Lipofectamine PLUS reagent (Invitrogen, Carlsbad, CA). The supernatants were collected 48 hours after transfection and filtered through a 0.4-μm filter. The transduction units of the lentiviral vector and proviral integration into the genomic DNA were measured as described earlier.[4]

Isolation of KSL cells, viral transduction, and stem cell transplantation. Mice with FVIII-deficient hemophilia A with targeted destruction of exon 16 of the *FVIII* gene were kindly provided by Dr H.H. Kazazian Jr. (University of Pennsylvania, Philadelphia, PA). C57BL/6 (Ly5.2) mice were purchased from Japan SLC (Shizuoka, Japan). C57BL/6 mice congenic for the Ly5 locus (Ly5.1) were purchased from Sankyo-Lab Service (Tokyo, Japan). All animal procedures were approved by the Institutional Animal Care and Concern Committee at Jichi Medical University (Tochigi, Japan), and animal care was in accordance with the committee's guidelines.

KSL HSCs were isolated as described earlier.[4] KSL cells or unfractionated bone marrow cells were precultured for 24 hours before viral transduction in Stem Pro Medium (Invitrogen, Carlsbad, CA) supplemented with 100 ng/ml stem cell factor, 10 ng/ml thrombopoietin, 100 ng/ml interleukin-6, 100 ng/ml Flt-3 ligand, and 400 ng/ml soluble interleukin-6 receptor. The cells were transduced with SIV vectors at a multiplicity of infection of 30 in the presence of the same cytokine combination, and incubated at 37 °C for 12 hours. The recipient mice (Ly5.2 mice or FVIII-deficient mice at 8–12 weeks of age) were irradiated with a single lethal dose of 9.5 Gy (Gamma Cell; Norton International, Ontario, Canada), and then administered either transduced KSL cells (1×10^4) and Ly5.2 competitor cells (2×10^5), or transduced whole bone marrow cells (2×10^6). The methods for transduction of UT-7/TPO and HEP-G2 cells by SIV vector are described in **Supplementary Materials and Methods.**

Immunogold electron microscopy. Washed murine platelets were obtained as described earlier.[25] The resting and phorbol 12-myristate 13-acetate–stimulated platelets were fixed in 0.1% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4) at 4 °C for 1 hour. The fixed platelets were transferred into Eppendorf tubes, centrifuged at 3,000 rpm for 3 minutes, and rinsed with phosphate-buffered saline five times at 4 °C. The specimens were sequentially immersed in 1 mol/l sucrose in phosphate-buffered saline for 1 hour, and then in 1.84 mol/l sucrose containing 20% polyvinylpyrrolidone (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline overnight at 4 °C.[26] The specimens were frozen in liquid nitrogen and ultra-thin frozen sections were prepared, incubated with biotin-labeled rabbit anti-hFVII antibody, washed with phosphate-buffered saline, and then incubated with goat anti-biotin antibody coupled to 15 colloidal gold (BBI International, Cardiff, UK). After being stained with uranyl acetate, the sections were examined using a JEM-1200EX electron microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

TEG and tail clipping. The principle of TEG is based on measurement of the physical viscoelastic characteristics of blood clots. Whole blood for TEG was drawn at 30–60 days after transplantation. Clot formation was monitored in whole blood at 37 °C, in an oscillating plastic cylindrical cuvette having a coaxially suspended stationary piston with 1-mm clearance between the surfaces, using a computerized TEG (ROTEG; Pentapharm, Munich, Germany). A sample (270 μl) of whole blood was carefully drawn from the superior vena cava of anesthetized mice using a syringe containing 30 μl sodium citrate. Whole-blood coagulation was initiated with the addition of 20 μl of 200 mmol/l $CaCl_2$, and TEG was used to assess the coagulation by measuring various parameters, such as the latency for clotting time, and the kinetics of clot development, as determined by the α-angle.[10] When blood coagulation was not observed within 30 minutes, the clotting time and α-angle were defined as 1,800 seconds and 0°, respectively. Phenotypic correction was tested in some of the transplant-recipient mice by anesthetizing them with diethyl ether and clipping 1.0 cm off the ends of their tails. The mice were then observed for 24 hours to determine the rate of mortality.

Circulating FVIII inhibitors. Antibodies against hFVIII were produced by administering weekly peritoneal injections of 0.05 U/g body weight of rhFVIII (Kogenate FS; Bayer AG, Wuppertal, Germany). In view of the possibility that lethal irradiation could cancel the circulating anti-FVIII antibodies (data not shown), we started immunization with hFVIII 1 month after transplantating bone marrow cells transduced with SIV-GPIbα-mFVII-2RKR. Analysis of neutralizing antibodies against hFVIII was performed using the Bethesda method described earlier.[27] After five immunizations, we could detect circulating inhibitors against hFVIII, as assessed in Bethesda units (**Figure 7a**).

SUPPLEMENTARY MATERIAL

Materials and Methods.

ACKNOWLEDGMENTS

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CLINICAL RESEARCH
Interventional cardiology

Determinants of thrombin generation, fibrinolytic activity, and endothelial dysfunction in patients on 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

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ticlopidine is 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

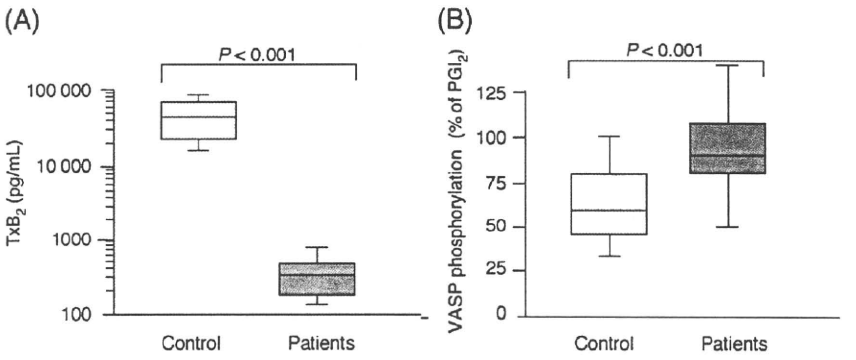

Figure 1 Serum thromboxane B_2 (TxB_2) concentration and vasodilator-stimulated phosphoprotein index in patients taking dual antiplatelet therapy. (*A*) The serum concentration of TxB_2 was measured by EIA. (*B*) The vasodilator-stimulated phosphoprotein phosphorylation was assessed by flow cytometry. 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,

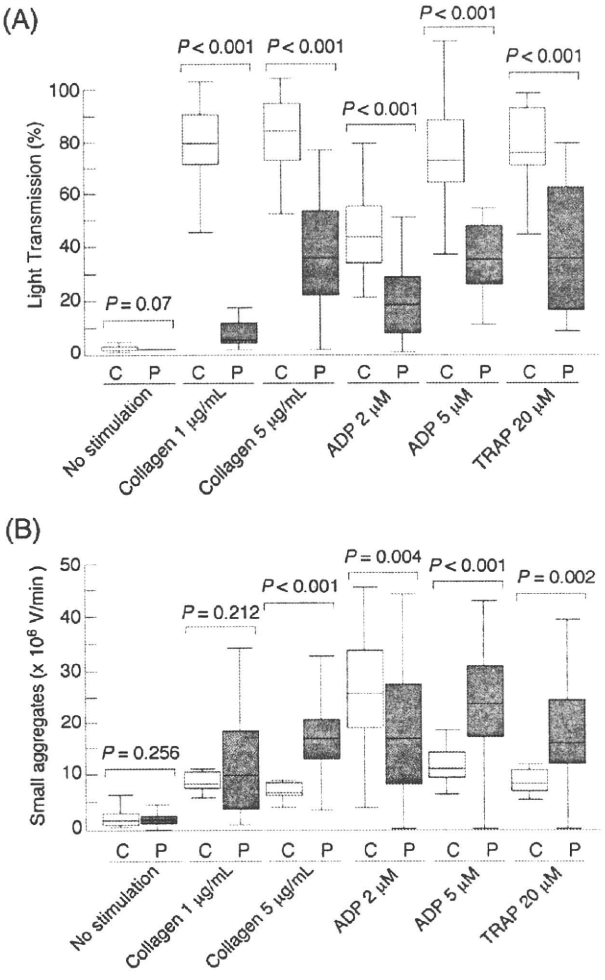

Figure 2 Platelet aggregation patterns in patients taking dual antiplatelet therapy. Platelets in platelet-rich plasma obtained from control subjects (*C*) or patients taking dual antiplatelet therapy (P) were stimulated with the indicated agonists for 5 min. (*A*) Changes in the maximum light transmission 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 ($P = 0.137$)	−0.17 7 ($P = 0.105$)	−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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INTERNAL MEDICINE

ORIGINAL ARTICLE

Molecular Defects Associated with Antithrombin Deficiency and Dilated Cardiomyopathy in a Japanese Patient

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Abstract

Objective The molecular basis for the antithrombin (AT) deficiency and dilated cardiomyopathy (DCM) combined in a Japanese patient was investigated.

Methods We analyzed candidate genes—*SERPINC1* for AT deficiency, and *TNNT2* and *LMNA* for DCM. In addition, we examined the characteristics of recombinant mutant AT and evaluated the *LMNA* mutation associated with DCM by molecular modeling.

Results Genome sequencing of *SERPINC1* revealed a C-to-A transversion in exon 6 that resulted in a p.Pro439Thr mutation of AT, which was previously reported as a pleiotropic effect type II AT deficiency (AT Budapest5). However, expression experiments with recombinant 439Thr-AT showed normal heparin affinity, slightly reduced secretion, and low specific activity, which suggested that this mutation exhibits an intermediate feature of type I and type II AT deficiencies. In a survey of gene abnormalities causing DCM, we found no causative gene defect in *TNNT2*; however, we identified a G-to-C transversion in *LMNA* that resulted in a novel p.Asp357His mutation in lamin A/C. This acidic-to-basic residue substitution might have impaired the head-to-tail association of two lamin dimers leading to DCM. Further, we identified both *SERPINC1* and *LMNA* mutations in the patient's daughter and son, both of whom had AT deficiency. These data suggested that a p.Pro439Thr mutation in *SERPINC1* and a p.Asp357His mutation in *LMNA* might have cosegregated in this family, associated with AT deficiency and DCM, respectively.

Conclusions We identified missense mutations in *SERPINC1* and *LMNA* genes to be associated with AT deficiency and DCM, respectively, which might have cosegregated in the family of the patient.

Key words: antithrombin (AT), pleiotropic effect, dilated cardiomyopathy (DCM), lamin A/C

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Introduction

Antithrombin (AT) is a plasma serine protease inhibitor that inhibits thrombin as well as other activated serine proteases of the coagulation system (1). Plasma AT plays a key role in the natural hemostatic balance to maintain blood fluidity. Therefore, patients with AT deficiency are susceptible to thromboembolic diseases, particularly deep vein thrombosis of the lower limb and pulmonary embolism. The human AT gene (*SERPINC1*) is located on chromosome 1q23-25 (2), and many defects of this gene have been reported in patients with AT deficiency (3). Congenital AT deficiency is usually heterozygous and classified into two types: quantitative deficiency (type I) and qualitative deficiency (type II). The latter includes reactive site defect, heparin binding site defect, and pleiotropic effect AT deficiencies.

Dilated cardiomyopathy (DCM), the most frequent form

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Table 1. Clinical Data of the Family Members Analyzed

Member	Age (yrs)	Antithrombin		Thrombosis	*EF (%)
		antigen (%)	activity (%)		
III2	47	49.7	52.0	**BI, 44yrs	38
IV1	27	58.6	43.9	None	65
IV3	22	59.0	59.8	None	***ND

*EF: ejection fraction (M.Simpson), **BI: brain infarction, ***ND: not done

of cardiomyopathy, is a myocardial disorder characterized by ventricular dilatation and impaired systolic function, which leads to congestive heart failure and sudden death (4). Familial cases of DCM were initially considered as quite rare; however, recent studies with systematic and careful screening of relatives of the patients have shown that up to 35% of patients with DCM have a familial disease (5). Familial DCM has been reported most commonly with autosomal dominant inheritance, and mutations in 16 autosomal genes have been the proximate cause until date (6). Among them, the most frequently reported genetic causes of DCM are mutations in the gene encoding lamin A/C proteins. The lamin A/C gene (*LMNA*), that is encoded on chromosome 1q21.2-q21.3 (7), has been reported to be involved in DCM associated with conduction system disease (8), and *LMNA* mutations can be found in up to 33% of cases of DCM in association with cardiac conduction disease (9).

AT deficiency is a risk of venous thromboembolism, and DCM is also a risk of systemic or pulmonary embolization, because blood stasis and low shear rate in the hypocontractile ventricle lead to the activation of coagulation processes (4). Therefore, the patient with both diseases in combination is likely to be in a highly thrombophilic state. Congenital AT deficiency and DCM are rare diseases inherited independently each other, and have never been reported in combination to date.

Here, we report a Japanese patient with combined congenital AT deficiency and DCM. We investigated the molecular defects associated with these two diseases, and identified the distinct missense mutations in *SERPINC1* and *LMNA* genes that might be responsible for AT deficiency and DCM, respectively.

Materials and Methods

Patient and sample preparations

The patient was a 47-year-old man, who had a history of brain infarction associated with cardiac arrhythmia at the age of 44, and was treated with an oral anticoagulant. He showed cardiomegaly on the chest radiography and left ventricular dilatation as well as hypokinesis (EF=38%) on the echocardiography, and was diagnosed as having DCM. A transesophageal echocardiography revealed a smoke-like echo that appeared to be a thrombus, although he had been taking warfarin. As the result of more intense warfarization, the thrombus disappeared. Finally, he was implanted a cardioverter defibrillator (ICD) to treat the sustained ventricular tachycardia. He was also diagnosed as having AT deficiency by blood coagulation tests (Table 1). In the family history, his monozygotic twin brother and maternal cousin had been also diagnosed as having AT deficiency and implanted a pacemaker because of complete AV-block (Fig. 1).

Ethical approval for the study was obtained from the Ethics Committee of the Nagoya University School of Medicine, as well as from the Ethics Committee of the Saga University Faculty of Medicine. Blood samples were obtained with an informed consent from the patient in accordance with the Declaration of Helsinki. Genomic DNA was isolated from peripheral blood leukocytes (10). We obtained DNA samples only from the proband and his two children, who were also diagnosed as having AT deficiency (Table 1).

Identification of gene abnormalities in the patient

All exons including splice junctions of the *SERPINC1* gene were amplified by polymerase chain reaction (PCR) and analyzed by direct sequencing (11). Similarly, *TNNT2* and *LMNA* genes were also analyzed by direct sequencing, after PCR amplification of all exons including splice junctions using the primer sets listed in Table 2.

Transient expression of recombinant ATs

We prepared a mutant human AT expression vector (pcDNA/439Thr-AT: the initial Met residue is denoted amino acid +1) using the recombinant PCR method (11). For heparin affinity experiments, we used the wild type AT (pcDNA/WT-AT) and the heparin affinity deficient AT Nagasaki (pcDNA/148Pro-AT) vectors as positive and negative controls, respectively (11).

Human embryo kidney 293 (HEK293) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5% CO_2, at 37 ℃. The cells were cultured in 60-mm dishes until they became 50% confluent and then transiently transformed with 10 μg of the expression plasmid vectors by the calcium phosphate method (12). After a 24 hours incubation in FCS-free DMEM, the cell culture media and cell lysates that had dissolved in the Reporter lysis buffer (Promega; Madison, WI, USA) were collected, centrifuged at 1500× g for 10 minutes, and used as Western blot samples. Western blot analysis was performed (13), using a polyclonal anti-AT antibody (rabbit IgG) and a peroxidase-labeled anti-rabbit IgG

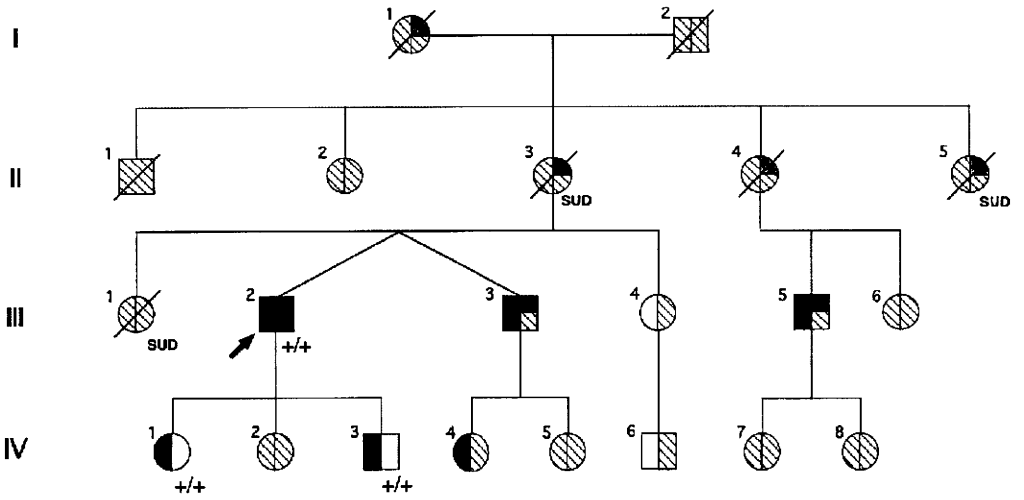

Figure 1. Pedigree of the family. Squares and circles show males and females, respectively. The proband is indicated by an arrow. Left half-black symbols, AT deficiency members; right upper quarter-black symbols, cardiac conduction disease members implanted with pace maker or ICD (proband); right upper quarter-gray symbols, unspecified heart disease members; right lower quarter-black symbol, DCM member; white symbols, unaffected members; shaded symbols, members of unknown clinical status; diagonal lines, dead members; SUD, sudden death, +/+, presence of *SERPINC1* p.Pro439Thr and *LMNA* p.Asp357His heterozygous mutations. Genotypes of 3 members were tested, but no sample from other members was available.

Table 2. Oligonucleotide Primers of PCR Amplifying *TNNT2* and *LMNA* Genes

Gene	Exon	Sense (5' → 3')	Antisense (3' → 5')	Product (bp)	Annealing (°C)
TNNT2	2	TTTTGTTGCAGGTCACACAG	AGGGGTACAGGAGTGGAAAG	367	56
	3+4	GCAACAAGGGAAAAGAAAGG	GAAGGCACTGTTGTTGGAGG	391	56
	5	AATGCCGGCCTAACTCCAA	GGAGGCAGGGGAGGAAAC	308	59
	6+7	TGCTCTGGGTTCTGCCTG	CTGCTGTGAGGGGTTCCTT	578	56
	8	GTGCAGATGGGGAAATGGA	CCTTAGGAAGAGACGCTTGTG	313	56
	9+10	TCAGTCCCTGGGTCCAGAA	GGATGGAGGACAGACTGGG	590	59
	11	AAAGTGGAGGCCCTTGGA	GATGAATAGAGAGGGGCCTG	315	59
	12	CAAGCTTCAGCCCAGAATCA	CAGTCTTCCACCCACAGCA	314	56
	13	TTGGTCTTTTTCTATGGGCCT	AGAGCAGATGCGGGCAGT	574	56
	14	TTGGCAGGCCTGGAGGT	GGCAGATGCAGGAGCTGA	347	59
	15+16	GCCAGTCAGCTCCAGCGT	GCGAGGAGCAGATCTTTGGT	537	59
LMNA	1	GCACTCCGACTCCGAGCA	CGCCGCCCTCTCCACTC	502	64
	2	GACCTCCTGGGAGCCTG	GGAGGGCCTAGGTAGAAGAGT	306	61
	3	GCAGCAGCCCACCTCTC	AAGGCGAGCTCTGCACAC	301	61
	4 1st	GACAGGGAGTTGGGGGTGG	TGACTGGGAGGGGTGGAGG	510	63
	2nd	GAGTAGGGCTGGGCAGG	AGCGTGGGTAAGGGTAGG	330	60
	5	CCTGGGGCTGTAGCAGTGA	CTGTGGTTGTGGGGACACTTT	354	60
	6	CTACACCGACCCACGTCC	CCAAGTGGGGGTCTAGTCAA	374	59
	7	GGGAGGTGCTGGCAGTGT	CTCTCTCCACATGCCATCCTT	378	59
	8+9	TGAGCCTCCCCGACCTT	TCCGATGTTGGCCATCAG	413	59
	10	AAAGGGCAGGCCACAAGA	CAGAGTAGGGCACCCAGACA	394	64
	11	TTGGGCCTGAGTGGTCAGT	CTCGTCCTACCCCTCGATG	398	59
	12	GAGGGGTAGGACGAGGTGG	TGAGGTGAGGAGGACGCAG	354	64

antibody (Behringwerke AG; Marburg, Germany).

Specific anticoagulant activities and heparin affinities of recombinant ATs

We established stable transformants of HEK293 cells highly expressing the recombinant AT molecules and measured their antigen levels in the culture media by enzyme-linked immunosorbent assay (11). Heparin cofactor activity of the AT was measured using Ntest AT III-S kit (Nittobo; Tokyo, Japan). Progress activity of the AT was measured in the absence of heparin. The specific activity of each recombinant AT molecule was calculated as a percentage of the wild type activity.

We also performed affinity chromatography of each re-

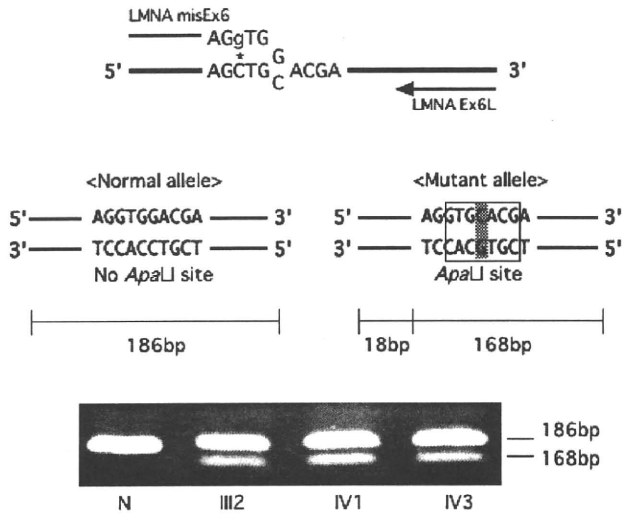

Figure 2. Mismatch PCR-*Apa*LI RFLP detected *LMNA* mutation. *Apa*LI-RFLP (PCR-mediated) using partially mismatched sense primer (LMNA misEx6) to detect a c.1,069G>C mutation in exon 6. PCR products from mutant allele DNA will yield a 168-bp fragment after being digested by *Apa*LI, whereas those from normal alleles yield an uncleaved 186-bp size band. Lane N shows a normal subject.

combinant AT molecule on a heparin-sepharose column as described previously (11). The bound AT was eluted by a stepwise increasing concentration of NaCl (0.25, 0.5, 0.75, 1.0, and 1.5 mol/l). The concentration of AT in each fraction was determined by dot blot assay, which showed data similar to ELISA.

Mismatch PCR-ApaLI-Restriction Fragment Length Polymorphism

We analyzed the identified mutation in *LMNA* gene by PCR-mediated restriction fragment length polymorphism (RFLP) as described previously (14). Thus, we designed a mismatch PCR strategy to introduce a new restriction enzyme (*Apa*LI) site into the PCR products through the mutant allele (Fig. 2). We used a partially mismatched sense primer (LMNA mis6U: 5′-CAAGGATGCAGCAGCAGgTG), which introduced an *Apa*LI site only into the mutant allele PCR products, to amplify a part of exon 6 fragment of *LMNA* gene. Subsequently, the PCR products were digested with *Apa*LI, and resolved by 3% agarose gel electrophoresis with ethidium bromide.

Results

We analyzed *SERPINC1* gene in the proband by PCR-mediated direct sequencing, and identified a C-to-A transversion in exon 6 [c.1315C>A; according to recommendations for the description of DNA sequence variants by Human Genome Variation Society (15)] in the heterozygous state, which was previously known as AT Budapest 5 (p.Pro439Thr) mutation with a pleiotropic effect phenotype (16). We also detected the same mutation in both his daughter and son in the heterozygous state, which was confirmed by *Stu*I RFLP analysis (data not shown).

In this study, we investigated the influences of p.Pro439-Thr mutation on secretion and function of the recombinant AT molecule. We observed a decrease in the secretion of the mutant 439Thr-AT (74%), and a slight decrease in progressive activity (84%) as well as heparin cofactor activity (83%) of the mutant, as compared with the wild type AT (Fig. 3). We also compared the heparin affinity of the recombinant ATs using heparin-sepharose affinity chromatography (Fig. 3C). We observed an unexpected normal heparin affinity in the recombinant 439Thr-AT, whereas the recombinant 148Pro-AT (AT Nagasaki) showed an impaired affinity to heparin as reported previously (11, 17).

Subsequently, we attempted to analyze two candidate genes causing DCM (*TNNT2* and *LMNA*), which are located on the same chromosome as *SERPINC1* (chromosome 1). In *TNNT2*, a 5bp deletion polymorphism in intron 3, which was reported as a risk of left ventricular hypertrophy in the homozygous state (18), was detected in the heterozygous state, although no other sequence alteration was found (data not shown). In *LMNA* gene, a G-to-C transversion in exon 6 (c.1069 G>C), resulting in a novel p.Asp357His mutation of the lamin A/C molecule, was identified in the heterozygous state. We also detected a heterozygous missense mutation in both his daughter and son, which was confirmed by the mismatch PCR-*Apa*LI-RFLP (Fig. 2). Additionally, the PCR-*Apa*LI-RFLP assay of 72 samples from healthy volunteers showed a single band (186 bp), suggesting that the c.1069 G >C mutation would not be a single nucleotide polymorphism (data not shown).

Discussion

We investigated the molecular basis of the AT deficiency and DCM in a family with both the diseases and identified p.Pro439Thr missense mutation in *SERPINC1* gene and p. Asp357His missense mutation in *LMNA* gene.

The p.Pro439Thr mutation in *SERPINC1* gene has been reported as AT Budapest 5 with a pleiotropic effect, which altered the reactive site and heparin-binding properties of the variant (16). It was reported that the patient with AT Budapest 5 had a normal level of AT antigen and a slightly decreased AT activity in the plasma; however, the proband in this study showed half levels of both plasma AT activity and antigen. The reason for this discrepancy is not clear, but it might be possible that some different environmental circumstances or other genetic factors influenced circulating levels of ATs. Some type II AT mutations were reported as having very low levels of AT antigen like a type I AT mutation, even though they had an identical mutation (3). In addition, the secretion of recombinant 439Thr-AT in our expression experiments was moderately reduced, whereas its heparin affinity was normal, and its progressive activity and heparin cofactor activity were mildly impaired. These data suggest

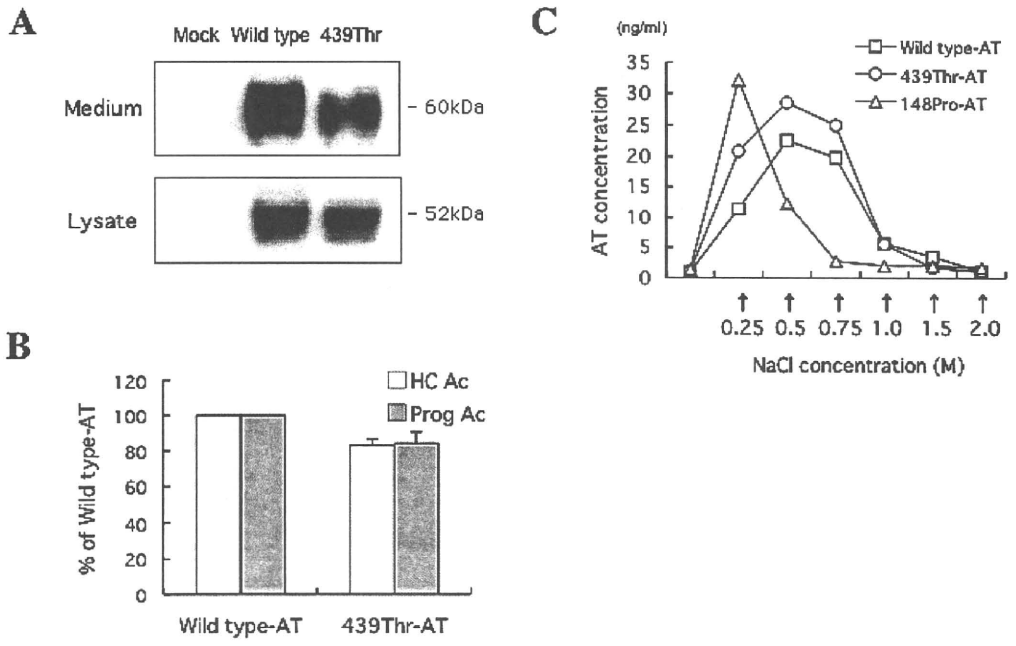

Figure 3. Molecular characterization of recombinant ATs. (A) Western blot analysis of the recombinant AT molecules. Wild type-AT and 439Thr-AT were transiently expressed in HEK293T cells, and the culture media and lysates were analyzed. Ten micrograms of each sample was loaded to normalize total protein. (B) Progressive AT activities (Prog Ac) and heparin cofactor AT activities (HC Ac) of the recombinant proteins stably expressed in the culture media were examined as described in Materials and Methods. Specific AT activities were calculated as a percentage of wild type-AT activity (mean±SD %, n=3). (C) Affinity chromatography of the recombinant AT molecules was performed on a heparin-sepharose column as described in Materials and Methods.

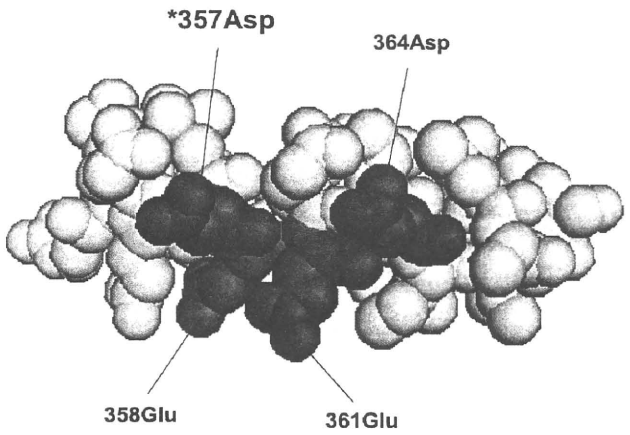

Figure 4. Molecular model of human lamin A/C coil 2B domain. Three-dimensional model of the partial peptide of the lamin A/C coil 2B domain (residues 351 to 370; protein data bank ID, 1×8y). The amino acids were shown as the spheres, and the acidic residues are colored in gray and others in white. The figure was designed by using MacPyMoL 0.99 (DeLano Scientific LLC, Palo Alto, CA, USA; http://pymol.sourceforge.net/).

that the p.Pro439Thr mutation would cause intermediate features of type I and type II AT deficiencies in the patients.

We subsequently tried to determine the cause of DCM in the same patient. We analyzed two candidate genes causing DCM (*TNNT2* and *LMNA*), located on the same chromosome as *SERPINC1* (chromosome 1), and identified a novel p.Asp357His mutation in *LMNA* gene, although no causative abnormality in the *TNNT2* was detected. There is no direct evidence that this *LMNA* mutation causes DCM, however, some relatives including his monozygotic twin brother suffered from cardiac conduction disturbance, which is common in DCM caused by *LMNA* mutations, suggesting that the *LMNA* p.Asp357His mutation could be associated with DCM.

Amino acid alignments of a part of the lamin A/C coil 2B fragment around an Asp357 in several species, including human, monkey, dog, mouse, chicken, frog and fish, shows that amino acid sequences are highly conserved among all species. In crystallographic modeling approach, the Asp357 lies within the highly conserved and exclusive acidic C-terminal sequences of the rod domain, which form one pronounced patch of negative electrostatic potential (Fig. 4). It was hypothesized that this acidic patch may be electrostatically attracted by a net positively charged cluster of Arg residues of the *N*-terminal head domains, and this interaction is important in the linear assembly of lamins (19). Therefore, we assumed that the substitution of an acidic aspartate with a basic histidine residue would impair normal lamina formation; hence, p.Asp357His mutation in *LMNA* gene