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Dual Therapy with Aspirin and Cilostazol May Improve Platelet Aggregation in Noncardioembolic Stroke Patients: A Pilot Study

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Abstract

Objective Some previous studies have found clinical benefit of dual antiplatelet therapy with aspirin and cilostazol for prevention of secondary stroke, but the physiological mechanism involved remains unknown. We aimed to clarify the effects of aspirin/cilostazol therapy on the platelet and endothelial functions of patients with acute noncardioembolic ischemic stroke, in comparison to patients who were treated with aspirin alone.

Methods The present randomized prospective pilot study enrolled 24 patients within a week after the onset of noncardioembolic ischemic stroke. The patients were randomly allocated to receive aspirin (100 mg/day) (A group; 11 patients) or cilostazol (200 mg/day) plus aspirin (100 mg/day) (CA group; 13 patients). We measured platelet aggregation, platelet activation, and the thrombomodulin (TM), highly sensitive C-reactive protein (hs-CRP), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and von Willebrand (vWF) antigen levels and vWF activity over a 4-week period after enrollment.

Results There was no significant difference in the platelet functions of the A and CA groups. However, the platelet aggregation induced by adenosine diphosphate (ADP) was decreased at 2 and 4 weeks ($p<0.05$) after treatment in comparison to the pre-treatment values in the CA group, but not in the A group. Platelet activation, and the hs-CRP, TM, ICAM-1, VCAM-1 and vWF values did not significantly decrease after treatment in either group.

Conclusion Although there were no significant differences in platelet aggregation, platelet activation or the endothelial biomarker levels of the A and CA groups, dual therapy with aspirin and cilostazol inhibited platelet aggregation in comparison to the pre-treatment values, similarly to patients who received aspirin alone. This may suggest the clinical usefulness of dual therapy with aspirin and cilostazol in the treatment of patients with noncardioembolic ischemic stroke.

Key words: cilostazol, aspirin, platelet aggregation

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Introduction

Patients with ischemic stroke are at high risk of recurrent stroke. Antiplatelet therapy reduces the risk of vascular events after the index stroke or transient ischemic attack (TIA) (1). Thus, current guidelines recommend antiplatelet therapy for the prevention of a recurrence of stroke and other vascular events in these patients (2-5). Although aspirin is widely recommended for the treatment of acute ischemic stroke (6), it fails to inhibit platelet aggregation in 5-55% of individuals. Clinical aspirin resistance is known to be an important cause of treatment failure (7-10).

Cilostazol, a selective antagonist of phosphodiesterase 3, also inhibits platelet aggregation (11). The plasma concentration of cilostazol after oral administration increases within

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1 hour, reaching a peak at approximately 3.6 hours; the maximal effect on platelet aggregation is seen at approximately 6 hours after administration (12). The CSPS II (cilostazol for prevention of secondary stroke) trial, an aspirin-controlled, double-blind, randomized Japanese trial, showed that cilostazol significantly lowered the risk of stroke in comparison to aspirin and that it was associated with significantly fewer hemorrhagic events (13). The trial also showed that cilostazol is superior to aspirin for the prevention of secondary vascular events, including stroke, transient ischemic attack, angina pectoris, myocardial infarction, heart failure, and hemorrhage requiring hospital admission (13). Otsuki et al. (14) showed that cilostazol significantly repressed the levels of cell-surface vascular cell adhesion molecule-1 (VCAM-1); this protein mediates mononuclear leukocyte-selective adhesion to vascular endothelium, which is enhanced by tumor necrosis factor (TNF)-α in endothelial cells. Thus, cilostazol may be a good option for the acute treatment of ischemic stroke. Dual antiplatelet therapy with aspirin and clopidogrel, but not aspirin and cilostazol, has been a standard treatment for acute noncardioembolic ischemic stroke and TIA. Nakamura et al. designed a randomized study to compare the effects of aspirin alone and aspirin plus cilostazol in stroke patients with noncardioembolic ischemic stroke (15). Their study demonstrated that the combined treatment resulted in a significant decrease in early neurological deterioration in comparison to aspirin alone in the first 14 days after the start of medication. Cilostazol not only has inhibitory effects on platelet aggregation, but also has vasodilating, endothelial-protecting and anti-inflammatory effects (15), which might contribute to the favorable results. However, the effects of cilostazol on platelet aggregation and the endothelium remain to be fully clarified. In the present study, we aimed to examine the effects of dual antiplatelet therapy with aspirin and cilostazol on the biomarkers associated with platelet aggregation or endothelial protection in patients with acute noncardioembolic ischemic stroke, in comparison to patients who were treated with aspirin alone.

Materials and Methods

Enrollment

This was an open-labeled, randomized prospective pilot study that enrolled patients with noncardioembolic ischemic stroke. We recruited patients who had suffered MRI-confirmed noncardioembolic ischemic stroke within the previous 1 week and who had been hospitalized in Tokai University Hospital. The subtype of ischemic stroke was diagnosed by experienced neurologists according to the NINDS-III (National Institute of Neurological Disorders and Stroke, 1990) criteria (16). The patients were randomly allocated to receive aspirin (100 mg/day) (A group) or cilostazol (200 mg/day) plus aspirin (100 mg/day) (CA group). The patients were randomized using the RAND function of Microsoft Excel 2010 (USA). Patients were excluded if they had contraindications to antiplatelet agents. This study was approved by the ethics committee of Tokai University, and written informed consent was obtained from either all of the patients or from their close relatives.

Clinical evaluation

All of the enrolled patients were evaluated at admission for risk factors for atherosclerosis, including hypertension, dyslipidemia, diabetes mellitus and smoking. Hypertension was defined as a blood pressure of ≥140/90 mmHg (17). A diagnosis of dyslipidemia required all of the following conditions to be met: a low-density lipoprotein cholesterol level of ≥120 mg/dL, a high-density lipoprotein cholesterol level of <40 mg/dL, and a triglyceride level of ≥150 mg (18). A diagnosis of diabetes mellitus required any one of the following conditions: a morning fasting blood sugar level of ≥126 mg/dL and a HbA1c level of ≥6.5% (19).

Blood sampling

In all cases, blood sampling was performed at around 10 a.m. under non-fasting conditions. Blood was obtained from the antecubital vein with the aid of a light tourniquet. The first 2 mL of blood was discarded, 4.5 mL of blood was then slowly collected into a plastic syringe fitted with a 21-gauge needle (Terumo, Tokyo, Japan), containing 0.5 mL of 3.14% sodium citrate (20).

All enrolled patients received a platelet function assay before, and at 2 and 4 weeks after the administration of antiplatelet agents. The platelet function was examined immediately after blood sampling.

The measurement of platelet aggregates

Platelet aggregation was detected by means of a particle counting method using a light scattering technique (21). Briefly, an optical device (PA-200, Kowa, Nagoya, Japan) designed to focus on a limited area of platelet-rich plasma was used to measure the intensity of light scattered by particles passing through the area, in order to minimize multiple light scattering. The use of polystyrene spheres of different diameters confirmed that the light scattering intensity increased in proportion to the particle size in a suspension. Platelet activation was induced by several agonists, i.e., collagen, arachidonic acid (AA) and adenosine diphosphate (ADP), which resulted in higher-intensity light scattering, which correlated closely with the number and size of aggregates observed by microscopy. These findings confirmed that the light scattering intensity measured with this device provided information on the number and size of aggregates in a suspension.

The concentrations of collagen, AA, and ADP were set at 0.5 μg/mL, 1,000 μM and 1 μM, respectively, and the platelet aggregation effects were evaluated according to algorithms modified from the PA-200 standard protocol (20). The extent of the effect on platelet aggregation was classified into 3 classes, +1 to -1, according to the proportions of

aggregate sizes in the induced platelet aggregates. Class +1 was defined by large platelet aggregates that were predominantly located in the target area (rather than small platelet aggregates). Class -1 was defined by small platelet aggregates predominating over large platelet aggregates. Class 0 was defined by the presence of similar amounts of large and small platelet aggregates in the target area. In some cases, it was difficult to make these visual classifications; thus, the platelet aggregation effect was further evaluated using a combination of 0.25 μg/mL of collagen and 0.5 μM ADP. In this case, increased platelet aggregation was defined as subclass +1, which indicated an increased platelet function in response to collagen and ADP, while unchanged or decreased platelet aggregation was defined as subclass 0 or -1, indicating a normal platelet function in response to collagen and ADP. Similarly, the cases that were determined to be class 0 or -1 in the initial evaluation were reevaluated with the combination of 1 μg/mL of collagen and 2 μM ADP, and increased or unchanged platelet aggregation was defined as subclass +1 or 0, indicating a normal platelet function in response to collagen and ADP. Decreased platelet aggregation was defined as subclass -1, which indicated the suppression of the platelet function in response to collagen and ADP.

To evaluate the platelet aggregation by AA, 500 μM AA was initially added, and the effect was divided into two groups according to whether large or small platelet aggregates were formed. To confirm the results, cases in the small platelet aggregate group were reevaluated using 1,000 μM AA. In this second evaluation, the effect was again divided into subgroups based on the size of the platelet aggregates that formed. Finally, the large and small platelet aggregate formation subgroups were defined as showing an increased or normal platelet function in response to AA, respectively. In the cases that were categorized into the small platelet aggregate group in the initial evaluation, AA was considered to suppress the platelet function.

The measurement of platelet activation using flow cytometry

Aliquots of 2.5 μL of blood were placed in microcentrifuge tubes containing 10 μL of fluorescein isothiocyanate (FITC)-conjugated PAC-1 (monoclonal antibody to fibrinogen receptors, Becton Dickinson Biosciences, San Jose, CA, USA), 10 μL of phycoerythrin (PE)-conjugated MoAb-CD62P (monoclonal antibody to P-selectin, Becton Dickinson Biosciences), and 10 μL of peridinin chlorophyll protein (perCP)-conjugated MoAb-CD61 (monoclonal antibody to GP IIIa, Becton Dickinson Biosciences) to identify platelets (22). To assess the extent of nonspecific protein binding, we used one tube with 10 μL of 5 mg/mL arginine-glycine-aspartic acid-serine (RGDS; Sigma Aldrich, St Louis, MO, USA) solution in the staining mixture. The reaction mixture was gently stirred without vortexing, followed by incubation for 15 minutes at room temperature in the dark. Subsequently, the platelets were fixed in 500 μL of cold 1% paraformaldehyde. The samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson Biosciences), using standard 488 nm excitation. Activation-dependent antibody binding was expressed as the percentage of platelets that were positive for the antibody. Antibody-positive cells were defined as platelets with a fluorescence intensity of >99.0% in comparison to platelets that were treated with isotype IgG of fibrinogen receptor-blocking tetrapeptide, RGDS, as a negative control. The total platelet populations were displayed, including any light scatter-gated subpopulations, as two-color dot plots (22).

The measurement of other biomarkers

The following factors were measured by commercial test kits. 1) Plasma von Willebrand factor (vWF) activity was assayed by platelet agglutination. 2) The plasma vWF antigen level was assayed by latex agglutination. 3) The levels of plasma thrombomodulin (TM), VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) were assayed by an ELISA. 4) The high-sensitivity CRP (hsCRP) level was assayed by latex agglutination turbidimetry.

Statistical analysis

All of the biomarkers were measured consecutively before treatment, and at 2 and 4 weeks after starting the administration of antiplatelet agents. The Mann-Whitney *U* test and Wilcoxon-signed rank-sum test were used for comparisons among groups. Statistical analyses were performed using SPSS 23.0 (SPSS, Chicago, IL, USA). The data are presented as the mean ± SD. The significance level was set at $p<0.05$.

Endpoint

The primary endpoints of this study were the differences in platelet aggregation, platelet activation, and biomarkers at 2 weeks and 4 weeks after the start of treatment between the A and CA groups, as well as adverse effects, such as the recurrence of stroke, and intracranial and gastrointestinal bleeding. The secondary endpoint was the extent of the inhibition of platelet aggregation at 2 weeks and 4 weeks after the start of treatment in each group, in comparison to the pre-treatment value.

Results

Baseline characteristics

During the study period, 32 patients (aspirin alone, n=14; aspirin and cilostazol, n=18) were enrolled. Twenty-four of these patients (aspirin alone, n=11; aspirin and cilostazol, n=13) met the inclusion criteria, and the platelet aggregation test was repeated 2 and 4 weeks after the initial test. The clinical characteristics of these patients are summarized in Table 1. The A group included 6 patients with atherothrombosis, 5 with lacunar infarction and 1 with transient ischemic attack (TIA). The CA group included 2 patients with atherothrombosis, 8 with lacunar infarction, 1 with TIA

Table 1. Clinical Characteristics.

	A group (n=11)	CA group (n=13)
Age at onset (mean ± SD)	63.6±9.1	60.5±10.0
Male/Female (n)	8/3	9/4
Subtype of stroke		
Large artery atherosclerosis (n)	5	2
Small vessel occlusion (n)	5	8
TIA (n)	1	1
Stroke of undetermined etiology (n)	0	2
Concomitant risk factors		
Smoking (n)	7	7
Alcohol intake (n)	6	5
Hypertension (n)	9	10
Dyslipidemia (n)	10	11
Diabetes mellitus (n)	6	6

and 2 with unclassified stroke. No significant differences were observed between the 2 groups with regard to age, gender, or the proportions of co-existing risk factors for ischemic stroke (Table 1).

The effects on platelet aggregation

There was no significant difference in the platelet aggregation of the A and CA groups at pretreatment, or at 2 or 4 weeks after the start of treatment.

Figure shows the time course of the changes in platelet aggregation. The platelet aggregation induced by collagen in the A group was significantly decreased at 2 and 4 weeks after treatment, in comparison to the pre-treatment values ($p<0.05$). The platelet aggregation induced by collagen in CA group was also significantly decreased in a similar manner ($p<0.01$). The platelet aggregation induced by AA in the A group was significantly decreased at 2 and 4 weeks after treatment in comparison to the pre-treatment value ($p<0.01$), while that in the CA group was also significantly decreased at 2 and 4 weeks ($p<0.01$). The platelet aggregation induced by ADP in the A group was not decreased at either 2 or 4 weeks in comparison to the pre-treatment value; however, it was significantly decreased in the CA group ($p<0.05$).

The effects of dual therapy on platelet activation and other biomarkers

We found that dual therapy had no significant effects on platelet activation as measured by flow cytometry, hs-CRP, TM, ICAM-1, VCAM-1 or vWF in either of the groups (Table 2).

Adverse effects during the study period

No adverse effects, including recurrent ischemic or hemorrhagic stroke, or gastrointestinal hemorrhage, occurred in either group during the 4-week study period.

Discussion

This is the first prospective pilot study to examine the effects of dual antiplatelet therapy with aspirin and cilostazol on platelet aggregation in patients with acute noncardioembolic ischemic stroke, in comparison to aspirin alone. There was no significant difference in the platelet function or the endothelial biomarker levels of the A and CA groups. Although we found that dual therapy with aspirin and cilostazol inhibited the induction of platelet aggregation by ADP at 4 weeks in comparison to the pre-treatment values, it is difficult to conclude the dual therapy is more effective than mono-therapy with aspirin because there was variation in the levels of ADP-induced platelet aggregation in the CA group.

Cilostazol is an antiplatelet drug that inhibits phosphodiesterase 3, thereby increasing the Cyclic adenosine monophosphate (cAMP) concentration and consequently inhibiting platelet aggregation (23). It inhibits arachidonic acid-induced platelet aggregation more effectively than aspirin (24). Cilostazol inhibits both the primary and secondary platelet aggregation induced by collagen, ADP, arachidonic acid, and epinephrine (24). In a previous study comparing the effects of cilostazol and ticlopidine on porcine platelet aggregation, cilostazol significantly inhibited ADP- and collagen-induced platelet aggregation, whereas ticlopidine showed no effect (25). These previous studies are consistent with our finding that the ADP-induced platelet aggregation, in addition to collagen- and AA-induced platelet aggregation, might have been inhibited in the CA group.

ADP-induced platelet aggregation plays a predominant role in the occurrence of recurrent vascular events after acute ischemic stroke (26). Thus, it is possible that persistent elevated ADP-induced platelet aggregation after acute ischemic stroke might remain unregulated by aspirin alone (27). We therefore designed a study to investigate the relationship between extent of ADP-induced platelet aggregation and the occurrence of vascular events or death within a 90-day follow-up period in acute ischemic stroke patients who were treated with aspirin. An increase in ADP-induced platelet aggregation in patients receiving aspirin was associated with a poor outcome after acute ischemic stroke. In such cases, it is recommended that aspirin be replaced with another class of anti-platelet agent (or that such an agent should be administered in addition to aspirin) (28, 29). However, those trials focused on the effectiveness of dual therapy with aspirin plus clopidogrel. On the other hand, cilostazol is also known to be effective for limiting the platelet function and reducing the likelihood of recurrent vascular events. Our present results indicate that the dual therapy with aspirin plus cilostazol could inhibit platelet aggregations the same as mono-therapy with aspirin. Thus, this dual therapy might be an option for the treatment of patients with recurrent vascular events after acute ischemic stroke.

Cilostazol is a phosphodiesterase inhibitor that has anti-inflammatory potential in addition to vasodilator and anti-

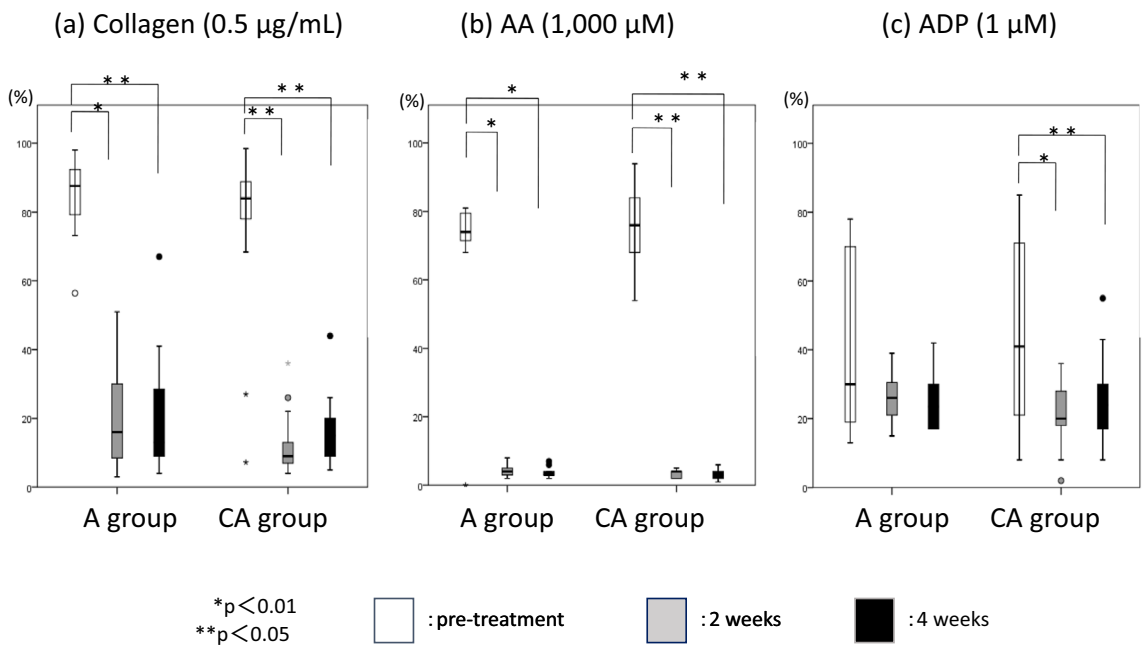

Figure. **The time course of the changes in platelet aggregation in each of the groups. The time course of the changes in platelet aggregation induced by 0.5 μg/mL collagen (a), 1,000 μM AA (b) and 1 μM ADP (c) pre-treatment, and at 2 weeks and 4 weeks after the start of medication. The values are expressed as the median and 25th and 75th percentiles. Asterisks indicate a statistically significant difference between the pre- and post-treatment values; *: p<0.01, **: p<0.05**

Table 2. **Biomarkers of Platelet Function and Vascular Endothelium.**

	A Group			CA Group		
	pre- treatment	2 weeks	4 weeks	pre- treatment	2 weeks	4 weeks
vWF activity (%)	136.8±44.3	136.8±51.3	136.2±55.0	141.7±52.2	141.7±46.5	128.9±43.6
vWF antigen (%)	140.7±43.5	147.3±58.9	132.1±43.0	140.1±53.1	136.2±45.7	120.2±37.5
Thrombomodulin	2.7±0.9	2.9±0.8	2.9±0.9	3.6±2.3	3.6±2.2	4.0±2.8
VCAM-1 (ng/mL)	694.8±215	660.7±185.5	747.7±230.3	798.5±281.9	859.7±356.9	824±307.2
ICAM-1 (ng/mL)	245.4±117.9	238.4±56.4	244.1±82.1	199.4±75.9	188.9±77.9	190.4±69.4
hs-CRP (mg/dL)	3.7±4.0	2.1±2.6	1.8±2.3	7.8±2.1	2.1±2.9	0.5±0.3
ADP(1μM)	42.8±27.0	26±7.2	26.4±9.0	46±26.9	20.5±9.2	25.9±13.0
Collagen (0.5 μg/mL)	84.6±12.2	20.6±15.6	21.4±19.6	75.5±27.5	12.6±9.6	16.5±10.5
AA (1000 μM)	68.6±23.2	4.1±1.7	3.7±1.6	75.2±11.2	3.5±1.2	3.3±1.4
PAC1	32.3±13.0	31.3±18.6	30.1±16.1	26.1±16.9	35.0±21.8	24.9±13.6
CD62P	3.4±2.0	4.2±3.2	4.4±4.0	3.9±4.8	7.4±9.8	4.0±4.1

platelet effects (30-33). It also inhibits VCAM-1 via the suppression of nuclear transcription factor kappa B (NF-kB) in cultures of endothelial cells taken from the human umbilical cord (34, 35). However, VCAM-1 was not significantly decreased in either of the groups in our study. Aspirin might inhibit adhesion molecules such as VCAM-1 and ICAM-1, as effectively as aspirin plus cilostazol. Unfortunately, we did not observe a direct anti-inflammatory effect in this study, which is in contrast to previous studies. It is possible that differences in the background characteristics of patients, such as the extent of brain damage, may account for this, since our patients were less severely affected.

A major limitation of our study is the small sample size. However, the sample size was considered adequate for a pilot study. Additionally, the platelet functional assays used in the present study may not be generally employed; however, the use of a battery of assays and the results regarding the effectiveness of treatment for the secondary prevention of stroke suggest that the present platelet functional assay methodology is reliable. Nevertheless, further large-scale and multicenter studies will be required to confirm the usefulness of dual anti-platelet therapy with aspirin and cilostazol for the secondary prevention of stroke.

Conclusion

This is the first study to examine the effect of dual therapy with aspirin and cilostazol in patients with acute-phase

noncardioembolic ischemic stroke. Although we found no differences between patients who received dual therapy with aspirin and cilostazol and those who received mono-therapy with aspirin with regard to platelet aggregation, platelet activation, or biomarkers levels, our results suggest that it might be useful - at least in some patients. The incidence of complications did not increase in the patients who received dual therapy.

The authors state that they have no Conflict of Interest (COI).

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