Comparison of Platelet P2Y$_{12}$ ADP Receptor-Mediated Pathway Inhibition in Triple Versus Dual Antiplatelet Therapy as Assessed by VASP-Phosphorylation in Japanese Patients Undergoing Coronary Stenting

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Summary

Despite wide interindividual variability in response to clopidogrel, platelet P2Y$_{12}$ ADP receptor inhibition in Japanese patients has not been fully studied using specific methodology. This study compared platelet P2Y$_{12}$ ADP receptor inhibition during treatment with clopidogrel versus clopidogrel plus cilostazol in patients undergoing coronary stenting.

Forty-two patients in whom platelet function was measured within 2 months after coronary stenting were enrolled. All patients were treated with aspirin 100 or 200 mg/day, and were divided into a dual therapy group (aspirin plus clopidogrel 75 mg/day; $n = 34$) and a triple therapy group (aspirin plus clopidogrel 75 mg/day plus cilostazol 200 mg/day; $n = 8$). Vasodilator-stimulated phosphoprotein (VASP) phosphorylation analysis and 5 and 20 μmol/L-induced maximal platelet aggregation were assessed.

No differences were found in baseline characteristics except for a higher incidence of diabetes mellitus (DM) in the triple therapy group. Although there were no differences in platelet aggregation between the 2 groups, VASP index was significantly lower in the triple therapy group than in the dual therapy group ($23.1 \pm 15.3\%$ versus $51.2 \pm 19.9\%$; $P = 0.001$). The rate of low responsiveness to clopidogrel, defined by VASP index > 50%, was lower in the triple therapy group than in the dual therapy group (12.5% versus 55.9%; $P = 0.047$). Similarly, in DM patients the triple therapy group had a lower VASP index compared with the dual therapy group ($23.1 \pm 15.3\%$ versus $47.0 \pm 23.5\%$; $P = 0.015$).

Clopidogrel plus cilostazol is more effective in inhibiting the platelet P2Y$_{12}$ ADP receptor pathway than clopidogrel alone. This may be useful for reducing clopidogrel resistance in Japanese patients.   (Int Heart J 2010; 51: 303-307)

Key words: Platelet P2Y$_{12}$ ADP receptor, Vasodilator-stimulated phosphoprotein, Clopidogrel, Cilostazol

Dual antiplatelet treatment with aspirin and clopidogrel has been recommended as a standard therapy for the prevention of thrombotic events in patients undergoing percutaneous coronary intervention (PCI).[1] However, several studies have shown considerable interindividual variability in the response to clopidogrel, with an inadequate antiplatelet effect in approximately 5-30% of patients.[2,3] As a consequence, higher clopidogrel loading and maintenance doses, as well as administration of the novel thienopyridine agent prasugrel, which exerts a more potent antiplatelet effect than clopidogrel, have been employed.[4-6] It may be difficult to employ the same doses in Japanese patients as in western patients due to racial differences, and therefore other approaches are needed.

Previous investigations have demonstrated that triple antiplatelet therapy (the addition of cilostazol to aspirin and clopidogrel) causes greater platelet inhibition than dual antiplatelet therapy, and may result in decreased stent thrombosis in patients undergoing coronary stenting.[7,8] However, conventional optical aggregometry was used in most of these studies. Recently, vasodilator-stimulated phosphoprotein (VASP) analysis has been used as a specific method for evaluating platelet P2Y$_{12}$ ADP receptor inhibition.[2] Angiolillo,[9] *et al* showed that the P2Y$_{12}$ reactivity index was lower following cilostazol treatment than with placebo treatment by VASP analysis in patients with diabetes mellitus (DM). However, it remains uncertain whether triple antiplatelet therapy would also result in more potent platelet P2Y$_{12}$ ADP receptor-mediated pathway inhibition in Japanese patients. Furthermore, platelet P2Y$_{12}$ ADP receptor-mediated pathway inhibition levels in triple versus dual antiplatelet therapy using VASP analysis have never been compared in Japan. Accordingly, we carried out this study in Japanese patients undergoing stent implantation in order to compare the platelet inhibitory effects of dual versus triple antiplatelet therapy.

Methods

Study population: A total of 108 patients underwent VASP phosphorylation analysis and ADP-induced platelet aggregation studies after coronary stent implantation from October

2007 to May 2010. Patients who received both platelet function tests within 2 months after PCI were included. Exclusion criteria included patients with concomitant use of antithrombotic drugs such as warfarin and antiplatelet drugs other than aspirin, clopidogrel and cilostazol, those who had platelet function tests more than 2 months after PCI, and patients with active bleeding or a bleeding diathesis or with a platelet count < 100,000/μL. Of the total of 108 patients, 42 qualified for study and were included. All study patients were treated with aspirin 100 or 200 mg/day. Of the group of 42 patients, 34 patients received aspirin and clopidogrel 75 mg/day and 8 patients were treated with aspirin, clopidogrel 75 mg/day, and cilostazol 200 mg/day. Thus, the study subjects were divided into a dual therapy group (n = 34) and a triple therapy group (n = 8). This study was approved by the Ethics Committee of Teikyo University Hospital (No.10-025), and all patients provided informed consent.

Blood samples: Blood samples for platelet function assays were drawn into vacutainer tubes containing 0.5 mL of 3.14% sodium citrate from an antecubital vein using a 21-gauge needle by the double syringe technique 2 to 4 hours after antiplatelet therapy. In order to avoid spontaneous platelet activation, the first 2 mL of blood was discarded.

Platelet function testing: As indicated above, ADP-induced platelet aggregation and VASP phosphorylation analyses were performed within 2 months after coronary stent implantation. ADP-induced platelet aggregation was measured within 3 hours after blood sampling. Platelet maximal aggregation was assessed at 37°C with platelet rich plasma (PRP) by MCM HEMA TRACER 212 aggregometry (MC Medical, Tokyo) after stimulation with 5 or 20 μmol/L ADP. Platelet maximal aggregation curves were recorded for 5 minutes. PRP was obtained as a supernatant after centrifugation of citrated blood at 1000 rpm for 10 minutes. The isolated PRP was kept at room temperature before use. Platelet poor plasma (PPP) was obtained by a second centrifugation of the blood fraction at 3000 rpm for 15 minutes. Light transmission was adjusted to 0% with PRP and to 100% with PPP for each measurement. Platelet aggregation was determined as the maximal percent change in light transmittance from baseline using PPP as a reference.

VASP phosphorylation analysis was performed within 24 hours after blood sampling using platelet VASP/P2Y$_{12}$ kits (Biocytex, Marseille, France) according to the manufacturer's protocol.[10] Briefly, 10 μL blood samples were incubated with ADP (10 μM) and/or prostaglandin E1 (PGE1) at room temperature for 10 minutes. Reactions were stopped by addition of 10 μL of fixation reagent and fixed for 5 minutes at room temperature. Subsequently, cell suspensions were immunolabeled for 5 minutes using a VASP-P specific mouse monoclonal antibody or a negative isotypic control antibody and a CD61 phycoerythrin-labeled platelet specific antibody. Finally, platelets were counterstained with a FITC-conjugated polyclonal anti-mouse IgG antibody for 5 minutes. Flow cytometric analysis was performed using FACSCalibur (Becton Dickinson) and platelet events were analyzed for geometric mean fluorescence intensity (MFI). The MFI corresponding to each experimental condition (ADP, ADP+PGE1) was determined to establish a ratio directly correlated with the VASP phosphorylation state. A platelet reactivity index (PRI) or VASP index was calculated by measuring VASP phosphorylation levels after stimulation with PGE1 and also PGEI + ADP according to the formula:

PRI = [(MFI^{PGE1}-MFI$^{ADP+PGE1}$)/MFI^{PGE1}] ×100. A ratio of 100% indicated complete absence of a clopidogrel effect; and a decrease in the ratio indicated the presence of some degree of clopidogrel-induced inhibition of the platelet P2Y$_{12}$ ADP receptor.

Endpoints and definition: The definitive endpoint of this study proved to be responsiveness to clopidogrel assessed with VASP phosphorylation analysis within 2 months after coronary stent implantation. Low responsiveness to clopidogrel was defined by VASP-PRI > 50%, based on previous studies suggesting that patients with these values were at a higher risk of stent thrombosis.[11]

Statistical analysis: Variables were analyzed for a normal distribution with the Kolmogorov-Smirnov test. Continuous variables following a normal distribution are expressed as the mean ± SD. Categorical variables are expressed as frequencies and percentages. Differences between the two groups were determined by the Student t test for continuous variables. The χ^2 test or Fisher exact test was used as appropriate to compare categorical variables. Differences were considered significant at $P < 0.05$. Statistical analysis was performed with SPSS 16.0 software (SPSS, Inc. Chicago, IL, USA).

RESULTS

The baseline demographic and clinical characteristics of the study population are listed in Table I. A significantly higher incidence of DM was present in the triple therapy group (100% versus 53%; P = 0.016). However, there were no significant differences in age, sex, other risk factors, or administration of other medications between the two groups. There were also no significant differences between the two groups in angiographic and procedural characteristics (Table II). Stent thrombosis and bleeding complications did not occur in either group.

No significant differences were observed in 5 and 20 μmol/L ADP-induced platelet maximal aggregation between the two groups (Table III). However, the VASP index was sig-

Table I. Baseline Demographic and Clinical Characteristics

Variable	Dual therapy group (n = 34)	Triple therapy group (n = 8)	P
Age (years)	69 ± 8	70 ± 11	0.87
Male	26 (76%)	5 (63%)	0.41
Risk factor			
Diabetes mellitus	18 (53%)	8 (100%)	0.016
Hypertension	26 (76%)	6 (75%)	1.00
Hyperlipidemia	25 (74%)	6 (75%)	1.00
Smoking	9 (27%)	4 (50%)	0.23
Hemodialysis	7 (21%)	3 (38%)	0.37
Concomitant medications			
Statin	26 (76%)	6 (75%)	1.00
β-blocker	15 (44%)	4 (50%)	1.00
ACE-inhibitor/ARB	27 (79%)	4 (50%)	0.17
Calcium channel blocker	15 (44%)	3 (38%)	1.00
Proton pump inhibitor	21 (62%)	6 (75%)	0.69

Data are shown as the mean ± SD or number (%) of patients. ACE indicates angiotensin-converting enzyme and ARB, angiotensin receptor inhibitor.

Table II. Baseline Angiographic and Procedural Characteristics

Variable	Dual therapy group ($n = 34$)	Triple therapy group ($n = 8$)	P
Bifurcation	19 (56%)	3 (38%)	0.45
Calcification	23 (68%)	6 (75%)	1.00
Type B$_2$/C	32 (94%)	8 (100%)	1.00
Stent type			
Bare metal stent	6 (18%)	1 (13%)	1.00
Sirolimus-eluting stent	19 (56%)	5 (63%)	1.00
Paclitaxel-eluting stent	7 (21%)	2 (25%)	1.00
Zotarimus-eluting stent	1 (3%)	0 (0%)	1.00
Everolimus-eluting stent	1 (3%)	0 (0%)	1.00
Total stent length (mm)	45.3 ± 25.2	56.1 ± 26.1	0.28
Number of stents	2.1 ± 0.9	2.3 ± 0.9	0.60

Data are shown as the mean ± SD or number (%) of patients.

Table III. Maximal Platelet Aggregation Assessed by Light Transmittance Aggregometry

Variable (%)	Dual therapy group ($n = 34$)	Triple therapy group ($n = 8$)	P
5 μmol/L ADP	54.3 ± 9.8	51.5 ± 7.7	0.46
20 μmol/L ADP	59.4 ± 9.2	59.1 ± 7.7	0.94

Data are shown as the mean ± SD. ADP indicates adenosine diphosphate.

Figure. Comparison of VASP index (PRI) between dual and triple antiplatelet therapy.

Table IV. Platelet Reactivity in Patients With DM

Variable (%)	Dual therapy group ($n = 18$)	Triple therapy group ($n = 8$)	P
Maximal platelet aggregation (%)			
5 μmol/L ADP	53.7 ± 10.6	51.5 ± 7.7	0.60
20 μmol/L ADP	58.7 ± 11.2	59.1 ± 7.7	0.92
VASP index (PRI; %)	47.0 ± 23.5	23.1 ± 15.3	0.015

Data are shown as the mean ± SD. ADP indicates adenosine diphosphate; VASP, vasodilator-stimulated phosphoprotein; and PRI, platelet reactivity index.

nificantly lower in the triple therapy group compared with the dual therapy group (23.1 ± 15.3% versus 51.2 ± 19.9%; P = 0.001; Figure). Also, the rate of low clopidogrel responsiveness was significantly lower in the triple therapy group than in the dual therapy group (12.5% versus 55.9%; P = 0.047).

In addition, since patients with DM have high platelet reactivity and reduced responsiveness to antiplatelet drugs after antiplatelet treatment,[12] a subgroup analysis in DM patients was carried out. Among the 26 patients with DM in the entire study group (18 treated with dual therapy and 8 with triple therapy), there were no differences in ADP-induced platelet aggregation between dual and triple therapy, whereas the VASP index in the triple therapy group was significantly lower than in the dual therapy group (Table IV).

DISCUSSION

The results of the present study demonstrate that triple antiplatelet therapy (aspirin plus clopidogrel plus cilostazol) is more effective than dual therapy (aspirin plus clopidogrel) in inhibiting the platelet P2Y$_{12}$ ADP receptor-mediated pathway, based on the results of VASP phosphorylation analysis.

It is well-known that current standard dual antiplatelet therapy with aspirin and clopidogrel can effectively prevent thrombotic events in patients with acute coronary syndromes and/or undergoing PCI.[13,14] However, an inadequate response to clopidogrel is commonly observed, and is associated with a higher risk of thrombotic complications.[2] Hoshino, et al[15] reported that the rate of inadequate responsiveness to clopidogrel was 50% in Japanese patients. Moreover, the frequency of
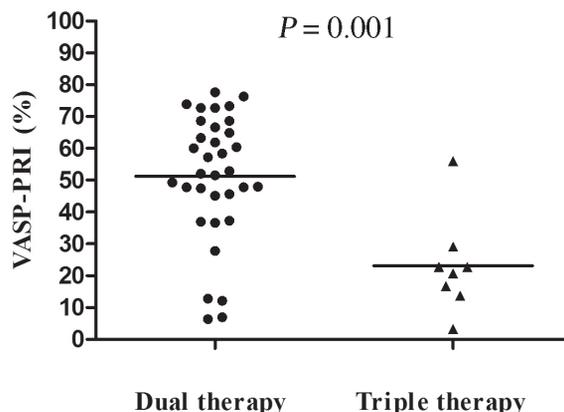
poor metabolism of clopidogrel caused by cytochrome P450 (CYP) 2C19*2 or *3 mutations in Japanese patients was approximately 20%, which is higher than that reported in a western population.[16] This defect may result in suboptimal platelet P2Y$_{12}$ ADP receptor inhibition.

Although inadequate responsiveness to clopidogrel can be treated by increasing the dose, this is not currently approved in Japan. A better approach in Japanese patients might be to improve clopidogrel responsiveness by combining this agent with other antiplatelet drugs, thereby producing more potent inhibition of the platelet P2Y$_{12}$ ADP receptor-mediated pathway. Cilostazol is a selective oral inhibitor of cyclic nucleotide phosphodiesterase 3 (PDE3), with associated antiplatelet, vasodilatory, and antimitogenic effects, which is used for secondary prevention of ischemic events, and is a candidate drug for this additional agent. The antiplatelet effects of cilostazol are considered to be due to an increase in intracellular cyclic adenosine monophosphate (cAMP) resulting from inhibition of PDE3 in platelets, an action similar to that seen with both ticlopidine and clopidogrel.[17-19] Recent research has demonstrated that the addition of cilostazol to clopidogrel therapy significantly increased ADP-induced platelet inhibition as compared with a high maintenance dose (150 mg/day) of clopidogrel alone in patients with acute myocardial infarction.[20] In our study, both the VASP index and the rate of suboptimal responsiveness to clopidogrel in the triple therapy group were lower than in the dual therapy group. Further ele-

vation of intracellular cAMP through inhibition of cAMP degradation by cilostazol[17] may contribute to platelet $P2Y_{12}$ ADP receptor pathway suppression and improved clopidogrel responsiveness. We also observed that the VASP index in triple therapy was markedly lower than in dual therapy in the DM subgroup, suggesting that triple therapy may be useful in this group also. Since antiplatelet drug resistance is associated with a higher risk of cardiovascular events including stent thrombosis in patients undergoing PCI,[21] the findings of this study may have broad implications favoring the value of triple therapy in patients with aspirin and clopidogrel resistance.

Enhanced platelet inhibition is often accompanied by an increased risk of bleeding, but was not observed with triple therapy in our study. This could be attributed to the protective effects of cilostazol in improving endothelial cell function and decreasing the interaction between activated platelets and endothelial cells.[17] Although in the present study we did not detect significant ADP-induced aggregation inhibition, a stronger inhibitory effect on the platelet $P2Y_{12}$ ADP receptor-mediated pathway using VASP phosphorylation analysis was observed in the triple therapy group. This discrepancy may be explained by the fact that VASP phosphorylation analysis more precisely reflects platelet $P2Y_{12}$ ADP receptor-mediated pathway inhibition, and has a higher sensitivity and specificity for monitoring clopidogrel treatment than ADP-induced platelet aggregation.[22,23]

Study limitations: The present study was an observational study that was retrospective in nature, and had a relatively small number of patients. It is, however, the first study to show the effect of triple antiplatelet therapy on the platelet $P2Y_{12}$ ADP receptor in Japanese patients undergoing stent implantation. Further large-scale multicenter studies are needed to confirm our results. In addition, the follow-up period of this study (< 2 months) was too short to evaluate the long-term efficacy and safety of triple antiplatelet therapy. Longer follow-up should be carried out to evaluate the preventive effect of triple antiplatelet therapy on late or very late thrombosis.

Conclusions: Our findings indicate that the combination of clopidogrel and cilostazol plus aspirin is more effective in inhibiting the $P2Y_{12}$ ADP receptor-mediated pathway than clopidogrel plus aspirin alone. These results support the combined use of cilostazol and clopidogrel to improve clopidogrel responsiveness in Japanese patients.

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