**Table 1**
Analytical recovery of FV:PLbound from FV depleted plasma.

| Theoretical values (%)* | Assay values (%) | Recovery (%) |
|---|---|---|
| 100 | 95 | 95 |
| 50 | 54 | 108 |
| 25 | 28 | 112 |
| 12.5 | 12.5 | 100 |
| 0 | 0 | |

*Amounts of normal plasma to FV depleted plasma (normal plasma concentration: 100, 50, 25, 12.5 and 0%).

x50, x100, x200, x400, x800 in 1.0% BSA-TTBS). The standard FV:PL-bound curve is shown in Fig. 1. When two samples were measured 8 times using this standard curve, the coefficient of variation was 2.98 (mean: 84.8%) and 12.08 (mean: 46.6%).

*Analytical recovery of FV:PL-bound from factor V depleted plasma*

To determine the accuracy of FV:PL-bound measurements, different amounts of normal plasma were added to FV depleted plasma (Dade Behring, Marburg, Germany) (reconstituting normal plasma concentrations of 100, 50, 25, 12.5 and 0%), and the analytical recovery of FV:PL-bound was measured (Table 1). In these experiments, the mean analytical recovery (range) of FV:PL-bound was 103% (95-112%) of the diluted normal plasma.

*Characteristics of the study subjects*

Characteristics of the study subjects are shown in Table 2. One hundred and twenty-three patients (53 men and 70 women) and 100 healthy subjects (45 men and 55 women) were recruited. Mean age ± SD (range) of patients and healthy subjects was 53.7 ± 15.4 (19-88) years and 53.2 ± 15.5 (19-81) years, respectively. There was no significant difference in the mean age or sex ratio between patients and healthy subjects ($p=0.883$ and $p=0.529$, respectively). A thrombophillic cause was found in 27 patients (22.0%), i.e., deficiency of antithrombin ($n=2$), protein C ($n=5$) or protein S ($n=20$). FV Leiden and the prothrombin G20210A mutation were not examined because of the extremely low prevalence of these mutations in the Japanese population. Thrombosis occurred in the absence of acquired

**Table 2**
Characteristics of patients with DVT and control subjects.

| | Patients n = 123 | Controls n = 100 |
|---|---|---|
| Age (y) | | |
| Mean (y) | 54 | 53 |
| Range (y) | 19-88 | 19-81 |
| Sex | | |
| Male (n) | 53 | 45 |
| Female (n) | 70 | 55 |
| Anti-thrombin deficiency (n) | 2 | 0 |
| Protein C deficiency (n) | 5 | 0 |
| Protein S deficiency (n) | 20 | 0 |

risk factors (oral contraceptives, recent trauma, cancer, pregnancy or child birth, and immobilization) in all cases, and none of the patients were on anticoagulants or in the acute phase of DVT at the time of blood sampling. Moreover, anticardiolipin-β2glycoprotein I antibodies were negative in all patients. Among these 123 patients, 18 had had two thrombotic episodes, and the remaining 105 had had one episode. Patients were also asked about the occurrence of venous thromboembolism in their families. One of them had a family history of venous thromboembolism, and 1 patient had a family history of myocardial infarction.

*Levels of FV:Ag and FV: PL-bound*

Fig. 2 shows the distribution of FV:Ag and FV:PL-bound in DVT cases and controls. A Gaussian distribution was noted for the FV:Ag and FV:PL-bound levels in the control group. The FV:Ag, and FV:PL-bound were significantly lower in DVT patients (FV:Ag, median, 89.3; Inter Quartile Range (IQR), 24.1; range, 24.3-222.2. FV:PL-bound, median, 78.0; IQR, 19.5; range, 22.6-165) than in control subjects (FV:Ag, median, 95.8; IQR, 11.6; range, 41.2-194.3. FV:PL-bound, median, 91.3; IQR, 13.3; range, 36.0-162) ($p<0.05$ and $p<0.005$, respectively). Few patients had low levels of FV:Ag and FV:PL-bound, and the distribution of values among patients were distinctly bimodal: 24.4% fell below the 5th percentile of control values, and 57.7% fell below the 50th percentile of control values for FV:Ag levels. 26% fell below the 5th

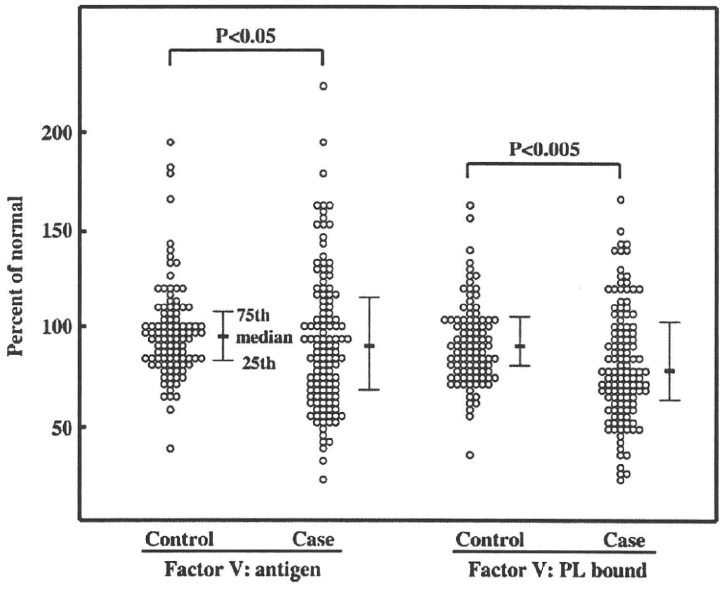


**Fig. 2.** Distribution of factor V antigen and PL-bound factor V levels in DVT patients (n = 123) and control subjects (n = 100). Values are shown as percentages of pooled normal plasma values. The median, 25th and 75th percentiles are indicated. Significance was determined by the Mann-Whitney U test.

**Table 3**
Factor V levels and risk of DVT.

| | Positive/total | OR | 95%CI |
|---|---|---|---|
| FV: Ag | | | |
| Control | 5/100 | | |
| Case | | | |
| Including deficiencies* | 30/123 (24.4%) | 6.1 | 2.3-16.5 |
| Excluding deficiencies* | 24/96 (25.0%) | 6.6 | 2.4-18.3 |
| FV: PL-bound | | | |
| Control | 5/100 | | |
| Case | | | |
| Including deficiencies* | 32/123 (26.0%) | 6.7 | 2.5-17.9 |
| Excluding deficiencies* | 27/96 (28.1%) | 7.4 | 2.7-20.3 |

FV:Ag and FV:PL-bound indicate the factor V antigen and the capability of factor V to bind to phospholipid. Odds ratios (ORs) are given as determinations of relative risk for developing DVT. The risk was evaluated using the 5th percentile (Antigen: 66.5%, PL bound: 63.0%) of the distribution found in plasma from controls.
*Deficiencies of antithrombin, protein C and protein S.

percentile of control values, and 64.2% fell below the 50th percentile of control concentrations for FV:PL-bound.

*Evaluation of FV:Ag and FV:PL-bound levels as predictors of risk for DVT*

It was examined whether a low FV level is a risk factor for development of DVT. Thirty five and 41% of patients had FV:Ag and FV:PL-bound levels that fell below the 10th percentile of control values, respectively, and individuals with FV:Ag and FV:PL-bound levels below the 10th percentile had a 3.3 and 6.2 fold increased risk of developing DVT compared to that of individuals with a FV:Ag and FV:PL-bound level above this cutoff value. Of the 123 patients, 30 (24.4%) showed FV:Ag levels below the 5th percentile of control values, 32 (26%) showed FV:PL-bound levels below this cutoff value, and the ORs were 6.1 (95%CI, 2.3-16.5) and 6.7 (95%CI,2.5-17.9) respectively. Six of the 30 patients with a low FV:Ag level and 5 of the 32 patients with low FV:PL-bound levels were deficient in protein S; other deficiencies (antithrombin or protein C) were not found. When patients deficient in natural anticoagulant were excluded from the analysis, 24 (25%) and 27 (28%) of the remaining 96 patients had FV:Ag and FV:PL-bound levels below the cutoff points, respectively, and the ORs (6.6 and 7.4) increased compared to that of the total patient group. ORs for development of DVT were higher for low FV:PL-bound levels than for low FV:Ag levels (Table 3).

*Correlation of FV:Ag and FV:PL-bound levels*

The present method for measuring FV:PL-bound was compared with the FV:Ag assay, using DVT patients and healthy subjects. Fig. 3 shows the correlation between FV:Ag and FV:PL-bound in cases and controls. The correlation coefficient (r) between FV:Ag and FV:PL-bound levels, in normal subjects and DVT patients, was 0.658 ($p<0.00001$) and 0.622 ($p<0.00001$), respectively. Twenty one (17%) of 123 DVT patients and 1 (1%) of 100 control subjects were below the cutoff point for FV:Ag and FV:PL-bound, (which was the 5th percentile for healthy subjects), and the OR was 21.6 (95%CI, 2.85-163.1). Three of 32 DVT patients, who had FV:PL-bound values below the 5th percentile for control values, had a discrepancy with their FV:Ag level (FV:PL-bound/FV:Ag level, 22.6/117.6, 35.9/196, and 48/222.2).

*Mixing study for discrepancy samples between FV:Ag and FV:PL-bound*

Three samples that had discrepancies between the FV:Ag and FV:PL-bound concentrations, were studied by a mixing test. We added different amounts of normal plasma to the patient's plasma (normal plasma concentrations of 100, 80, 60, 40, 20, and 0%) and FV deficient plasma, then measured the FV:PL-bound levels. When each concentration of normal plasma was added to the discrepancy sample, in all three of the samples, the assay values fell lower than theoretical values (Fig. 4).

## Discussion

The importance of the protein C pathway is underscored by the observation that protein C deficiency, protein S deficiency, and the FV Leiden mutation are associated with an increased risk of thromboembolic events. Deficiencies in natural anticoagulants (protein C, protein S, and antithrombin) were previously described in approximately 30% of Japanese DVT patients [13]. Moreover, the FV Leiden mutation and the prothrombin G20210A mutation that are found in Caucasian populations are not found in the Japanese population [14,15]. However, in Japanese patients with thrombosis, the prevalence of cases of unknown cause remains greater than 60%. FV has a two-way function (procoagulant and anticoagulant), and it is surmised that the balance of these two functions of FV influences the protein C pathway. The breakdown of this balance may lead to development of thrombosis.

The present study evaluated the relationship between FV levels and venous thrombosis. For comparison of FV levels between DVT patients and healthy subjects, an assay method was developed to

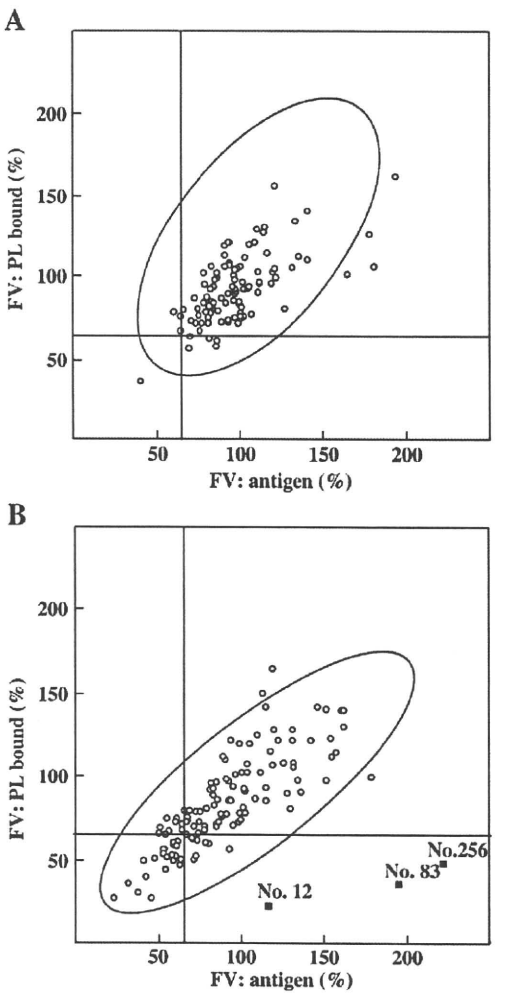


**Fig. 3.** Correlation between FV:antigen and FV:PL-bound levels in normal subjects and DVT patients. The horizontal and vertical lines indicate the cut-off values from the 5th percentile from normal controls. The ellipses represent 95% bivariate tolerance regions. (A) Normal subjects. (B) DVT patients. The closed square indicates the samples with discrepancies between FV:Ag and FV:PL-bound values.

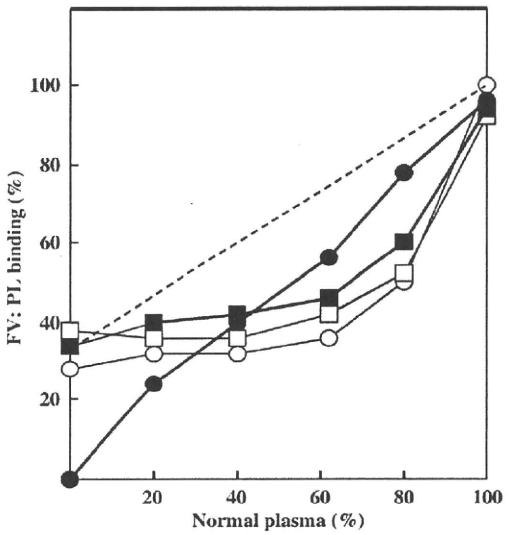


**Fig. 4.** Mixing study for discrepancy samples between FV:Ag and FV:PL-bound. Values of the mixing test in samples number 12 (○), 83 (■),256 (□), and Factor V deficient plasma (●) are shown. Dotted lines represent the average of the theoretical value in the 3 samples.

determine the capability of FV to bind to phospholipid. The correlation in healthy subjects, between FV activity and FV:Ag or FV:PL-bound was significant; the correlation coefficient (r) was 0.68 ($p<0.0001$) and 0.57 ($p<0.0001$), respectively.

Our results suggest that low FV levels are a risk factor for DVT in the Japanese population, and were not associated with deficiencies of natural anticoagulants. Studies have been performed to analyze whether low levels of circulating FV increase the risk of thrombosis. However, in contrast to other reports regarding FV levels and thrombosis, levels of plasma FV displayed a statistically significant association with thrombosis in this current study. Concerning mild FV deficiency in Japanese subjects, we had reported that the Japanese population might have a relatively high prevalence of mild FV deficiencies with 36-64% of normal antigen levels [16]. In this study, unfortunately, we were not able to carry out gene analysis of the patients who had low levels of FV. However, we think that a decrease of FV is not caused by consumption of this factor during DVT development because our patients did not demonstrate the acute phase of DVT. These results differ from those of Kamphuisen et al. [11], who reported that factor V antigen levels are not associated with a risk of venous thrombosis. This discrepancy might be due to the difference in location and institution where the studies were performed.

FV is the key in the procoagulant and anticoagulant pathways. From previous reports, the existence of two pathways has long been established [17–22] with formation of prothrombin activation via meizothrombin or prethrombin2. Accumulation of intermediate prethrombin2 is observed in the absence of FVa [17–19], and accumulation of the intermediate meizothrombin is observed in the presence of fully assembled prothrombinase [23]., and moreover, meizothrombin can bind thrombomodulin and activate protein C; following this reasoning, meizothrombin is approximately 6-fold more effective in activating protein C than is thrombin [24–26]. Therefore, it is surmised that the balance of these two pathways in prothrombin activation influences protein C activation. On the other hand, previous reports have described that the intact pro-cofactor, single chain FV, can act as a cofactor, contributing to the acceleration of inactivation of FVIIIa by APC [7,27]. Low FV levels are associated with a reduced APC cofactor activity in the inactivation of FVIIIa [7–9], which could be responsible for an APC-resistance phenotype [28]. These reports indicate the possibility that low FV levels impair the protein C pathway and result in development of thrombosis. For the verification of our speculation, in DVT patients who have low levels of FV, confirmation of accumulation of prothrombin2 during the coagulation process and low levels of activated protein C resistance are necessary in future studies.

In the patients studied here, three were observed with chronic rheumatoid arthritis (not having anti-cardiolipin antibody and lupus anticoagulant), and they had considerably-low levels of FV:PL-bound in comparison with FV:Ag. Furthermore, in the mixing study, all 3 of the samples were not revived by addition of normal plasma. These results suggest that an inhibitor to the reaction of FV and phospholipid (for example auto-antibodies against FV), have existed in these samples. Although antibodies against FV generally have no clinical consequences or induce a bleeding tendency, a number of cases exist in which the development of FV auto-antibodies was associated with thrombosis. In previous reports, spontaneous anti-FV antibodies have been associated with thromboembolic complications in 3 patients, and the presence of an inhibitor with both anti-FV and lupus like features has been described [29–31]. The molecular mechanisms that yield an increased risk of thrombosis in the rare patients with thrombosis are not known. From the results in this study, a depression of the binding capacity of FV to phospholipid by an inhibitor may be one of the causes of thrombosis development.

Measurement of FV's ability to bind PL and FV:Ag concentration are appropriate markers for further association studies investigating the relationship of FV levels and thrombotic disease. This hypothesis needs to be tested in large thrombophilic families and also a larger series of patients with DVT in another area.

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◆編集者への手紙◆

# Effect of Beraprost Sodium on Coagulation in Peripheral Arterial Disease

Tomio KAWASAKI [*1], Etsuji SUEHISA [*2], Masayuki TOKU [*2], Sumiko TORITA [*2], Masato MINAMI [*3]

**Key words:** beraprost sodium, D-dimer, coagulation, peripheral arterial disease, drug dosing interval

## 1. Introduction

Antiplatelet drugs are effective and are used in the management of peripheral arterial disease (PAD). A Japanese PAD cohort study (J-METHOD) revealed that use of beraprost sodium, a prostaglandin I2 analogue, inhibited the progression of arteriosclerosis[1]. D-dimer is considered to be a consistent marker of the risk of cardiovascular disease[2]. Here, we examine the possible inhibition of D-dimer formation by beraprost sodium.

## 2. Methods

Fifteen patients (10 men, 5 women) suffering from Fontaine stage II PAD and who had been in stable condition for at least several months comprised this retrospective study. Average age was 72±2 years (mean±SEM), and each patient took a daily dose of 120μg of beraprost sodium split into individual doses of 40μg after each meal. Diagnosis and staging were determined on the basis of a typical clinical history and decreased ankle systolic blood pressure by Doppler ultrasound examination. Co-existing disease present in the study patients included diabetes mellitus (n=6), coronary artery disease (n=4), cardiovascular disease (n=3), hypertension (n=10), and hyperlipidemia (n=6). Patients who developed apparent complications or deterioration of PAD over the course of this study were excluded because of the possibility of secondary thrombosis due to arterial occlusion.

Blood samples were collected in the morning after overnight fasting. Measurements of levels of prothrombin fragment 1+2 (F1+2) and D-dimer were performed on the day before beginning beraprost sodium therapy (baseline) and after taking the drug for 1 month. F1+2 levels were determined with Enzygnost F1+2 (normal range : 69-229 pmol/L ; Dade Behring Marburg GmbH, Marburg, Germany), and D-dimer levels were measured with LPIA-ACE D-dimer (normal range : 0-0.5 μg/ml ; Mitsubishi Chemical Medience, Tokyo, Japan). The data obtained at baseline and following 1 month of therapy with beraprost sodium were compared with Wilcoxon's signed-rank sum test. Differences were considered statistically significant at $p<0.05$.

[*1] 大阪大学大学院医学系研究科外科学講座心臓血管外科学〔〒565-0871 大阪府吹田市山田丘 2-2〕
Department of Cardiovascular Surgery, Osaka University Graduate School of Medicine
〔2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan〕
Tel: +81-6-6879-3154 Fax: +81-6-6879-3163 e-mail: kawasaki@ck9.so-net.ne.jp
[*2] Central Laboratory for Clinical Investigation, Osaka University Hospital
[*3] Department of General Thoracic Surgery, Osaka University Graduate School of Medicine
受付日：2010年9月24日，受理日：2010年11月9日

## 3. Results

Plasma levels of D-dimer decreased with statistical significance from baseline (1.03±0.17 μg/ml, mean ± SEM) to post-treatment with beraprost sodium (0.85±0.16 μg/ml) ($p<0.01$). However, plasma levels of F1+2 showed no remarkable change between baseline (260±30 pmol/L, mean±SEM) and post-treatment (287±32 pmol/L, $p>0.05$).

## 4. Discussion

Prostaglandin I2 does not inhibit fibrinolysis[3]. Because beraprost sodium is a prostaglandin I2 analogue, it may be accepted that a decrease in D-dimer is not the outcome of a decrease in fibrinolytic activity in this study. Thus, our results show that beraprost sodium inhibits coagulation in PAD patients, although the mechanism of inhibition of coagulation has not yet been fully elucidated. D-dimer concentration is an independent predictor of the progression of PAD[4]. The mechanism of activation of coagulation in stable PAD patients may be speculated from the results of Bini et al., who observed distribution of fibrinogen, crosslinked fibrin, and D-dimer in small flecks in fibrous plaque in atherosclerotic vessel wall, which suggests a possible involvement of macrophages and vascular smooth muscle cells located in the intima in fibrinogen-to-fibrin transition[5]. Further, the mechanism of inhibition of coagulation on these cells by beraprost sodium may be explained by the results of Niwano et al., who observed eNOS expression in vascular endothelial cells with treatment by beraprost sodium[6]. Expression of eNOS inhibits conversion of vascular endothelial cells into a procoagulant surface that recruits leukocytes and macrophages. The mechanism of the decrease in D-dimer may be explained by the cytoprotective effect of beraprost sodium on vascular endothelial cells that inhibits migration of leukocytes and macrophages into the intima.

One-month administration of beraprost sodium did not show significant effect on the blood levels of F1+2 in this study. The incompatible results of F1+2 compared with D-dimer may be explained by the drug dosing interval, half-life time, and the formation of thrombin accompanied by inactivation soon after its generation on vascular endothelial cells. In Japan, drugs indicated to be taken three times a day are usually administered after meals rather than every 8 hours, possibly due to the benefit of food in the mitigation of the side effects of drugs. The effect of these two different administration patterns on markers of coagulation has not been fully elucidated. When taking breakfast at 7 AM, lunch at 1 PM, and dinner at 7 PM, the interval from dinner to breakfast is about 12 hours, twice the time of each of the other two dosing intervals. Intermittent phase shift of drug dispensation may activate coagulation through the rebound phenomenon[7]. This can occur during the 12-hour period of cessation of beraprost sodium at nighttime, and levels of coagulation parameters may possibly increase during this period. The amount of D-dimer measured represents the summation of its variation throughout the day because of its longer half-life time of 8 hours[8], whereas the level of F1+2 represents activated coagulation in the early morning because of its shorter half-life time of 1.5 hours[8], when beraprost sodium becomes ineffective.

In PAD patients, tissue factor is expressed on the surface of the vascular endothelial cells that are activated by inflammatory cytokines[9]. F1+2 is a peptide generated during the conversion of prothrombin to thrombin that is immediately inactivated by binding to either thrombomodulin or antithrombin on the surface of the endothelial cells. Thus, the increase in the amount of F1+2 does not necessarily indicate the conversion of fibrinogen to fibrin on the vascular endothelial cells or an increase in the plasma level of D-

dimer. Beraprost sodium increases cyclic AMP that inhibits tissue factor expression[10] and enhances thrombomodulin expression on vascular endothelial cells[11]. The removal of beraprost sodium diminishes the sustained effect of the drug on thrombomodulin expression within 9 hours[11]. Thus, the amount of F1+2 measured in the morning does not reflect the effect of beraprost sodium on the vascular endothelial cells, and this may be the reason why the change in F1+2 between baseline and post-treatment was not consistent with that of D-dimer.

In conclusion, this study demonstrates for the first time, to our knowledge, that beraprost sodium inhibits coagulation in PAD patients as indicated by the decrease in D-dimer level measured after 1 month of therapy. The statistical insignificance of the change in F1+2 levels may be explained by its shorter half-life time and changes in coagulation activity through an uneven "after meals" method of drug administration. A limitation of this study is its small number of patients. Additional prospective and/or intervention studies comparing regular administration of beraprost sodium every 8 hours with irregular intermittent dosing adjusted to meals are required to determine a dosage method that maximizes the therapeutic effects while minimizing the side effects of the drug.

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## Disclosure of Conflict of Interests

The authors indicated no potential conflict of interest.

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ORIGINAL ARTICLE

# Usefulness of antithrombin deficiency phenotypes for risk assessment of venous thromboembolism: type I deficiency as a strong risk factor for venous thromboembolism

**Mana Mitsuguro · Toshiyuki Sakata · Akira Okamoto · Sachika Kameda · Yoshihiro Kokubo · Yoshiaki Tsutsumi · Michitaka Sano · Toshiyuki Miyata**

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**Abstract** Inherited antithrombin deficiency, an established risk factor for venous thromboembolism (VTE), can be classified into type I (quantitative deficiency) or type II (qualitative deficiency). In the present study, we assessed the VTE risk associated with the phenotypes of antithrombin deficiency in patients admitted to our hospital. We found that patients with type I deficiency ($n = 21$) had more VTE events and earlier onset of VTE than those with type II deficiency ($n = 10$). The VTE-free survival analysis showed that the risk for VTE in patients with type I deficiency was sevenfold greater than that in patients with type II deficiency (hazard ratio: 7.3; 95% confidence interval: 1.9–12.2; $P = 0.0009$). The prevalence of type I deficiency in the VTE group (5.6%, 6/108) was higher than that in the general population (0.04%, 2/4,517) (odds ratio: 132.8; 95% confidence interval: 26.5–666.1; $P < 0.0001$). However, the prevalence of type II deficiency was not different between the VTE group and the general population. Our study indicated that the risk for VTE in patients with type I deficiency was much higher than that in patients with type II deficiency. Thus, simple phenotypic classification of antithrombin deficiency is useful for assessment of VTE risk in Japanese.

**Keywords** Antithrombin deficiency · Deep vein thrombosis · Phenotype · Risk assessment · Venous thromboembolism

M. Mitsuguro · T. Sakata (✉) · A. Okamoto · S. Kameda · M. Sano
Laboratory of Clinical Chemistry,
National Cerebral and Cardiovascular Center,
5-7-1, Fujishirodai, Suita, Osaka 565-8565, Japan
e-mail: tsakata@hsp.ncvc.go.jp

Y. Kokubo
Department of Preventive Cardiology,
National Cerebral and Cardiovascular Center,
5-7-1, Fujishirodai, Suita, Osaka 565-8565, Japan

Y. Tsutsumi
Department of Medicine, National Cerebral and Cardiovascular Center, 5-7-1, Fujishirodai, Suita, Osaka 565-8565, Japan

T. Miyata
Research Institute, National Cerebral and Cardiovascular Center,
5-7-1, Fujishirodai, Suita, Osaka 565-8565, Japan

## 1 Introduction

Antithrombin is a serine protease inhibitor and functions as a potent natural anticoagulant by inactivating proteases in the coagulation cascade [1–3]. Since Egeberg's [4] first report of antithrombin deficiency in a Scandinavian family in 1965, numerous additional families with antithrombin deficiency have been reported [5, 6]. The majority of individuals with antithrombin deficiency are heterozygotes, and homozygotes are extremely rare.

Antithrombin deficiency is phenotypically classified into two types. In type I deficiency, both antithrombin activity and antigen levels are low in the plasma, indicating that the protein is not produced by the mutant allele. In type II deficiency, low antithrombin activity within normal antigen limits is observed, indicating a functional impairment of the molecule. Type II deficiency can be further divided into three subtypes: a heparin-binding site subtype, in which heparin binding is abnormal; a reactive site subtype, in which the reactive center loop is abnormal; and a pleiotropic effect subtype, in which the influence is pleiotropic [3, 5].

Antithrombin deficiency or low antithrombin levels are associated with increased incidence of VTE and are a risk for first and recurrent VTE [1–3, 7–12]. Asymptomatic antithrombin deficiency is present as frequently as 1:600 [9, 13, 14], and type II deficiency is more prevalent than type I deficiency in the general population [13]. In a cumulative analysis of the literature, more than half of the patients with antithrombin type I deficiency or the reactive site or pleiotropic effect subtypes of type II deficiency manifested VTE events [15–17]. However, the incidence of VTE events in patients with the heparin-binding site subtype of type II deficiency was very low (only 6%), although patients who were homozygous or compound heterozygous for the heparin-binding site subtype mostly manifested VTE events [15–18]. These observations were obtained from cumulative evidence collected worldwide from patients with antithrombin deficiency. However, the methods used for identifying antithrombin deficiency in each institution were different and not standardized. To date, there has been no direct comparison of the differential thrombotic risk associated with type I and type II antithrombin deficiency at a single institution.

We have measured hemostatic factors including antithrombin, protein C, and plasminogen in patients admitted to the National Cerebral and Cardiovascular Center Hospital [9, 19, 20]. We have thus far identified 31 patients with antithrombin deficiency based on their heparin-dependent activity [9]. We also measured antithrombin activity in a Japanese general population consisting of more than 4,500 individuals, who were selected randomly from the municipal population registry stratified by sex and 10-year age group. Comparing the prevalence of antithrombin deficiency in the VTE patient group with that in the general population, we concluded that antithrombin deficiency is a strong risk factor for VTE with an odds ratio of 38 [9].

Here, we extend our previous study to clarify the VTE risk associated with the antithrombin deficiency phenotypes (type I and type II) using two studies. In the first study, 31 patients with antithrombin deficiency admitted to our hospital were classified as type I or type II and their VTE events were retrospectively collected and compared between the two types. In the second study, we compared the prevalence of type I and type II antithrombin deficiency in the VTE patient group and that in the general population and assessed whether the phenotypes of antithrombin deficiency affected VTE events. Both studies showed that type I antithrombin deficiency was a stronger risk factor for VTE than type II deficiency. Thus, the phenotypic classification of antithrombin deficiency is important for assessing VTE risk, and a simple phenotypic classification for type I and type II is useful for assessment of VTE risk in Japanese.

## 2 Materials and methods

### 2.1 Study population: retrospectively followed patients with antithrombin deficiency classified by phenotypes

We measured antithrombin and protein C activities, in response to a doctor's request, in addition to routine hemostatic parameters including prothrombin time and activated partial thromboplastin time in patients admitted to the National Cerebral and Cardiovascular Center Hospital from January 1986 to May 2006. We ultimately identified 31 patients with antithrombin deficiency from 30 families on the basis of antithrombin and protein C activities and the family study. We classified them into type I or type II antithrombin deficiency groups based on the antithrombin activity/antigen ratio. These patients were admitted to our hospital due to cardiovascular problems and did not always have VTE. We, therefore, retrospectively followed them for VTE events and VTE onset age by their medical records for assessment of thrombotic risk. Information on hypertension, hyperlipidemia, diabetes mellitus, and current smoking status was also collected. The diagnosis of deep vein thrombosis was based on radioisotope venography, contrast venography, magnetic resonance imaging, and/or continuous-wave Doppler ultrasonography. The diagnosis of pulmonary embolism was confirmed by pulmonary angiography, computed tomography, and/or magnetic resonance imaging.

### 2.2 Study populations: the VTE group and the general population

We previously enrolled 108 outpatients with VTE (54 men and 54 women), who were admitted to the National Cerebral and Cardiovascular Center Hospital between 1994 and 1998, and identified 6 patients with antithrombin deficiency [9]. These 6 patients were included in the 31 patients with antithrombin deficiency described above. We also previously enrolled 4,517 individuals (2,090 men and 2,427 women), who were randomly selected from the residents of Suita city in Japan and stratified by sex and 10-year age group as a general population, and identified 7 individuals with antithrombin deficiency [9, 21, 22]. Here, we measured the antigen levels of antithrombin in these 13 patients (6 with antithrombin deficiency described above and 7 from Suita city), classified them as having type I or type II deficiency, and compared the prevalence of the phenotypes of antithrombin deficiency in the VTE patient group with that in the general population.

### 2.3 Assay methods

Blood samples were collected in siliconized plastic vacuum tubes containing a 1/10 volume of 3.13% trisodium citrate.

The tubes were centrifuged at 4,000 rpm for 10 min at room temperature and the plasma samples were obtained. Antithrombin activity was determined as heparin-cofactor activity based on the antithrombin assay by the use of chromogenic substrate S-2238 (CHROMOGENIX AB, Stockholm, Sweden) [9, 19]. Protein C activity was measured using the chromogenic substrate S-2236 (CHROMOGENIX AB) after activation by Protac (Pentapharm, Basel, Switzerland) [9, 19]. Antithrombin antigen levels were determined using a latex photometric immunoassay kit (Mitsubishi Chemical Medience Co., Tokyo, Japan) and LPIA-A700, a fully automated quantitative latex photometric immunoassay instrument (Mitsubishi Chemical Medience Co.). The activity and antigen levels were expressed as percentages of the levels obtained from commercially available standard human plasma (Siemens AG, Bayern, Germany). As measured in our laboratory, the inter-assay coefficients of variation were 2.2% for the antithrombin activity assay, 1.9% for the antithrombin antigen assay, and 2.4% for the protein C activity assay. Antithrombin deficiency was defined by the antithrombin and protein C activities and the family study. In this study, patients having an antithrombin activity <3 standard deviations below the mean (70%) and a protein C/antithrombin activity ratio >3 standard deviations above the mean (1.65) [9] and at least one relative with antithrombin deficiency were considered to have antithrombin deficiency.

### 2.4 Statistical analysis

Statistical analysis was performed using Prism (GraphPad Software, La Jolla, CA, USA). Quantitative data were assessed using the Mann–Whitney test. Associations between qualitative variables were analyzed with the Chi-square test. Odds ratios with 95% confidence intervals were used to assess the differences between prevalence rates in the different groups. VTE-free curves in patients with type I or type II antithrombin deficiency were created using the method of Kaplan–Meier and compared using the log-rank test.

## 3 Results

### 3.1 Classification of type I and type II antithrombin deficiency

To classify antithrombin deficiency into type I and type II, the antithrombin activity/antigen ratio (AT-act/AT-ag ratio) was calculated. Figure 1 shows the distribution and box plots of the AT-act/AT-ag ratio in the 31 patients with antithrombin deficiency. We arbitrarily divided the patients into two groups based on this ratio; the median (range) of the ratio in the two groups was 0.98 (0.84–1.29) and 0.52 (0.42–0.62), respectively. We considered 0.75 as the cut-off value for discrimination between the two groups. This cut-off value was calculated as the mean of the median in the two groups. There was no overlap of the AT-act/AT-ag ratio between the two groups. Based on these criteria, 21 patients with type I (circles) and 10 patients with type II (triangles) were identified.

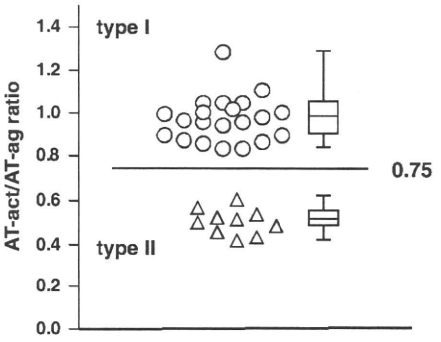


**Fig. 1** Discrimination of antithrombin deficiency phenotypes using the antithrombin activity/antigen ratio. Thirty-one patients with antithrombin deficiency admitted to our hospital were classified into type I (*circles*, $n = 21$) or type II (*triangles*, $n = 10$) deficiency groups using the antithrombin activity/antigen ratio (AT-act/AT-ag) of 0.75. In the box plots, the *error bars* represent minimum and maximum values, the *horizontal rules* indicate median values, and the *ends of the boxes* indicate interquartile ranges

### 3.2 Retrospective follow-up study to compare VTE-free curves in patients with type I and type II antithrombin deficiency

We retrospectively collected the VTE events of the 21 patients with type I antithrombin deficiency and the 10 patients with type II antithrombin deficiency (Table 1). In the type I group, 33 VTE events occurred in 18 of the 21 patients (85.7%), and 16 patients (76.2%) experienced a VTE event before 50 years of age. Recurrent VTE events were observed in 6 of the 18 patients with VTE. The median age for VTE events was 37 years with a range of 15–89 years of age. In contrast, in the type II group, only 3 VTE events occurred in 2 of the 10 deficient patients (20.0%), and the first VTE events occurred at the ages of 57 and 70. There were no significant differences in the risk factors for coronary artery disease, including hypertension, hyperlipidemia, diabetes mellitus, and smoking, between the type I and type II groups.

To evaluate the VTE risk for patients with type I and type II antithrombin deficiency, we compared the VTE-free curves between the groups (Fig. 2). The VTE risk was increased by approximately sevenfold in patients with type I

**Table 1** Characteristics of patients with type I or type II antithrombin deficiency

| | Phenotypes | | *P* value |
|---|---|---|---|
| | Type I | Type II | |
| Number of patients | 21 | 10 | |
| Sex (male/female) | 11/10 | 4/6 | 0.52 |
| Antithrombin levels | | | |
| Activity, median (range) (%) | 54 (29–69) | 62 (40–68) | 0.27 |
| Antigen, median (range) (%) | 55 (30–71) | 116 (87–127) | <0.0001 |
| Activity/antigen ratio, median (range) | 0.97 (0.84–1.29) | 0.51 (0.42–0.61) | <0.0001 |
| Onset of VTE events | | | |
| Number of total events | 33 | 3 | |
| Number of patients (%) | 18/21 (85.7) | 2/10 (20.0) | <0.0001 |
| Number of patients, age < 50 years (%) | 16/21 (76.2) | 0/10 (0.0) | <0.0001 |
| Recurrence of VTE onset | | | |
| Number of patients (%) | 6/18 (33.3) | 1/2 (50.0) | 0.64 |
| Age at VTE onset (years) | | | |
| Median (range) | 37 (15–89) | 64 (57–70) | 0.09 |
| Risk for coronary artery disease | | | |
| Hypertension (%) | 2/21 (10) | 3/10 (30) | 0.30 |
| Hyperlipidemia (%) | 2/21 (10) | 3/10 (30) | 0.30 |
| Diabetes (%) | 0/21 (0) | 2/10 (20) | 0.10 |
| Current smoking (%) | 8/21 (38) | 5/10 (50) | 0.70 |

*VTE* venous thromboembolism, *Hypertension* systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg or antihypertensive medication, *Hyperlipidemia* total cholesterol ≥ 5.68 mmol/L (220 mg/dL) or antihyperlipidemia medication, *Diabetes* fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dL) or non-fasting plasma glucose ≥ 11.1 mmol/L (200 mg/dL) or HbA1c ≥ 6.5% or antidiabetic medication

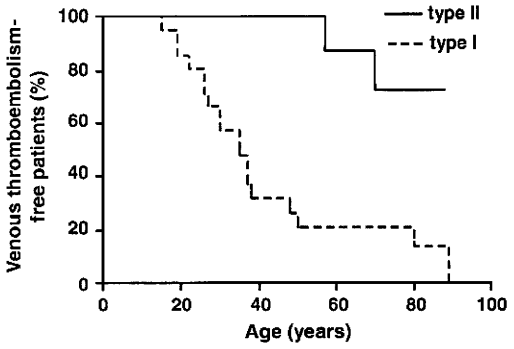


**Fig. 2** VTE-free curves of patients with type I or type II antithrombin deficiency. In type I antithrombin deficiency, 18 out of 21 patients developed VTE. In type II antithrombin deficiency, only 2 out of 10 patients developed VTE. For the Kaplan–Meier analysis, the remaining three or eight patients were treated as the censored patients

deficiency relative to those with type II deficiency (hazard ratio: 7.3; 95% confidence intervals: 1.9–12.2; $P = 0.0009$).

### 3.3 Comparison of the prevalence of type I and type II antithrombin deficiency in the VTE patient group and the general population

We previously identified 6 patients with antithrombin deficiency among 108 consecutive outpatients with VTE [9]. We also identified 7 individuals with antithrombin deficiency in a general population consisting of 4,517 individuals. Comparing these frequencies, we reported that antithrombin deficiency was a strong risk factor for VTE with an odds ratio of 38 [9]. To extend this previous work, we measured the antithrombin antigen levels in these patients in the present study. We found that all 6 VTE patients with antithrombin deficiency were type I (Table 2). In the general population, two individuals were type I and the remaining 5 were type II (Table 2). The prevalences of type I antithrombin deficiency in the VTE group (6/108, 5.56%) and the general population (2/4,517, 0.04%) were significantly different, and the odds ratio between the two groups was 132.8 (95% confidence intervals: 26.5–666.1; $P < 0.0001$). In contrast, the prevalences of type II antithrombin deficiency in the VTE group (0/108, 0.00%) and the general population (5/4,517, 0.11%) were not significantly different (odds ratio: 3.8; 95% confidence interval: 0.2–68.9; $P = 0.7294$).

## 4 Discussion

In the present study, we identified 21 patients with type I antithrombin deficiency and 10 patients with type II antithrombin deficiency. Among these patients, more than 75% with type I deficiency had VTE events before the age of 50; in contrast, only 2 out of 10 patients with type II deficiency had VTE events, and these occurred at the ages of 57 and 70. The VTE-free curves showed that type I antithrombin

**Table 2** Prevalence of type I and type II antithrombin deficiency in the VTE patient group and the general population

| Phenotype of antithrombin deficiency | Number of individuals (%) | | Odds ratio (95% CI vs. general population) | *P* value |
|---|---|---|---|---|
| | VTE patient group ($n = 108$) | General population ($n = 4,517$) | | |
| Type I | 6 (5.56%) | 2 (0.04%) | 132.8 (26.5–666.1) | <0.0001 |
| Type II | 0 (0.00%) | 5 (0.11%) | 3.8 (0.2–68.9) | 0.73 |
| Type I + II[a] | 6 (5.56%) | 7 (0.15%) | 37.9 (12.5–114.8) | <0.0001 |

*CI* confidence interval, *VTE* venous thromboembolism

[a] Data taken from Ref. [9]

deficiency was a strong risk factor for VTE, with a hazard ratio of 7.3 compared to type II deficiency. These findings were reinforced by a comparison of the prevalence of type I and type II deficiency in the VTE group and the general population, which showed very high odds ratio for VTE for type I, but not for type II.

Based on a cumulative analysis of the literature, type I antithrombin deficiency as well as both the reactive site and the pleiotropic subtypes of type II deficiency have an increased risk for VTE [15–17]. However, the heparin-binding site subtype of type II deficiency showed a relatively low risk for VTE [15–17]. Thus, the VTE risk conferred by type I antithrombin deficiency was consistent between our study and the cumulative analysis. However, the risk assessment of type II antithrombin deficiency for VTE is in part debatable. In our analysis, we could not classify our patients with type II deficiency into three subtypes, a heparin-binding site subtype, a reactive site subtype, and a pleiotropic effect subtype, due to the sample limitations. Therefore, we could not evaluate the risk assessment for VTE by subtypes. Despite this limitation, we demonstrated that type I antithrombin deficiency was a greater risk for VTE than type II.

The plausible explanation for type II deficiency as a low VTE risk would be that mutations responsible for a low VTE risk, the heparin-binding defects, would be more common than those for a high VTE risk, the reactive site defects and pleiotropic effect defects, in a Japanese population. This could explain the clearly differentiated risk between type I and type II deficiency in our study. In the public database, the cases of at least three Japanese VTE patients homozygous for the R47C mutation, that causes a heparin-binding defect, have been deposited (Japanese Thrombophilia Mutation Database, http://hes.met.nagoya-u.ac.jp/KENSA WEB/labo/blood/bunshi_hp/mutation.html, Antithrombin Mutation Database, http://www1.imperial.ac.uk/medicine/divisions/olddivisions/is/haematology/coag/antithrombin/) [18, 23, 24], suggesting the prevalence of this mutation in the Japanese population. As for the reactive site defects in the Japanese population, two heterozygous patients, one with the R425C mutation [25] and another with the R425H mutation [26] have been deposited in the public database. Thus, the frequency of patients with the reactive site defects seemed lower than those with the heparin-binding defects.

We identified 2 individuals with type I antithrombin deficiency and 5 individuals with type II deficiency out of 4,517 individuals, who were selected randomly from the municipal population registry stratified by sex and 10-year age group. Thus, the prevalences of type I and type II deficiency in the Japanese general population were 0.04% (2/4,517) and 0.11% (5/4,517), respectively, which were similar to those obtained in western Scotland (type I: 0.02%, type II: 0.15%) [13], although the selection criteria for antithrombin deficiency were different. The Japanese population is now about 128 million. Extrapolating from the prevalence, we estimate that as many as 57,000 Japanese have type I antithrombin deficiency and are at risk of developing VTE.

In conclusion, considerable differences in VTE risk were observed between Japanese individuals with type I and type II antithrombin deficiency and a simple phenotypic classification for type I and type II antithrombin deficiency was shown to be useful for assessment of VTE risk in Japanese.

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ORIGINAL ARTICLE

# Impact of RGS2 deficiency on the therapeutic effect of telmisartan in angiotensin II-induced aortic aneurysm

Sachiko Matsumoto[1], Kei Kamide[1,2], Fumiaki Banno[1], Nobutaka Inoue[1,3], Naoki Mochizuki[1], Yuhei Kawano[4] and Toshiyuki Miyata[1]

**Regulator of G-protein signaling 2 (RGS2) negatively regulates the signaling of G-protein-coupled receptors, such as the angiotensin II (AngII) type 1 receptor by accelerating the inactivation of Gαq. Rgs2-deficient mice show increased sensitivity and prolonged responsiveness to vasoconstrictors, and genetic variations in the *RGS2* gene are associated with hypertension in humans. This study aimed to clarify whether Rgs2 deficiency contributes to the development of vascular remodeling and therapeutic efficacy of the angiotensin receptor blocker telmisartan on atherosclerotic vascular damage. We treated *Rgs2*$^{+/+}$, *Rgs2*$^{+/-}$ and *Rgs2*$^{-/-}$ mice with saline (control group), AngII (1000 ng per kg per min, AngII group) or low-dose telmisartan (0.3 mg per kg per day) with AngII infusion (AngII+Telmi group) for 4 weeks. For all genotypes, the AngII groups exhibited significantly higher blood pressure, a higher mortality rate and a higher incidence of aortic aneurysm than the respective control group. Interestingly, aneurysm incidence was decreased in the AngII+Telmi group compared with the AngII group in *Rgs2*$^{-/-}$ mice (6.7 *vs.* 42.9%, $P<0.05$), but not in *Rgs2*$^{+/+}$ mice (38.9 *vs.* 40.0%). Moreover, in *Rgs2*$^{-/-}$ mice, the AngII+Telmi group exhibited significant improvement in survival, reduction of enlarged aortic diameter, inhibition of superoxide production and suppression of NAD(P)H oxidase activity compared with the AngII group. Thus, Rgs2 deficiency potentiates the vascular protection effect of low-dose telmisartan. Our results suggest that angiotensin receptor blocker may be useful for protection from cardiovascular events in hypertensive subjects with risk alleles in the *RGS2* gene.**
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**Keywords:** angiotensin II; aortic aneurysm; oxidative stress; RGS2; telmisartan

## INTRODUCTION

The renin–angiotensin system has an important role in the regulation of blood pressure and vascular structure. Angiotensin II (AngII) is a potent vasoconstrictor that elevates blood pressure through a G-protein-coupled receptor, angiotensin type 1 receptor (AT1R). AngII generates aldosterone at the adrenal gland and activates the sympathetic nervous system, leading to blood pressure elevation. In addition to the effects of AngII on the elevation of blood pressure, evidence has revealed that it has a role in atherogenesis. In animal models, chronic infusion of AngII promotes the formation of atherosclerotic lesions and aneurysms.[1,2] It is widely known that AngII-induced NAD(P)H oxidase activation increases the production of reactive oxygen species from various cell types,[3] including endothelial cells, vascular smooth muscle cells and monocytes/macropharges, and promotes inflammation in atherosclerotic lesions.[4]

Regulator of G-protein signaling 2 (RGS2) is present in many cardiovascular tissues, including the heart, kidney and blood vessels, and it is required for normal vascular function and regulation of blood pressure.[5] RGS2 negatively regulates the signaling of G-protein-coupled receptors, such as $AT_1R$, by accelerating the inactivation of Gαq by its guanosine triphosphatase-activating protein activity. RGS2 also mediates the nitric oxide–cyclic guanosine monophosphate pathway to decrease vascular resistance and attenuate vasoconstrictor signaling in vascular smooth muscle cells.[5,6] Patients with Bartter's and Gitelman's syndromes have hypotension with an enhancement of RGS2 expression.[7] Taken together, silencing of the *RGS2* gene disrupts these pathways and enhances the vasoconstrictor signaling.

The first reported phenotypes of Rgs2-deficient mice were the reduction of T-cell activation, the control of synapse development in the hippocampus and an increase in anxiety responses.[8] With respect to the blood pressure regulation, Rgs2-deficient mice exhibit a hypertensive phenotype and persistent constriction of the resistance vasculature.[5,9] This hypertensive phenotype differs in degree according to conditions such as age in weeks, anesthesia, postoperative recovery, restrained stress, time zone and apparatus for blood pressure measurement.[5,9,10] Together with another group, we have reported that genetic polymorphisms within the human *RGS2* gene are associated with hypertension.[11–14] It has been speculated that genetic variations

[1]Research Institute, National Cerebral and Cardiovascular Center, Suita, Osaka, Japan; [2]Department of Geriatric Medicine and Nephrology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan; [3]Department of Cardiovascular Medicine, Kobe Rosai Hospital, Kobe, Hyogo, Japan and [4]Division of Hypertension and Nephrology, National Cerebral and Cardiovascular Center, Suita, Osaka, Japan
Correspondence: Dr K Kamide, Department of Geriatric Medicine and Nephrology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita city, Osaka 565-0871, Japan.
E-mail: kamide@geriat.med.osaka-u.ac.jp
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may reduce RGS2 function.[15,16] Because hypertension contributes to the pathogenesis of cardiovascular disease, it is hypothesized that genetic variations in RGS2 might be a risk factor for the development of atherosclerosis through enhancement of AngII signaling.

Telmisartan is an angiotensin receptor blocker (ARB) with a longer half-life and higher lipophilicity than other ARBs,[17] and it is a commonly used medication for the treatment of hypertension. In a recent clinical trial,[18,19] telmisartan resulted in the prevention of vascular events such as myocardial infarction and stroke. Therefore, telmisartan is expected to be effective for cardiovascular protection in Rgs2-deficient mice that have enhanced AngII signaling.

In this study, we investigated the effects of RGS2 deficiency on the development of vascular remodeling and the therapeutic efficacy of low-dose telmisartan on atherosclerotic vascular damage resulting from excessive stimulation of $AT_1R$ by RGS2 deficiency.

## METHODS

### Mice

Rgs2-deficient mice on the C57BL/6 background were provided by Dr Michael E Mendelsohn (Tufts University School of Medicine).[8] Mice were kept in a specific pathogen-free barrier under constant temperature conditions and housed on a 12-h light/12 h dark cycle. All experiments were approved by the Animal Care and Use Committees of the National Cardiovascular Center, Japan, and they were performed in accordance with the guidelines.

### Drug administration

We used 18-week old male Rgs2-deficient ($Rgs2^{-/-}$, $Rgs2^{+/-}$) and wild-type ($Rgs2^{+/+}$) mice and divided them into three treatment groups. Mice were subcutaneously infused with AngII (1000 ng per kg per min, AngII group), or saline containing 0.3% bovine serum albumin (control group) using an ALZET Micro-Osmotic Pump (model 1004, Durect, Cupertino, CA, USA) for 4 weeks. Mice were also treated with AngII (1000 ng per kg per min) and low-dose telmisartan (0.3 mg per kg per day, AngII+Telmi group). Telmisartan was administered in drinking water. We adopted a low dosage of telmisartan that does not affect blood pressure.[20]

### Hemodynamic analysis

Systolic blood pressure and heart rate were measured in conscious, pre-warmed, and restrained mice by the tail-cuff method using a non-invasive blood pressure measuring device (BP98-A, Softron, Tokyo, Japan) before treatment and on days 7, 14, 21 and 28 after treatment just around the same time of day. For stable measurement, tail-cuff pressures were obtained after a 2-week-training period to acclimatize the mice to the restraining device and cuff inflation. The pulse waveform was monitored in real time using the BP98AW software (version 2.12, Softron). The first 10 measurements were excluded from the analysis, and at least 5 measurements with an untroubled pulse waveform were collected.

### Biochemical examination

After 4 weeks of treatment, overnight fasting blood was collected from anesthetized mice and put into capillary blood collection tubes including a gel/clot activator (Capiject tube, Terumo Medical, Somerset, NJ, USA). Serum was obtained by the manufacturer's instructions and stored at −80 °C in aliquots before use. Serum levels of blood urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, low-density lipoprotein-cholesterol and glucose were measured using a Hitachi clinical analyzer (model 7180, Hitachi High-Technologies, Tokyo, Japan).

### Aortic tissue collection and morphometric analysis

Dissection was performed under anesthesia after blood collection. After thoracolaparotomy, the inferior vena cava was cut for exsanguination and the aorta was perfused with ice-cold saline through the left ventricle. The aortic root and heart were subsequently eviscerated, and the periadventitial tissue was dissected away under a stereomicroscope. The external diameters in the middle of the suprarenal abdominal aorta between the diaphragm and renal artery bifurcation were measured using the ImageJ software (version 1.40, National Institute of Health, Bethesda, MD, USA). After taking the images, the aorta was cut into thoracic and abdominal regions. The thoracic aorta was immediately frozen by liquid nitrogen and stored at −80 °C for the measurement of NAD(P)H oxidase activity. The suprarenal abdominal aorta was cut into two pieces. For detection of superoxide production, the superior half was immediately embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan) in liquid nitrogen and stored at −80 °C. For immunohistochemical staining, the inferior half was fixed with 4% paraformaldehyde overnight and paraffin embedded.

### Detection of aortic superoxide production

Aortic superoxide levels were measured with dihydroethidium (Invitrogen Molecular Probes, Carlsbad, CA, USA) on cross sections (9 μm) obtained from the unfixed frozen blocks of abdominal aorta, as previously described.[21] The unfixed frozen sections were stained by dihydroethidium (2 μM, 30 min, 37 °C) in a dark humidified chamber and washed briefly. The images were captured with a laser scanning confocal fluorescent microscope (FLUOVIEW system, Olympus, Tokyo, Japan). For the quantification of ethidium fluorescence, the mean fluorescence intensity (fluorescence intensity per unit area) in the aortic wall was calculated using the ImageJ software on high-power (x300) images.

### Measurement of NAD(P)H oxidase activity

The frozen aortic segments were homogenized using a Sample Grinding Kit (GE Healthcare UK, Buckinghamshire, England). After centrifugation, the supernatant was stored at −80 °C until use. Protein concentrations were measured by the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The enzymatic activity of NAD(P)H oxidase of the homogenates was measured by lucigenin-enhanced chemiluminescence, as previously described.[20] The assay solution contained lucigenin (250 μM) as an electron acceptor and NADH (100 μM) or NADPH (100 μM) as a substrate. After pre-incubation at 37 °C for 20 min, the reaction was started by adding 50 μg of homogenate. Photon emissions were continuously recorded for 15 min with a chemiluminescence reader (BLR-201, ALOKA, Tokyo, Japan). The chemiluminescent signals observed in the absence of homogenates were subtracted from the signals of the samples. The signal was corrected for the protein concentration of each homogenate and expressed as counts per minute by mg protein for a 15-min period. In some experiments, the homogenates were pre-incubated with 10 μM diphenyleneiodium, a selective NADPH oxidase inhibitor, for 20 min before the lucigenin-enhanced chemiluminescence measurements.

### Statistical analysis

Data are expressed as mean ± s.e.m. All statistical analyses were performed using the Prism software (version 5.0, GraphPad Software, La Jolla, CA, USA). Hemodynamic changes were analyzed by two-way analysis of variance during the period of administration. Differences between multiple groups were analyzed by one-way analysis of variance or Kruskal–Wallis test in the case of a non-Gaussian distribution, followed by the Bonferroni *post-hoc* test for comparison between treatment groups or genotype groups. The log-rank (Mantel–Cox) test was used for statistical analysis of survival curves, and the $\chi^2$-test was used to compare the incidence of aneurysm. Values of $P<0.05$ were considered statistically significant.

## RESULTS

### Changes in blood pressure

To elucidate the direct role of AngII signaling in Rgs2-deficient mice, we divided $Rgs2^{+/+}$, $Rgs2^{+/-}$ and $Rgs2^{-/-}$ mice into three treatment groups: the control group, the AngII group and the AngII+Telmi group. Drug administration was performed for 4 weeks, and hemodynamic changes were measured. In all Rgs2 genotypes, the AngII group exhibited ~50 mm Hg higher systolic blood pressure than the control group ($P<0.001$) (Figure 1). Rgs2 dysfunction was expected to enhance the AngII signaling, leading to higher blood pressure in

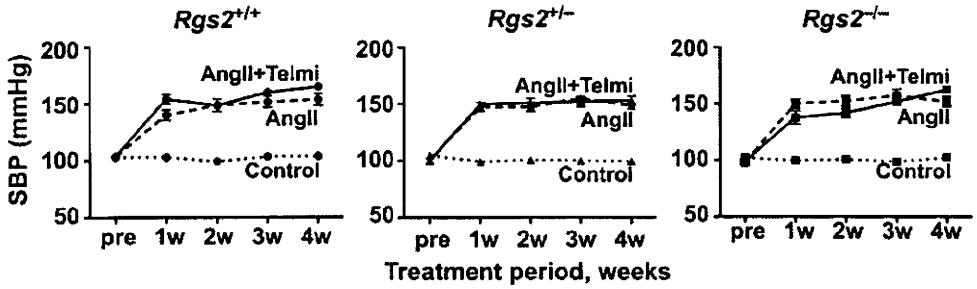


**Figure 1** Blood pressure changes in response to AngII or AngII plus telmisartan. Systolic blood pressure (SBP) variations were shown in *Rgs2*$^{+/+}$, *Rgs2*$^{+/-}$ and *Rgs2*$^{-/-}$ mice. Mice were divided into three groups, control group (saline), AngII group (AngII, 1000 ng per kg per min) and AngII+Telmi group (AngII, 1000 ng per kg per min, telmisartan, 0.3 mg per kg per day). Results are expressed as mean ± s.e.m. in the control group (*Rgs2*$^{+/+}$: $n$=8, *Rgs2*$^{+/-}$: $n$=8, *Rgs2*$^{-/-}$: $n$=8), the AngII group (*Rgs2*$^{+/+}$: $n$=17, *Rgs2*$^{+/-}$: $n$=25, *Rgs2*$^{-/-}$: $n$=15) and the AngII+Telmi group (*Rgs2*$^{+/+}$: $n$=14, *Rgs2*$^{+/-}$: $n$=15, *Rgs2*$^{-/-}$: $n$=15). The AngII and AngII+Telmi groups showed significantly higher SBP than the control group ($P$<0.001).

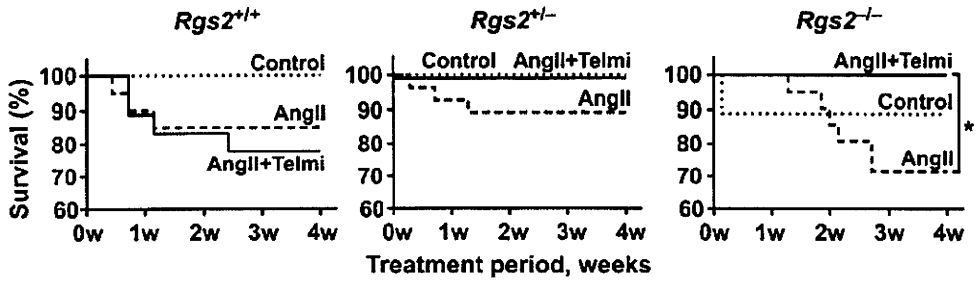


**Figure 2** Comparison of survival curves in mice treated with AngII or AngII plus telmisartan. The treatment design was the same as that in Figure 1. The survival rate is expressed as a percent of animals in the control group (*Rgs2*$^{+/+}$: $n$=8, *Rgs2*$^{+/-}$: $n$=8, *Rgs2*$^{-/-}$: $n$=9), the AngII group (*Rgs2*$^{+/+}$: $n$=20, *Rgs2*$^{+/-}$: $n$=28, *Rgs2*$^{-/-}$: $n$=21) and the AngII+Telmi group (*Rgs2*$^{+/+}$: $n$=18, *Rgs2*$^{+/-}$: $n$=15, *Rgs2*$^{-/-}$: $n$=15). *$P$<0.05.

*Rgs2*$^{-/-}$ mice, but there were no blood pressure differences among the Rgs2 genotypes in the AngII group. Moreover, the AngII+Telmi group did not show reduced blood pressure in any of the genotypes. Before drug administration, there were no significant differences in blood pressure among the Rgs2 genotypes in our experimental condition using the tail-cuff method. Both the AngII and the AngII+Telmi groups showed no differences in heart rate among the genotypes (data not shown). Although *Rgs2*$^{-/-}$ mice tended to show a lower body weight than the other genotypes, drug administration did not affect the body weight (data not shown).

### Survival rates and blood biochemical examinations

Some mice died during the period of administration mainly because of the cardiovascular events, including the rupture of aneurysms. Therefore, we compared the survival curves in mice with all Rgs2 genotypes and with the three treatment groups. As shown in Figure 2, AngII treatment decreased the survival rate for all the Rgs2 genotypes compared with the control group, but there were no significant differences in survival rate among the Rgs2 genotypes in the AngII group. Interestingly, *Rgs2*$^{-/-}$ mice treated with AngII+Telmi had significantly improved survival compared with *Rgs2*$^{-/-}$ mice treated with AngII alone ($P$<0.05).

As shown in the Table 1, blood urea nitrogen levels were significantly increased in the AngII and AngII+Telmi groups compared with the control group ($P$<0.05). Creatinine levels also tended to increase in the AngII group compared with the control group. These results indicated the exacerbation of renal function caused by AngII-induced hypertension in all Rgs2 genotypes. Recent studies have shown that telmisartan improves the metabolism of lipids and glucose.[22,23] Our study showed that low-density lipoprotein-cholesterol levels were significantly decreased in the AngII+Telmi group compared with the

**Table 1 Biochemical examinations of serum samples**

| | *Rgs2* | *Control* | *AngII* | *AngII+Telmi* |
|---|---|---|---|---|
| BUN (mg dl$^{-1}$) | +/+ | 27.2 ± 2.9 | 57.1 ± 4.6* | 51.7 ± 1.8* |
| | +/− | 23.8 ± 1.9 | 52.7 ± 2.2* | 48.8 ± 3.7* |
| | −/− | 29.4 ± 4.3 | 47.8 ± 2.3* | 51.0 ± 2.6* |
| CRE (mg dl$^{-1}$) | +/+ | 0.07 ± 0.00 | 0.12 ± 0.01* | 0.09 ± 0.01* |
| | +/− | 0.06 ± 0.01 | 0.13 ± 0.01* | 0.10 ± 0.01* |
| | −/− | 0.06 ± 0.01 | 0.10 ± 0.01 | 0.08 ± 0.01 |
| LDL-C (mg dl$^{-1}$) | +/+ | 2.0 ± 0.0 | 3.1 ± 0.3 | 4.9 ± 0.7 |
| | +/− | 2.4 ± 0.4 | 4.1 ± 0.4 | 2.8 ± 0.3 |
| | −/− | 5.0 ± 1.2 | 6.0 ± 1.4 | 3.1 ± 0.5† |
| GLU (mg dl$^{-1}$) | +/+ | 108.4 ± 14.4 | 93.6 ± 11.7 | 98.6 ± 14.1 |
| | +/− | 112.4 ± 23.8 | 103.5 ± 10.4 | 109.5 ± 10.7 |
| | −/− | 70.8 ± 7.2 | 72.2 ± 9.7 | 88.9 ± 8.1 |

Abbreviations: AngII, angiotension II; BUN, blood urea nitrogen; CRE, creatinine, LDL-C, low-density lipoprotein-cholesterol; GLU, glucose, Rgs2, regulator of G-protein signaling 2; Telmi, telmisartan.
Results are expressed as mean ± s.e.m. in the control group (*Rgs2*$^{+/+}$: $n$=5, *Rgs2*$^{+/-}$: $n$=5, *Rgs2*$^{-/-}$: $n$=5), the AngII group (*Rgs2*$^{+/+}$: $n$=11, *Rgs2*$^{+/-}$: $n$=21, *Rgs2*$^{-/-}$: $n$=12) and the AngII+Telmi group (*Rgs2*$^{+/+}$: $n$=14, *Rgs2*$^{+/-}$: $n$=15, *Rgs2*$^{-/-}$: $n$=15). *$P$<0.05, *vs.* the control group. †$P$<0.05, *vs.* the AngII group.

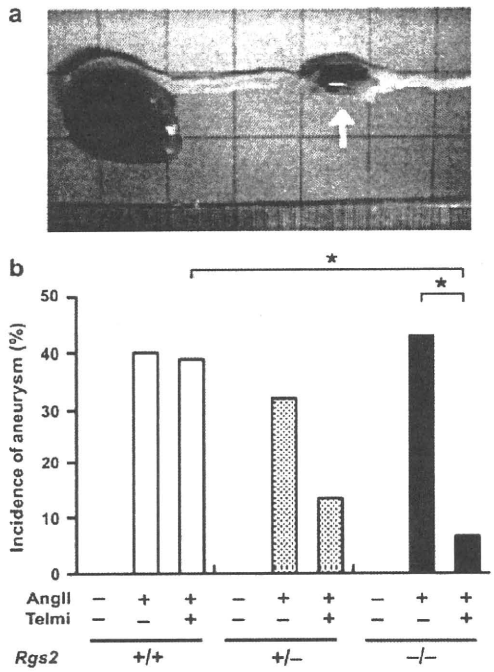


**Figure 3** Gross morphology and incidence of aortic aneurysms observed in mice treated with AngII or AngII plus telmisartan. (**a**) Representative photograph showing the macroscopic features of aneurysm induced by AngII treatment. Aneurysm is indicated by arrow. (**b**) The incidence of aneurysms is expressed as a percent of animals in the control group ($Rgs2^{+/+}$: $n$=8, $Rgs2^{+/-}$: $n$=8, $Rgs2^{-/-}$: $n$=9), the AngII group ($Rgs2^{+/+}$: $n$=20, $Rgs2^{+/-}$: $n$=28, $Rgs2^{-/-}$: $n$=21) and the AngII+Telmi group ($Rgs2^{+/+}$: $n$=18, $Rgs2^{+/-}$: $n$=15, $Rgs2^{-/-}$: $n$=15). *$P$<0.05.

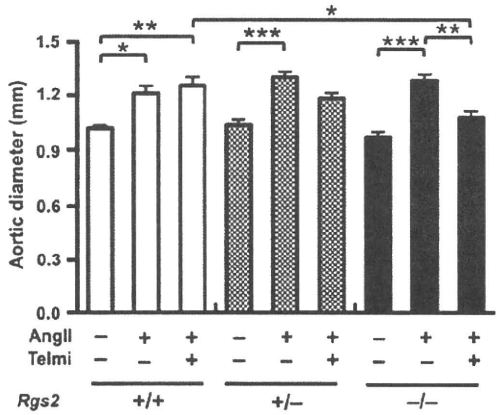


**Figure 4** Comparison of abdominal aortic diameters in mice treated with AngII or AngII plus telmisartan. Diameter was measured in the middle of the abdominal aorta between the diaphragm and the renal artery bifurcation, and was compared. Results are expressed as mean±s.e.m. in the Control group ($Rgs2^{+/+}$: $n$=8, $Rgs2^{+/-}$: $n$=8, $Rgs2^{-/-}$: $n$=8), the AngII group ($Rgs2^{+/+}$: $n$=12, $Rgs2^{+/-}$: $n$=19, $Rgs2^{-/-}$: $n$=11) and the AngII+Telmi group ($Rgs2^{+/+}$: $n$=10, $Rgs2^{+/-}$: $n$=13, $Rgs2^{-/-}$: $n$=14). *$P$<0.05, **$P$<0.01, ***$P$<0.001.

AngII group in $Rgs2^{-/-}$ mice ($P$<0.05), whereas no changes in glucose levels were observed. Liver function, as indicated by aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase levels, was normal (data not shown).

### Vascular remodeling

In the AngII group, ~30–40% of mice had aortic aneurysms, as shown in Figure 3a. The incidence of aortic aneurysm was not different among Rgs2 genotypes in the AngII group. $Rgs2^{-/-}$ mice treated with AngII+Telmi had a significantly lower rate of aneurysm formation than the AngII group ($P$<0.05, Figure 3b). In $Rgs2^{+/-}$ mice, the AngII+Telmi group had a lower rate of aneurysm than the AngII group. Moreover, the incidence of aortic aneurysms in $Rgs2^{-/-}$ mice was significantly lower than that in $Rgs2^{+/+}$ mice in the AngII+Telmi group ($P$<0.05).

Next, we compared the diameters of the abdominal aorta (Figure 4). The AngII group showed significantly more enlarged aortic diameters than the control group for all Rgs2 genotypes ($Rgs2^{+/+}$: $P$<0.05, $Rgs2^{+/-}$, $Rgs2^{-/-}$: $P$<0.001). Although AngII+Telmi treatment did not reduce blood pressure in any of the genotypes, it significantly reduced enlargement of the aortic diameter compared with the AngII treatment of $Rgs2^{-/-}$ mice ($P$<0.01). In addition, in $Rgs2^{+/-}$ mice the AngII+Telmi treatment tended to reduce the enlargement of the aortic diameter compared with AngII treatment alone.

### Oxidative stress in vascular walls

To assess oxidative stress in the aortic wall, we measured superoxide production and NAD(P)H oxidase activity. Figure 5A shows the *in situ* detection of superoxide in the abdominal aorta using dihydroethidium staining in $Rgs2^{-/-}$ mice. The red fluorescence intensity of the aorta in $Rgs2^{-/-}$ mice tended to be more intense in the AngII group and was obviously suppressed in the AngII+Telmi group. Quantitative analysis showed that superoxide production of the aorta in $Rgs2^{-/-}$ mice was significantly decreased in the AngII+Telmi group compared with the AngII group ($P$<0.001), whereas production in the other Rgs2 genotypes was not statistically different (Figure 5B). Furthermore, the NAD(P)H oxidase activity of aorta in $Rgs2^{-/-}$ mice was also decreased in the AngII+Telmi group compared with the AngII group ($P$<0.001, Figure 5c).

## DISCUSSION

This study showed that the AngII group exhibited higher systolic blood pressure, a higher mortality rate, a higher aortic aneurysm incidence, and a more enlarged aortic diameter than the control group for all Rgs2 genotypes. Interestingly, in $Rgs2^{-/-}$ mice, the AngII+Telmi group showed a significant improvement in the survival rate as well as in the suppression of vascular remodeling compared with the AngII group, although blood pressure was not changed. In parallel with this improvement of vascular phenotypes in $Rgs2^{-/-}$ mice, the NAD(P)H oxidase activity and superoxide production of the aorta in $Rgs2^{-/-}$ mice was decreased in the AngII+Telmi group. Thus, low-dose telmisartan could prevent AngII-induced vascular remodeling via the suppression of oxidative stress in the vascular wall of $Rgs2^{-/-}$ mice.

Rgs2 dysfunction is expected to enhance the AngII signaling through $AT_1R$, leading to blood pressure elevation, atherosclerotic vascular remodeling and organ damage. However, our results did not show significant differences among Rgs2 genotypes in the AngII group, including blood pressure, mortality rate, aneurysmal formation, aortic diameter and aortic oxidative stress (Figures 1–5). The reason for the lack of differences among the Rgs2 genotypes may have been because the concentrations and dosing period of AngII may be excessive and outside the capability of Rgs2 regulation. Nevertheless, AngII was given in doses sufficient to cause the

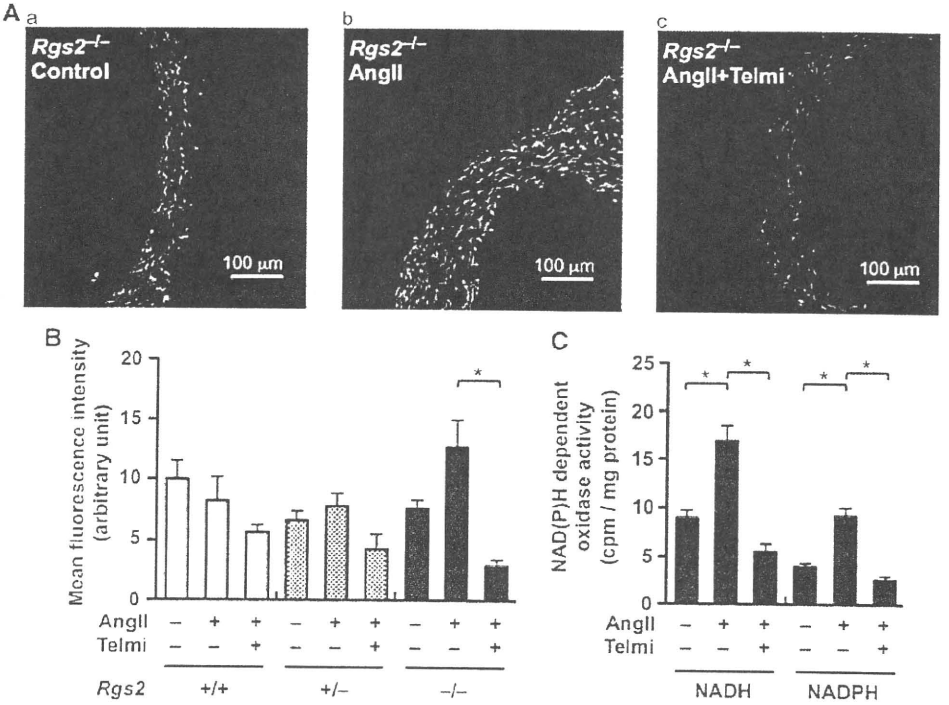


**Figure 5** Comparison of oxidative stress in the aortic wall in mice treated with AngII or AngII plus telmisartan. (**A**), Representative photographs of *in situ* superoxide production in the aortic wall using dihydroethidium staining in $Rgs2^{-/-}$ mice of the Control group (a), the AngII group (b) and the AngII+Telmi group (c). (**B**), Quantitative analysis of superoxide production in the aortic wall. For quantification of ethidium fluorescence at the aortic wall, the fluorescence intensity was calculated using the ImageJ software and is expressed in arbitrary units. (**C**), Effect of telmisartan on NAD(P)H dependent oxidase activity of aorta homogenates using lucigenin-enhanced chemiluminescence in $Rgs2^{-/-}$mice. NAD(P)H oxidase activity was measured as described in Methods section. As a control, NADPH oxidase inhibitor, diphenyleneiodium, reduced NADH and NADPH oxidase activities below measurable limits. Results are expressed as mean ± s.e.m. in the control group ($Rgs2^{+/+}$: $n$=5, $Rgs2^{+/-}$: $n$=5, $Rgs2^{-/-}$: $n$=5), the AngII group ($Rgs2^{+/+}$: $n$=11, $Rgs2^{+/-}$: $n$=21, $Rgs2^{-/-}$: $n$=12) and the AngII+Telmi group ($Rgs2^{+/+}$: $n$=14, $Rgs2^{+/-}$: $n$=15, $Rgs2^{-/-}$: $n$=15). *$P$<0.001.

exacerbation of vascular phenotypes in all genotypes under our experimental condition. Thus, we examined the impact of Rgs2 deficiency on the therapeutic effect of low-dose telmisartan in AngII-infused mice.

The most interesting aspect of our study was the observed therapeutic efficacy of telmisartan. Low-dose telmisartan significantly improved survival, inhibited vascular remodeling such as aneurysmal formation and enlargement of aortic diameter, and decreased aortic oxidative stress in $Rgs2^{-/-}$ mice. These effects, mostly observed in the aorta, were independent of blood pressure reduction and were not observed in $Rgs2^{+/+}$ mice (Figures 2–5). In $Rgs2^{+/-}$ mice, low-dose telmisartan exhibited partial therapeutic effects such as improvement of survival, inhibition of aneurysm formation and reduction of enlarged aortic diameter, although there were no significant differences (Figures 2–4). These Rgs2 deficiency-dependent vascular protective effects of low-dose telmisartan could be explained by the following mechanisms. Some reports have shown that telmisartan and another ARB, valsartan, prevent vascular remodeling through inhibition of oxidative stress, inflammation and degradation of the extracellular matrix independent of their antihypertensive effects.[20,24–26] Heximer *et al.*[9] have reported that responsiveness to another ARB, candesartan, is more sensitive in $Rgs2^{-/-}$ mice than $Rgs2^{+/+}$ mice with regard to its antihypertensive and organ protection effects. Thus, vascular protective effects through inhibition of oxidative stress by low-dose ARB may be exaggerated in $Rgs2^{-/-}$ mice as a result of its antagonism for excessive $AT_1R$ signaling. Moreover, some reports have characterized new functions of telmisartan as a partial agonist for peroxisome proliferator-activated receptors (PPARs).[22,23,27] Activation of PPARα by agonists or telmisartan induces an anti-inflammatory response through the repression of nuclear factor-κB signaling in umbilical vein endothelial cells and aortic smooth muscle cells *in vitro*[27,28] and inhibits macrophage infiltration and reduces aortic dilatation in a mouse model of aortic aneurysm.[29] PPARα and PPARγ improve lipid and glucose metabolism, respectively.[30] Low-dose telmisartan in $Rgs2^{-/-}$ mice improved lipid metabolism but did not affect glucose metabolism, as shown in the Table 1. Therefore, these protective effects of telmisartan in $Rgs2^{-/-}$ mice might be dependent on the anti-inflammatory response via PPARα activation, and Rgs2 deficiency might affect enhancement of the anti-inflammatory effect of telmisartan. Taken together, these results show that the therapeutic effect of low-dose telmisartan might be higher in the aorta of $Rgs2^{-/-}$ mice than in that of $Rgs2^{+/+}$ mice through both $AT_1R$ blockade and PPARα activation.

Hypertension is a major risk factor of cardiovascular disease. Human *RGS2* genetic polymorphism is associated with the pathogenesis of hypertension in different races[11–14] that may result from Gαq-signal acceleration by RGS2 dysfunction. Our mouse study did not indicate a relationship between Rgs2 deficiency and the development of atherosclerosis and aneurysm *in vivo*. Instead, we found that low-dose telmisartan would be beneficial in AngII-induced vascular remodeling, dependent on Rgs2 deficiency and dysfunction. This

study suggests that ARB might be more useful for protection from cardiovascular events in hypertensive subjects with risk alleles in the *RGS2* gene than other antihypertensive drugs. This concept might be applicable for personalized medicine on the basis genetic information.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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◆特集：ITP：最近の話題◆

# ITP に関する用語の標準化：国際作業部会からの報告

Standardization of terminology in immune thrombocytopenic purpura : report from an international working group

冨山佳昭 *
Yoshiaki TOMIYAMA *

**Key words:** primary ITP, terminology, definition, outcome criteria

## 1. はじめに

わが国において広く ITP という用語が日常臨床で用いられているが，その正式な名称に関してはいまだ統一されていないのが現状である．具体的には，厚生労働省の特定疾患である ITP は，idiopathic thrombocytopenic purpura（特発性血小板減少性紫斑病）と呼ばれているが，一方その自己免疫機序が明らかになるに従い，autoimmune thrombocytopenic purpura（自己免疫性血小板減少性紫斑病），さらには chronic immune thrombocytopenic purpura（慢性免疫性血小板減少性紫斑病）などの用語も用いられている．さらに，各薬剤に関する治療効果に関しても，前向き無作為試験の報告はほとんど無く，また治療効果の判定に関しても論文によりその判定基準は異なっていた．最近，トロンボポエチン（TPO）受容体アゴニストが開発され，難治性 ITP を対象として前向き無作為臨床試験が行なわれ，その有効性がエビデンスとして示されるようになってきた．この進歩を踏まえ，ITP に関する用語や治療効果の判定を標準化する必要性が叫ばれるようになり，国際作業部会（International Working Group：IWG）が編成され議論された．本稿では，作業部会が提案している内容を紹介する．詳細は Blood **113** : 2286-2393, 2009 に掲載されているので参照されたい[1]．

## 2. 国際作業部会について

ITP に関して，専門家による国際作業部会はイタリアのヴィチェンツァで 2007 年 10 月に 2 日間にわたり開催された（the Vicenza Consensus Conference）．欧州の 7 名の幹事と地域性を加味した 13 名のメンバーにより構成されており，小児 ITP も含んで議論するため International Childhood ITP study（ICIS）Group からのメンバーも入っている．残念ながら，日本からはこの作業部会には参加していない．以下に IWG からの提言を紹介する．

## 3. Primary ITP および secondary ITP の定義と血小板数（表 1）

IWG においては，ITP に関する用語に関しては idiopathic（特発性）と言う表現は避け，immune（免疫性）を用いることを推奨している．血小板減少を来たしうる明らかな原因や疾患は存在しないという意味で idiopathic ではなく primary を用いる．Purpura の表現に関しては，多くの症例において紫斑が認められるわけではないため，この用語は病名から削除している．ただ，

*大阪大学医学部附属病院輸血部〔〒 565-0871 吹田市山田丘 2-15〕
Department of Blood Transfusion, Osaka University Hospital
〔2-15, Yamadaoka, Suita, Osaka 565-0871 Japan〕
Tel: 06-6879-5887 Fax: 06-6879-5889 e-mail: yoshi@hp-blood.med.osaka-u.ac.jp