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Influence of PAR-1 in patients with non-valvular atrial fibrillation: The antiplatelet effect of dabigatran

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ABSTRACT

Introduction: Dabigatran, a direct thrombin inhibitor, has been widely used in patients with non-valvular atrial fibrillation (NVAF) and is considered to have an antiplatelet effect. However, the mechanisms remain unclear. We evaluated protease-activated receptor-1 (PAR-1) expression and activation by thrombin on platelets from NVAF patients, before and after dabigatran treatment, in addition to the expression of platelet activation marker CD62P.

Materials and methods: The study included 18 NVAF patients. We used flow cytometry to measure the binding of PAR-1 monoclonal antibodies (SPAN12 and WEDE15) and the expression of CD62P with and without thrombin stimulation, before, 14 days after, and 28 days after treatment with dabigatran. Coagulation fibrinolysis markers were also measured.

Results: PAR-1 expression was significantly lower in NVAF patients than in healthy controls (HC); it was further reduced by thrombin stimulation. CD62P expression was almost absent on the platelets in NVAF patients, but was significantly increased by thrombin stimulation. PAR-1 expression was not significantly different before and after treatment; CD62P expression was inhibited by dabigatran. The levels of coagulation markers were significantly higher in NVAF patients than in HC, and decreased after treatment.

Conclusions: Lower expression of PAR-1 in NVAF patients resulted from the cleavage of PAR-1 on some platelets, by exposure to small amounts of thrombin *in vivo*. The therapeutic effect of dabigatran in NVAF patients was demonstrated by inhibition of CD62P expression on the platelet upon thrombin stimulation *in vitro*. Our results indicate that dabigatran may reveal antithrombotic activity with antiplatelet and anticoagulant effects.

1. Introduction

Platelets are stimulated with thrombin mainly by activating protease-activated receptor-1 (PAR-1) and -4, members of the G-protein-coupled seven-transmembrane receptor family [1–3]. In particular, PAR-1 seems to play a major role in the initiation of platelet activation. Therefore, the degree of cleavage and internalization of PAR-1 on the platelet membrane may represent a marker of thrombin-induced platelet activation *in vivo*. A previous study reported that the antibodies SPAN12 and WEDE15 could be used to evaluate this [4]. SPAN12, an antibody that binds to uncleaved PAR-1, binds to PAR-1 at a lower rate when cleaved; the cleaved PAR-1 is then internalized. Conversely, WEDE15, which recognizes both cleaved and uncleaved, but not internalized PAR-1, is a marker whose expression is decreased on PAR-1 internalization. In ischemic stroke, the reduction of PAR-1 expression may result from exposure to a large amount of thrombin, *i.e.*, thrombin-induced platelet

Abbreviations: PAR-1, protease-activated receptor-1; NVAF, non-valvular atrial fibrillation; HC, healthy controls; CD62P, P-selectin; DOACs, direct oral anticoagulants; MI, myocardial infarction; APTT, activated partial-thromboplastin time; PT, prothrombin time; FMC, fibrin monomer complex; PLG, Plasminogen; TAT, Thrombin-antithrombin; PIC, plasminogen inhibitor complex; PBS, phosphate buffered saline; GPRP, Gly-Pro-Arg-Pro; PE, phycoerythrin; TRAP, thrombin receptor activating peptide.

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activation [4]. In contrast, few reports have evaluated thrombin-induced platelet activation *in vivo* in patients with non-valvular atrial fibrillation (NVAF) [5–7].

P-selectin (CD62P) expression on platelets is often used to detect activated platelets. We have previously reported that activated platelets were enhanced in patients with transient ischemic attack [8], cerebral infarction [9], and carotid artery stenosis [9]. Enhanced surface expression of CD62P has shown platelet degranulation during stroke [10]. Expression of CD62P has also been associated with spontaneous echo contrast, presence of left atrial thrombi or embolic events, and silent cerebral infarction in patients with AF [7,11].

Dabigatran, which is one of the direct oral anticoagulants (DOACs), is being widely used among patients with NVAF in recent years [12–14]. It significantly reduces embolic events at a dose of 300 mg/day compared to warfarin, showing good inhibitory effect on embolism [15,16]; it also has antiplatelet effects [17]. Reports suggest that thrombin receptor activating peptide (TRAP)-induced platelet aggregation is reduced in cardiovascular patients 2 h after administration of dabigatran [17]. However, reports have also suggested that dabigatran enhances platelet reactivity [18] and platelet thrombin receptor expression in patients with AF, 12 h after administration of dabigatran [18,19]. The landmark trial of dabigatran in patients with AF showed higher rates of myocardial infarction (MI) in dabigatran treated patients [20]. Since then, the question as to whether dabigatran increases the risk of MI has been much debated.

We have previously demonstrated the antiplatelet effects of dabigatran using thrombin-induced platelet aggregation on automated coagulation analyzers *in vivo* [21,22]; TRAP-induced platelet aggregation, that directly stimulates PAR-1 was also measured simultaneously. Low-concentration TRAP -induced platelet aggregation appeared to be non-significantly enhanced 14 days after treatment with dabigatran in patients with NVAF, compared to that in the pre-treatment period [22].

Hence, we hypothesized that NVAF increased the risk of thrombosis by thrombin generation and platelet activation. Furthermore, we hypothesized that direct thrombin inhibition by dabigatran enhances thrombin receptor expression on platelets, and continuously enhances PAR-1 direct reactivity to platelets, while exerting an antiplatelet effect by inhibiting thrombin activity. The aim of this study was to evaluate the inhibition of platelet activation upon dabigatran administration in detail, by analyzing the state of exposure to thrombin and the direct change of PAR-1 in patients with NVAF.

2. Materials and methods

2.1. Subjects

We prospectively enrolled consecutive patients with NVAF, who had never received treatment with anticoagulants before enrollment, from outpatient clinic or hospital wards off the Iwate Medical University Hospital between April 2017 and April 2018. Seven patients with acute ischemic stroke, who had been admitted to the Department of Neurology, were included in the study. The etiology of their stroke was assessed after admission by ECG, Holter-ECG, Doppler and color-coded duplex sonography of the cerebral arteries and transesophageal echocardiography. The exclusion criteria were as follows: 1) active bleeding or high risk of bleeding, 2) coagulation disorders, 3) advanced renal dysfunction, 4) spinal and epidural catheters *in situ*, 5) concurrent itraconazole therapy, 6) therapy with other DOACs or warfarin, and 7) otherwise considered inappropriate. Finally, we enrolled 18 patients (12 males and 6 females; age range: 53–89 years; median age: 75 years). In addition, we recruited 12 healthy volunteers (healthy controls; HC) (5 males and 8 females; age range: 26–54 years; median age: 39 years) who had no physical signs or symptoms of disease, and were not taking any medications. They were also recruited from the Iwate Medical University Hospital.

This study was performed after obtaining approval from the institutional ethics committee (H27–32). Written informed consent was obtained from all participants, and this study was conducted in accordance with the guidelines of the Declaration of Helsinki.

2.2. Sample collection

Dabigatran was administered in twice-daily (7:00 AM and 7:00 PM) doses of either 150 mg (D150) or 110 mg (D110). Blood samples were collected thrice: before dabigatran treatment (pre), 14 days after treatment (post 14 days), and 28 days (post 28 days) after treatment. Samples were collected from the cephalic vein with a 21-G needle at approximately 11:00 AM, 4 h after taking dabigatran. The initial 2 mL drawn was discarded, and the remaining volume was collected in a tube with 3.13% sodium citrate for aggregation testing.

Citrated blood was gently mixed by inverting the tubes, followed by centrifuging at 3000 ×g for 15 min at 4 °C. The plasma was immediately stored at below −80 °C before analysis.

2.3. Assay of coagulation markers

The prothrombin time (PT) and activated partial-thromboplastin time (APTT) were measured by the clotting method (Thromborel S, SIEMENS, and Dade Actin FS Activated PTT Reagent, SIEMENS). D-dimer and fibrin monomer complex (FMC) were measured by the immunoassay method (Auto LIA FM, Sysmex, and Lias Auto D-Dimer NEO, Sysmex). Plasminogen (PLG) was measured by the chromogenic method (Berichrom Plasminogen, SIEMENS). Levels of thrombin-antithrombin (TAT) and plasminogen inhibitor complex (PIC) were determined using the chemiluminescence enzyme immunoassay method (HISCL TAT, Sysmex, and HISCL PIC, Sysmex). PT, APTT, D-dimer, FMC, PLG, TAT, PIC were measured using the CS-5100 (Sysmex Corp, Kobe, Japan), and PT was measured by the one-step method of Quick (Thromboplastin C kit, Baxter Dade). Levels of D-dimer and PIC were determined using the enzyme immunoassay method, and PLG was measured by the thrombin method (Fibrinogen a-BMY, Boehringer Mannheim). These data were estimated as indices of the coagulation system.

Expression of PAR-1 and platelet activation marker on platelet membranes by flow cytometry.

Whole blood, diluted 10-fold with phosphate buffered saline (PBS; Wako pure chemical industries, Ltd. Osaka, Japan) to minimize the effects of antithrombin in blood, was stimulated with thrombin (Sigma-Aldrich Co., St Louis, MO, USA). *In vitro* thrombin stimulation was performed in the presence of Gly-Pro-Arg-Pro (GPRP, 1.25 mmol/L; Zedira GmbH, Darmstadt, Germany) to prevent fibrin polymerization. Sample preparation and measurement were performed by modifying previous methods [4,23]. For PAR-1 and P selectin detection, 25 μL of diluted whole blood was mixed in microcentrifuge tubes containing 10 μL of phycoerythrin (PE)-conjugated mouse anti-thrombin receptor monoclonal antibody (clones WEDE15 or SPAN12; Beckman-Coulter, Brea, CA, USA) or monoclonal antibody to P-selectin (MoAb-CD62P; Becton Dickinson Biosciences, San Jose, CA, USA), and 10 μL of peridinin chlorophyll protein-conjugated MoAb-CD61 and MoAb-CD41a (monoclonal antibodies to glycoprotein IIIa and IIb, respectively; Becton Dickinson Biosciences, San Jose, CA) to identify all the platelets. This procedure was performed within 15 min of blood collection, since addition of citrated whole blood to the cocktail of MoAbs within this time had no effect on the results. To assess the extent of nonspecific protein binding to platelets, 0.62 μL of isotype control was added instead of PE-conjugated MoAb-CD62P. The reaction mixture was stirred gently without vortexing, following incubation for 15 min at room temperature in the dark. Subsequently, platelets were fixed in 500 μL of cold 1% paraformaldehyde. Fixed cells were stored at 4 °C in the dark for at least 2 h. The samples were analyzed using a flow cytometer.

Data acquisition and analysis.

Samples were analyzed by flow cytometry with BD accuri™ (C6; BD

Biosciences, Heidelberg, Germany). Platelets were identified on the basis of the scattering profile and platelet-specific antibodies, MoAb-CD61 and MoAb-CD41a. Isotype immunoglobulin G was used as a negative control.

2.4. Statistical analysis

All data were analyzed using SPSS version 25 (IBM Japan, Ltd., Tokyo, Japan). The effects of the duration from dabigatran administration in NVAF patients on coagulation were analyzed by two-way repeated measures analysis of variance. In the nonparametric population, all values are described as the median (25th–75th percentiles) throughout the results.

The Mann-Whitney *U* test was used to compare the expression of PAR-1 and CD62P between samples collected from NVAF patients and HC. The Friedman test was used to compare the expression of PAR-1 and CD62P between samples collected from NVAF patients, before and after taking dabigatran. Similarly, the Friedmann (> 3 groups) or the Wilcoxon test (2 groups) were used to compare the expression of PAR-1 between after thrombin stimulation and no stimulation. Further, we compared the expression of PAR-1 in diluted samples stimulated *in vitro* by thrombin among the three groups (pre, post 14 days, and post 28 days). Statistical values of $p < 0.05$ were considered significant.

3. Results

The clinical backgrounds of all the patients are summarized in Table 1. Six patients had a history of ischemic disease, and 12 patients did not have a history of stroke. Among them, 11 and 7 patients were prescribed D110 and D150 twice daily, respectively.

Coagulation markers in patients with NVAF

Table 1
Characteristics of patients with non-valvular atrial fibrillation (NVAF).

Patient characteristics	Number of patients or value ($n = 18$)		
Age, years (median age)	53–89 (75)	Medication	
Male sex	12 (66.6%)	Anti-platelet agent	5 (27.8%)
		Clopidogrel	5 (27.8%)
Acute ischemic stroke	8 (44.4%)	Aspirin	0
Atherosclerosis	0	Cilostazol	0
Cardioembolic[a]	7 (38.9%)	Anti-coagulant agent	0
Lacunar	0	Statin	5 (27.8%)
TIA	1 (5.5%)	Hypoglycemic medication	1 (5.5%)
		Insulin	0
History		ACE/AT-II receptor inhibitor	7 (38.9%)
Myocardial infarction	1 (5.5%)	Calcium channel inhibitor	8 (44.4%)
Cerebral infarction	5 (27.8%)	β-blocker	2 (11.1%)
		NSAIDs	1 (5.5%)
Cerebrovascular risk factor			
Hypertension	12 (66.6%)		
Diabetes mellitus	4 (22.2%)		
Hyperlipidemia	4 (22.2%)		
Heart failure	2 (11.1%)		
Chronic kidney disease	0		
Smoking	3 (16.7%)		

Abbreviations: ACE, angiotensin-converting enzyme; AT, angiotensin; n, number; NSAIDS, non-steroidal anti-inflammatory drugs; SD, standard deviation; TIA, transient ischemic attack.

[a] After admission, Stroke etiology was assessed by examination.

To establish the influence of NVAF on the coagulation system such as the inhibition of thrombin, the data were compared between NVAF patients and HC. Table 2 shows various coagulation markers, that qualitatively describe the biological differences in pre-treatment risk in NVAF patients, and the effect at 14 and 28 days after treatment with dabigatran. The coagulation markers in HC are also shown.

The D-dimer, FMC, TAT, and PIC levels were significantly higher in NVAF patients than in the HC (all $p < 0.001$). However, the PLG in NVAF patients did not differ from that in the HC. The PT and APTT in NVAF patients were also not different than those in the HC. These results showed that NVAF itself enhanced coagulation.

To clarify the effect of dabigatran on coagulation such as thrombin generation, the data were analyzed before treatment, and at 14 and 28 days after treatment with dabigatran in the NVAF patients. The PT and APTT at 14 and 28 days after treatment with dabigatran were significantly longer than those before treatment (*vs.* 14 days $p < 0.001$, $p < 0.001$; *vs.* 28 days $p < 0.001$, $p < 0.001$, respectively). Their PT and APTT were also significantly different from those of the HC (*vs.* 14 days $p < 0.001$, $p < 0.001$; *vs.* 28 days $p < 0.001$, $p < 0.001$, respectively). In NVAF patients, the D-dimer and TAT at 14 days after treatment with dabigatran were significantly lower than those before treatment ($p < 0.05$ and $p < 0.001$, respectively). The D-dimer levels at 28 days after treatment with dabigatran were significantly lower than those before treatment ($p < 0.05$). PIC levels at days 14 and 28 after treatment with dabigatran in NVAF patients were significantly different from those of the HC ($p < 0.001$ and $p < 0.001$, respectively). However, FMC and PLG at 14 and 28 days after treatment in NVAF patients did not differ from those before treatment. These results showed that dabigatran treatment in NVAF patients inhibited the coagulation system.

PAR-1 expression on the platelet surface in NVAF.

Fig. 1 shows the relative binding of the MoAbs, recognizing PAR-1 cleavage (SPAN12; Fig. 1A) or PAR-1 internalization (WEDE15; Fig. 1B), on the platelets from HC and NVAF patients. The percentage of SPAN12 binding was 25.6% (21.2–30.7%) in samples from HC and 16.9% (14.3–22.2%) in the samples from NVAF patients. The percentage of SPAN12 binding in patients with NVAF was significantly lower than that in HC ($p < 0.001$). Conversely, the percentage of WEDE15 binding was 41.3% (31.7–50.9%) in samples from HC and 29.8% (26.6–41.4%) in samples from NVAF patients. The percentage of WEDE15 binding in patients with NVAF was also significantly lower than that in the HC ($p < 0.05$). Compared to HC, both SPAN12 and WEDE15 were lower in patients with NVAF, indicating PAR-1 cleavage and internalization.

Dabigatran effects on PAR-1 cleavage and internalization.

Fig. 2 shows the relative binding of the MoAbs in NVAF patients before and after treatment with dabigatran. The percentage of SPAN12 binding was 18.8% (12.4–22.2%) in samples taken from patients 14 days after treatment with dabigatran, and 13.0% (10.2–18%) in samples taken from patients 28 days after treatment (Fig. 2A). The percentage of SPAN12 binding 14 days or 28 days after treatment with dabigatran were not significantly different.

The percentage of WEDE15 binding was 34.8% (22.5–43.2%) in samples taken from patients 14 days after treatment with dabigatran, 29.7% (22.7–33.7%) in samples taken 28 days after treatment, and 29.8% (26.6–41.4%) in samples taken before treatment (Fig. 2B). There was no statistically significant difference in the percentage of PAR-1 antibody (WEDE15) binding in the samples taken before and after treatment with dabigatran (Fig. 2B).

Dabigatran effects on PAR-1 cleavage and internalization stimulated by thrombin *in vitro*.

Fig. 3 shows the fluctuation in the percentage of PAR-1, stimulated by thrombin *in vitro*. The results of pretreatment in NVAF patients are listed above. After stimulation with 200 mU/mL thrombin, the percentage of SPAN12 binding was 5.9% (3.8–9.3%) in samples taken from HC, 6.6% (5.0–8.9%) in samples taken from patients before treatment with dabigatran, 6.4% (4.2–10.4%) in samples taken from patients 14 days after treatment, and 6.0% (3.2–6.9%) in samples taken from

Table 2
Coagulation markers in NVAF patients treated with dabigatran.

parameter	HC	NVAF pre on dabigatran	NVAF post 14 days on dabigatran	NVAF post 28 days on dabigatran
PT [sec] (median)	10.70–11.80 (11.30)	11.10–11.80 (11.30)	11.90–14.58 (12.80)***, §§§	11.75–13.50 (12.50)***, §§§
APTT [sec] (median)	26.20–31.05 (29.05)	23.25–28.08 (25.20)	35.80–53.10 (41.60)***, §§§	32.20–48.65 (37.70)***, §§§
D-Dimer [μg/mL] (median)	0.10–0.35 (0.20)	0.45–2.35 (0.80)***	0.10–0.73 (0.30)§	0.10–0.50 (0.20)§
FMC [μg/mL] (median)	1.25–1.80 (1.60)	1.63–2.70 (2.10)***	1.30–1.85 (1.60)	1.45–2.35 (1.80)
PLG [%] (median)	96.80–111.60 (104.75)	98.68–111.3.35 (106.80)	98.83–107.85 (103.10)	97.45–109.60 (102.90)
TAT [ng/mL] (median)	0.50–1.00 (0.70)	1.30–3.48 (1.80)***	0.58–1.05 (0.80)§§§	0.55–1.60 (1.00)
PIC [μg/mL] (median)	0.43–0.60 (0.51)	0.74–1.22 (0.95)***	0.61–1.10 (0.77)***	0.53–0.98 (0.81)***

Data are presented as median (25–75% inter-quartile range).
PT, prothrombin time; APTT, activated partial thromboplastin time; FMC, fibrin monomer complex; PLG, plasminogen; TAT, thrombin-antithrombin complex; PIC, plasmin-α2 plasmin inhibitor complex.
*** $p < 0.001$ in comparison to healthy controls (HC).
§§§ $p < 0.001$.
§ $p < 0.05$ compared to before treatment in non-valvular atrial fibrillation (NVAF) patients.

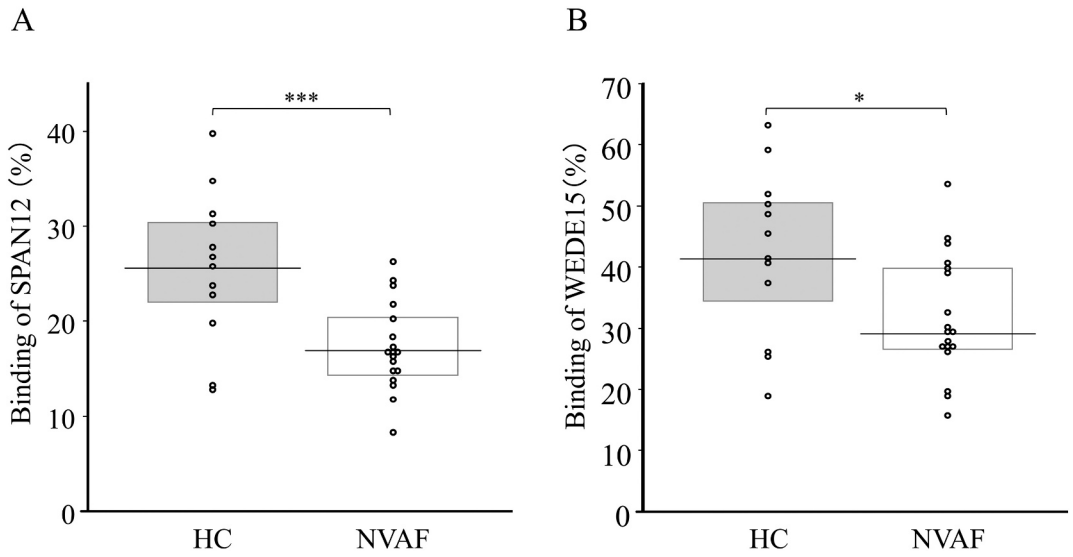

Fig. 1. Expression rate of protease activated receptor-1 (PAR-1) in patients with non-valvular atrial fibrillation (NVAF) assessed by flow cytometry. The data are expressed as medians (25th–75th percentiles). (A) The percentage of SPAN12 in patients with NVAF was lower than that in healthy controls (HC). (B) The percentage of WEDE15 in patients with NVAF was also lower than that in HC. Testing for differences between HC and patients with NVAF before treatment with dabigatran was performed using the Mann-Whitney *U* test. $^{**}p < 0.01$. $^{*}p < 0.05$.

patients 28 days after treatment (Fig. 3A). In addition, the percentage of WEDE15 binding was 14.0% (5.9–15.6%) in samples taken from HC, 13.3% (8.8–17.6%) in samples taken from patients before treatment with dabigatran, 18.2% (10.6–28.3%) in samples taken from patients 14 days after treatment, and 17.0% (8.8–23.5%) in samples taken from patients 28 days after treatment (Fig. 3B). After thrombin stimulation, the expression of PAR-1 (binding of SPAN12 or WEDE15) in HC was significantly reduced compared to that in the unstimulated state ($p < 0.001$ and $p < 0.001$, respectively). Furthermore, the expression of PAR-1 after thrombin stimulation was significantly reduced in patients with NVAF before, 14, and 28 days after treatment with dabigatran, as compared to the unstimulated state (All $p < 0.001$). However, the levels of 14- or 28-day post-treatment WEDE15 binding were not the same as those of NVAF patients before treatment. In particular, analysis after 200 mM/mL thrombin stimulation showed that WEDE15 binding in NVAF patients was significantly higher ($p < 0.01$) 14 days after treatment with dabigatran, than that before treatment.

CD62P expression in NVAF patients and the effect of dabigatran on platelet activation by thrombin *in vitro*.

The percentage of expression of CD62P in HC was 1.1% (0.5–1.5%). After 50 mU/mL thrombin stimulation *in vitro*, the percentage of CD62P expression was 40.4% (17.5–60.8%). After 200 mU/mL thrombin stimulation *in vivo*, the percentage of expression of CD62P was 96.6% (91.9–98.5%). The percentage of CD62P expression after stimulation by 50 or 200 mM/mL thrombin was significantly higher in samples from healthy subjects ($p < 0.001$ and $p < 0.001$, respectively).

Fig. 4 shows the expression of CD62P in patients with NVAF. The percentage of CD62P expression was 0.5% (0.4–1.2%) in samples taken from patients before treatment with dabigatran, 0.6% (0.5–1.0%) in samples taken 14 days after treatment, and 0.7% (0.4–1.5%) in samples taken 28 days after treatment. CD62P expression levels did not differ between the pre-treatment NVAF patients and healthy subjects. Furthermore, there were no differences in the percentage of CD62P expression between the samples taken before and after treatment (post 14 or 28 days) with dabigatran. Therefore, expression of CD62P was not observed in patients with NVAF without thrombin stimulation before or after treatment with dabigatran (post 14 days or post 28 days).

After stimulation with 50 mU/mL thrombin *in vitro*, the percentage of CD62P expression of was 32.9% (11.1–53.5%) in samples taken prior to treatment with dabigatran, 1.6% (0.9–2.6%) in samples taken 14 days after treatment, and 1.65% (0.8–2.7%) in samples taken 28 days after treatment.

After 200 mU/mL thrombin stimulation *in vitro*, the percentage of CD62P expression was 97.2% (96.4–97.8%) in samples taken before treatment with dabigatran, 46.3% (8.1–91.8%) in samples taken 14 days after treatment, and 34.9% (7.4–83.1%) in samples taken 28 days after

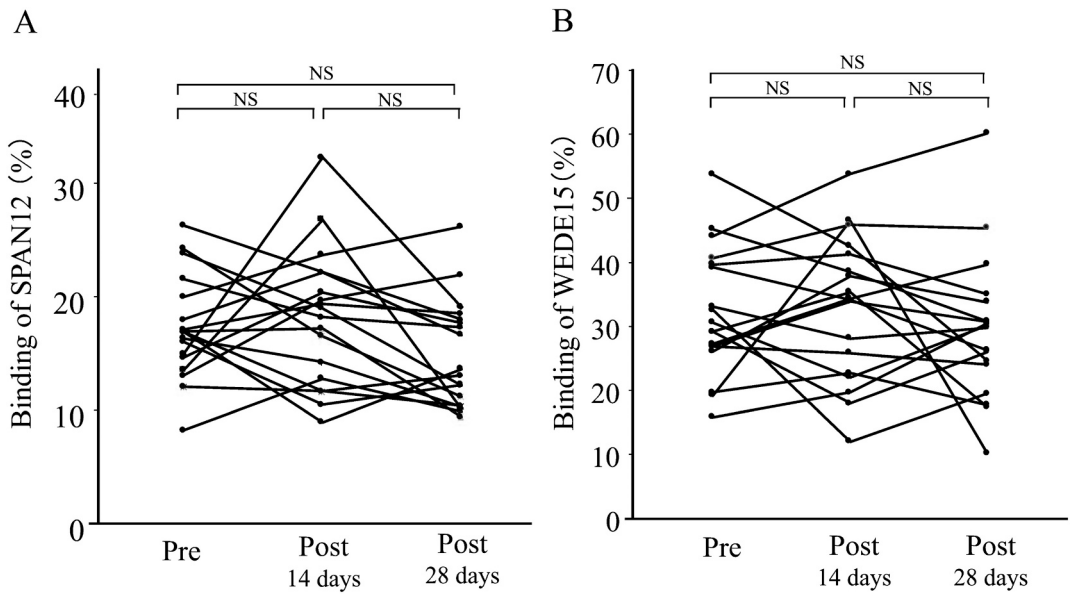

Fig. 2. Expression rate of protease activated receptor-1 (PAR-1) following treatment with dabigatran in patients with non-valvular atrial fibrillation (NVAF) assessed by flow cytometry. The individual data are represented as a line graph over time. (A) The percentage of SPAN12 in samples taken before treatment with dabigatran (Pre) and 14 days after treatment (Post 14 days) was not significantly different. The percentage of SPAN12 28 days after treatment with dabigatran (Post 28 days) was not significantly different from that at Pre and Post 14 days. (B) There was no significant difference in the percentage of WEDE15 in samples taken at Pre, Post 14 days, and Post 28 days. Testing for differences in Pre, Post 14 days, and Post 28 days was performed using the Friedman test. Abbreviation: NS, no significant difference.

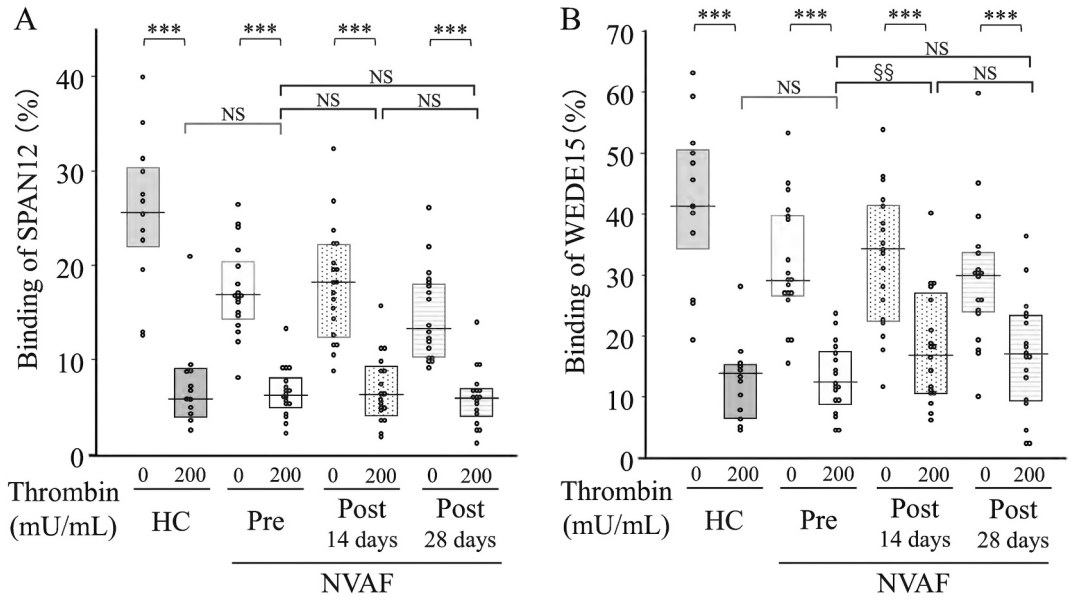

Fig. 3. Modification of protease activated receptor-1 (PAR-1) stimulated by thrombin *in vitro* following treatment with dabigatran in patients with non-valvular atrial fibrillation (NVAF) assessed by flow cytometry. The data are expressed as medians (25th–75th percentiles). (A) Binding of SPAN12. (B) Binding of WEDE15. Among the samples taken from healthy controls (HC) and from patients with NVAF before treatment with dabigatran (Pre), 14 days after treatment (Post 14 days), and 28 days after treatment (Post 28 days), the percentage of PAR-1 expression after thrombin stimulation significantly reduced compared to that after no stimulation. There were no statistically significant differences among the four groups. Testing for differences between samples from HC and Pre was performed using the Mann-Whitney U test. Testing for differences among samples taken at Pre, Post 14 days, and Post 28 days was performed using the Friedman test (***$p < 0.001$). Testing for differences among samples taken at Pre, Post 14 days, and Post 28 days at 200 mM/mL thrombin stimulation was also performed using the Friedman test (§§ $p < 0.01$). NS, no significant difference.

treatment.

Similar to HC, the percentage of CD62P expression in NVAF patients increased in a concentration-dependent manner of thrombin stimulation ($p < 0.001$ *vs.* thrombin 50, $p < 0.001$ *vs.* thrombin 200) before treatment with dabigatran. The percentage of CD62P expression on days 14 and 28 after treatment with dabigatran also increased in these patients after thrombin stimulation. However, the degree of CD62P expression after thrombin stimulation was clearly different from that before treatment. The percentage of CD62P expression after treatment with dabigatran was lower after thrombin stimulation than before treatment. (50 mU/mL for days 14 and 28, and 200 mU/mL for days 14 and 28).

The results therefore suggest that dabigatran treatment inhibited the

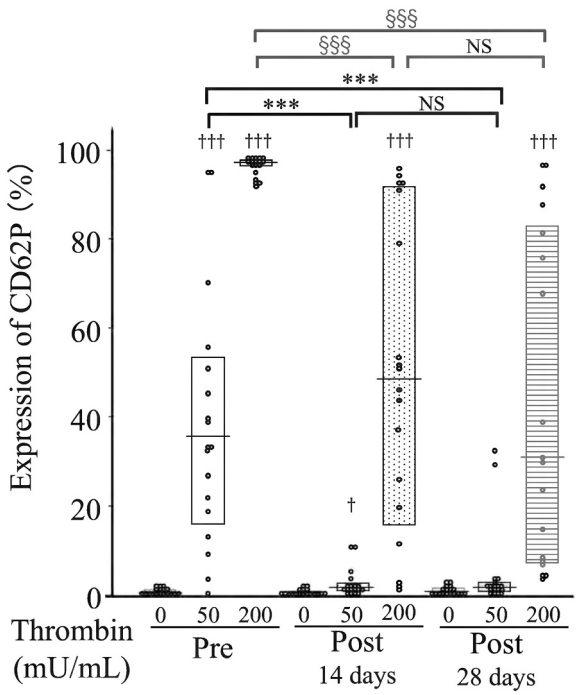

Fig. 4. Antiplatelet effects of dabigatran on platelets from patients with non-valvular atrial fibrillation (NVAF). The data are expressed as medians (25th–75th percentiles). Baseline expression of CD62P in patients with NVAF was no different among samples taken from NVAF patients before treatment with dabigatran (Pre), 14 days after treatment (Post 14 days), and 28 days after treatment (Post 28 days). After thrombin stimulation, the expression of CD62P in samples taken at Pretreatment significantly increased. In contrast, the expression of CD62P after thrombin stimulation was lower in samples taken after treatment with dabigatran than before treatment. The CD62P was suppressed by dabigatran treatment; thus, this result confirmed that dabigatran has an antiplatelet effect. Testing for differences between samples taken at Pre, Post 14 days, and Post 28 days was performed using the Friedman test.
*** $p < 0.001$ in comparison to before treatment in non-valvular atrial fibrillation (NVAF) patients at 50 mM/mL thrombin stimulation. §§§ $p < 0.001$ compared to before treatment in NVAF patients at 200 mM/mL thrombin stimulation. †††$p < 0.001$, †$p < 0.05$ compared to before thrombin stimulation in NVAF patients. NS, no significant difference.

expression of CD62P, which reflects platelet activation.

4. Discussion

This study assessed the details of the inhibition of platelet activation upon dabigatran administration, by analyzing the state of thrombin exposure and the direct change of PAR-1 in patients with NVAF. We found that there was minimal thrombin exposure, that did not cause platelet activation in the blood of NVAF patients, resulting in cleavage and internalization of PAR-1 in platelets. The findings also suggest that treatment with dabigatran *in vitro* and *in vivo* attenuated platelet activation and the coagulation-fibrinolysis system stimulated by thrombin.

Firstly, both SPAN12 and WEDE15 in patients with NVAF were lower than those in the HC group, indicating PAR-1 cleavage and internalization. Furthermore, D-dimer, TAT, and PIC, which are indicators of coagulation and fibrinolysis, were significantly higher in NVAF patients than in the normal control group; this suggests that the coagulation/fibrinolysis system is in an enhanced state in the NVAF patient group. Our findings are consistent with those of other studies [11]; NVAF patients were therefore considered to be in a thrombogenic state. Lim et al. [11] reported acute elevation in thrombin generation specifically in the left and right atrium at the onset of AF and rapid atrial rates; this was not seen in the peripheral circulation. It was therefore considered that thrombin generation was occurring in the atrium of NVAF patients, and platelets in these patients were clearly but not strongly exposed to thrombin *in vivo*; the stage of thrombus formation in NVAF patients was therefore unclear.

Secondly, to confirm whether PAR-1 on platelets of NVAF patients still had the ability to cleave, we examined the expression of PAR-1 after thrombin stimulation *in vitro*. The activated platelet status of NVAF patients after dabigatran treatment was also examined.

The expression rate of PAR-1 on platelets (SPAN12 and WEDE15 binding) treated with 200 mU/mL thrombin was significantly reduced in platelets of NVAF patients, compared to that in untreated platelets (*in vitro* study in Fig. 3).

Jurk et al. [4] found no further significant reduction in binding epitopes for SPAN12 and WEDE15 on *in vitro* stimulation of platelets of acute ischemic stroke patients with thrombin, supporting the notion that these had already strongly been activated by thrombin *in vivo*. This suggests that the platelets in NVAF patients had PAR-1 cleavage/internalization by exposure to thrombin *in vivo*; however, further cleavage/internalization by thrombin stimulation *in vitro* was sufficiently maintained. In summary, we speculated that PAR-1 cleavage/internalization *in vivo* was restricted. This finding was different from that reported in acute ischemic stroke patients [4].

Thirdly, the effect of dabigatran on PAR-1 expression and platelet activation in NVAF patients was examined. The short-term study by Sohara et al. [24] reported the expression of PAR-1 and CD62P within 12 h of dabigatran treatment in the AF patient group. The study showed that thrombin receptor expression and platelet response were enhanced during dabigatran treatment. Several studies [18,25] have shown that dabigatran treatment increases platelet reactivity by increasing PAR-1 expression. Dabigatran-bound thrombin suppresses thrombin activity, but also can bind stably to PAR-1. Further, the inability to cleave and internalize PAR-1 amplifies their number on the platelet membrane [19]. The present study examined changes in PAR-1 and CD62P expression in patients with NVAF after 14 days and 28 days of long-term continuous treatment with dabigatran. The results showed that the binding rate of SPAN-12 in NVAF patients was not significant after 14 days of treatment, compared with that before treatment. The reason was that PAR-1 expression varied significantly among individuals at days 14 days and 28 after treatment. After treatment with dabigatran, the amount of *in vivo* active thrombin in NVAF patients, as evaluated from the results of the measured coagulation markers (D-dimer, FMC, and TAT) was not different from that of the normal control group. It could be inferred that *in vivo* thrombin activity was strongly inhibited by dabigatran treatment. Our results suggest that the presence of dabigatran-bound thrombin may have caused the change in PAR-1 on platelets, 14 days after dabigatran treatment. Recent findings on the effect of dabigatran on PAR-1 are conflicting [15–20]. Future studies including larger numbers of cases need to be performed for further evaluation.

Fourthly, we investigated whether thrombin stimulation affects PAR-1 on platelets in NVAF patients after dabigatran treatment *in vitro*.

The groups of thrombin stimulation *in vitro* at 14 days after treatment showed that the binding to WEDE15 was slightly higher. This was probably because a part of the thrombin added *in vitro* lost its activity by binding with dabigatran, and the dabigatran-thrombin could not cleave PAR-1. Nevertheless, it was possible that dabigatran-thrombin stably bound to PAR-1, and inactive PAR-1 could not be internalized. Another possibility is that the expression of PAR-1 on platelets was increased by ubiquitination of PAR-1. Chen et al. [19] reported that PAR-1 exhibited a modest amount of ubiquitination when endothelial cells were exposed to dabigatran alone. As a result, prolonged exposure of endothelial cells to dabigatran-bound thrombin promoted PAR-1 ubiquitination and enhanced PAR-1 expression. The expression of PAR-1 in NVAF patients was probably significantly higher 14 days after treatment with dabigatran than before treatment, as moderate ubiquitination occurred with dabigatran alone.

Fifthly, we investigated the effect of dabigatran on platelets by

thrombin exposure *in vitro* in patients with NVAF. Platelets in NVAF patients were not activated; subsequent activation occurred with thrombin exposure. This suggests that platelets of NVAF patients were not activated by thrombin exposure *in vivo* and degranulation did not occur; however, subsequent *in vitro* stimulation with low concentrations of thrombin, ADP, and collagen, among others could cause degranulation.

Dabigatran conclusively suppressed CD62P expression due to thrombin stimulation, and our study suggested an antiplatelet effect of dabigatran. However, the inhibitive effect of dabigatran was not complete, because stimulation with a large amount of 200 mU/mL thrombin activated some platelets that could not bind to the former. Hence, our results suggest that dabigatran may not be able to inhibit platelet activation in the presence of large amounts of thrombin, such as when a thrombin burst occurs *in vivo;* the troughs during treatment with dabigatran are therefore a major issue. Hawes et al. [26] reported that trough concentrations within the therapeutic range of dabigatran were 18–206 ng/mL. The patients with increased PAR-1 expression on platelets may be affected by decreased blood levels of dabigatran, which may increase platelet sensitivity and activation. Therefore, patients should be compliant with dabigatran. In our previous studies, dabigatran had an inhibitory effect on thrombin-induced platelet aggregation *in vitro* in a dose-dependent manner [21], and we were able to demonstrate the antiplatelet effect of dabigatran in patients with NVAF using thrombin-induced platelet aggregation on automated aggregometry [22]. The thrombin-induced CD62P expression on platelets in the present study was consistent with the results of thrombin-induced platelet aggregation, and we speculated that thrombin-dependent platelet activation indirectly detected the inhibitory effect of dabigatran. Thus, it may be necessary to monitor platelet CD62P expression in patients with NVAF, who are undergoing dabigatran treatment.

This study had several limitations. First, we could only enroll a small number of patients, mainly because we performed the study at a single center. Moreover, strict exclusion criteria limited recruitment for this study. Further studies with a larger number of patients with NVAF from several institutions are needed for validation. A *se*cond limitation is that the antiplatelet effect of dabigatran was evaluated for several weeks after treatment. However, in patients with NVAF, long-term treatment with dabigatran is needed for preventing thrombosis. Thus, further studies with longer follow-up are required.

5. Conclusions

In patients with NVAF, lower expression of PAR-1 on platelets resulted from cleavage of PAR-1 on some platelets on exposure to small amounts of thrombin *in vivo*. Treatment with dabigatran strongly inhibited thrombin activity, which in turn inhibited thrombin activity on platelets. The antiplatelet effect of dabigatran was revealed by the inhibition of CD62P expression on the platelet membrane of patients with NVAF. Dabigatran may be an antithrombotic drug with both, antiplatelet and anticoagulant effects.

Declarations of competing interest

None.

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