Magnitude and time course of platelet inhibition with extended release dipyridamole with or without aspirin in healthy Japanese volunteers

The AGgrenox versus Aspirin Therapy Evaluation (AGATE-Japan)

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Summary

Randomized trials showed greater stroke prevention with extended release dipyridamole in combination with low dose aspirin than with either aspirin or dipyridamole alone. However, most studies with this formulation (Aggrenox®) were carried out in Europe and North America. Considering potential inter-racial differences in drug response, we conducted a small randomized study in healthy Japanese volunteers to compare antiplatelet regimens with regard to the changes in the platelet biomarkers. Thirty healthy volunteers (18–40 years old, 15 male and 15 female) of Japanese descent were randomized to Aggrenox (n=17) or aspirin 81 mg (n=13 volunteers) for 30 days. Platelet function was assessed at baseline, and on days 15, and 30 by conventional aggregometry, whole blood flow cytometry, and cartridge-based analyzer. Both Aggrenox and aspirin provided sustained platelet inhibition at Day 15 and Day 30. Therapy with Aggrenox, however, was associated with more prominent and significant inhibition of collagen-induced aggregation (p=0.08, Day 15), as well as prolongation of the closure time (p=0.001, Day 30); diminished expression of platelet endothelial cell adhesion molecule-1 (PECAM-1) (p=0.02, Day 30), glycoprotein IIb (GPIIb) antigen (p=0.001 and 0.024 for Day 15 and Day 30), and GPIIb/IIIa activity by PAC-1 antibody (p = 0.014 and 0.03), CD62 (P-selectin) (p = 0.03 for Day 15 and Day 30), as well as inhibition of protease activated receptors (PAR-1) associated with intact WEDE-15 (p = 0.002 and 0.003) and SPAN-12 (p = 0.002 and 0.04) thrombin receptors when compared with aspirin. The magnitude and durability of platelet response after Aggrenox in healthy Japanese is similar to those effects observed in Caucasians and African-Americans. A larger study to assess drug efficacy and safety in the Japanese post-stroke patients is warranted.

Keywords

Randomized trials, aspirin, Aggrenox®, dipyridamole, platelets, Japanese

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Introduction

The benefit of a small dose of aspirin (25 mg) and extended release dipyridamole (200 mg) combination (ERD/A, or Aggrenox®) has been proven by many studies ranging from the in-vitro and ex-vivo experiments performed in healthy volunteers to multicenter international randomized clinical trials in patients after ischemic stroke (1–4). The results of European Stroke Prevention Study 2 (ESPS-2) clearly demonstrated that ERD/A was twice as effective as either aspirin or dipyridamole alone in secondary stroke prevention (5), and those results were not outclassed for more than ten years, despite desperate attempts to apply more potent antiplatelet strategies (6). Moreover, the combination of small dose aspirin and extended release dipyridamole was found to be safest among the known anti-thrombotic regimens, and associated with the lowest bleeding risks (7, 8). Presently, American and European Stroke Associations recommend Aggrenox as the preferred drug for secondary stroke prevention (9, 10). However, due to the several reasons, all current antiplatelet recommendations for secondary stroke prevention were established based on the results of clinical trials performed predominantly in Europe and North America. Therefore, it is a legitimate concern that inter-ethnic differences between the Asian population in general, including Japanese patients in particular, and Caucasians exist, and may potentially account for the different safety and efficacy profile of ERD/A. Moreover, there

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is a lack of data pertaining to the antiplatelet efficacy of ERD/A in Japanese populations, which is urgently needed in order to justify and facilitate the introduction of ERD/A to this emergent patient cohort.

We designed and conducted this randomized small study to determine the effect of ERD/A compared to low dose aspirin alone on the measures of platelet activity in healthy Japanese volunteers. Platelet biomarkers were serially assessed with conventional optical aggregometry, rapid cartridge-based analyzer, and whole blood flow cytometry.

Methods

Healthy volunteers

The current randomized study was approved by the Quorum Institutional Review Board, Seattle, WA, USA (QR Protocol #23350/1). Written informed consent was obtained from all participants, who were aware of the strict compliance rules, and compensated for office visits and blood draws. Healthy Japanese passport holders aged 18–40 years were eligible if they had not received any drugs within the last 14 days. Participants were excluded from the study if they had a platelet count <100,000; Ht <30; a history of bleeding disorders; serum creatinine ³3 µg/dl, liver impairment defined as ALT/AST > 3 times the normal upper limit; glomerular filtration rate <50 ml/min; and a known allergy to or intolerance of the study drugs.

Using a table of random numbers by an independent statistical center, 30 consenting and eligible participants were assigned at random to Aggrenox (17 volunteers) twice a day or aspirin (13 participants) 81 mg once daily. The follow-up visits were scheduled at baseline, two weeks (Day 15), and four weeks (Day 30) after randomization.

Samples

Blood samples were obtained using a 19-gauge needle by direct venipuncture and drawn into four 4.5 ml vacutainer tubes containing 3.8% trisodium citrate at room temperature. The vacutainer tube was filled to capacity and gently inverted three to five times to ensure complete mixing of the anticoagulant. The first 4–5 ml of blood were discharged. All samples were labeled with coded number and analyzed by blinded technicians. Platelet studies were performed at baseline as well as at two and four weeks after treatment assignment.

Platelet aggregation

The blood-citrate mixture was centrifuged at 1200 g for 5 min. The resulting platelet-rich plasma was kept at room temperature for use within 1 hour. The platelet count was determined in the platelet-rich plasma sample and adjusted to 3.5 x 10 8/ml with homologous platelet-poor plasma. Platelets were stimulated with 5 mg/ml collagen, 0.75 M arachidonic acid, and 5 µM ADP (Chronolog, Havertown, PA, USA) and aggregation was assessed as previously described using a Chronolog Lumi-Aggregometer (model 560-Ca) with the AggroLink software package. Aggregation was expressed as the maximum percent change in light transmittance from baseline, using platelet-poor plasma as a reference. Curves were analyzed according to international standards (11).

Platelet function analyzer (PFA-100™)

Using the PFA-100 instrument (Dade Behring Inc., Miami, FL, USA), the blood-citrate mixture is aspirated under a constant negative pressure and contacts an epinephrine and collagen coated membrane. The blood then passes through an aperture that induces high shear stress and simulates primary hemostasis after injury to a small blood vessel under flow conditions. The time to aperture occlusion (the closure time) is recorded in seconds and is inversely related to the degree of shear-induced platelet activation (12).

Whole blood flow cytometry

The surface expression of platelet receptors was determined by flow cytometry using the following monoclonal antibodies: CD31 (platelet/endothelial cell adhesion molecule -PECAM-1), CD 41 antigen (GP IIb/IIIa), PAC-1 antibody (GP IIb/IIIa activity), CD 51/CD 61 (vitronectin receptor), CD 62p (P-selectin), CD151+14 (platelet-leukocyte interaction) (PharMingen, San Diego, CA, USA); and platelet thrombin PAR-1 receptors (WEDE-15 and SPAN-12) (Beckman Coulter, Brea, CA, USA). The blood-citrate mixture (50 µl) was diluted with 450 µl Tris buffered saline (TBS) (10 mM Tris, 0.15 M sodium chloride) and mixed by twice inverting an Eppendorf tube gently. The appropriate primary antibody was then added (5 µl) and incubated at 37°C for 30 min, and then a secondary antibody was applied if needed. After incubation, 400 µl of 2% buffered paraformaldehyde was added for fixation. The samples were analyzed on the FACScan flow cytometer (Becton Dickinson, San Diego, CA, USA) calibrated to measure fluorescent light scatter as previously described (13). All parameters were collected using four-decade logarithmic amplification. The data were collected in list mode files and then analyzed. P-selectin was expressed as per cent positive cells as previously described (14). Other antigens were expressed as *log* mean fluorescence intensity.

Statistics

The significance of differences between treatment arms was calculated by Fisher's exact tests for discrete variables, and Wilcoxon rank-sum test for continuous variables. The significance of differences between individual flow cytometric histograms was calculated using the Smirnov-Kolgomorov test incorporated in the CELLQuest' (San Diego, CA, USA) software. Statistical analyses were performed using SPSS/E11.5 (SPSS, Inc., Chicago, IL, USA). To control for any baseline differences analysis of variance was used. All *P* values are two sided.

Results

Participants

Thirty volunteers were screened in, randomized and completed the 30-day trial. There were no serious adverse events, neither were any missing samples reported. Three participants from the aspirin group and three from ERD/A arm reported minor bleedings related to teeth brushing, or shaving. Five participants from the Aggrenox group and two from the aspirin group experienced mild headaches during the first few days of therapy. The nature of all adverse events was explained to participants, and all of them continued to take the assigned medication. Table 1 shows base-

Table 1: Baseline characteristics.

Parameter	ERD/A (n=17)	Aspirin (n=13)
Demographics		
Age (mean ± SD)	27.6±7.4	24.0±2.9
Male	10 (58%)	5 (38%)
Known risk factors and history		
Obesity	1 (6%)	1 (7.6%)
Sedentary life-style	5 (29)	1 (7.6%)
Smoking	5 (29%)	3 (23%)
Family history of vascular disease	7 (41%)	5 (38%)

All – non significant (*P*>0.05) between groups by Fisher's exact test.

line pretreatment distribution of demographics, risk factors for both arms. Although there were no significant differences between ERD/A and aspirin groups, the patients that were treated with Aggrenox were slightly older, with a prevalence of men. The majority of enrolled volunteers was recruited from local colleges and were young and healthy, therefore risk factors for vascular disease were rarely reported. The most commonly prevalent risk factor was a family history of vascular disease. Participants from the ERD/A group more frequently mentioned sedentary life-style, but this difference was non-significant.

Platelet data

The data on platelet characteristics in the two treatment groups are presented in Table 2. At baseline, platelet activity was not remarkably different from the healthy control group previously observed in our center (data not shown). Therapy with aspirin and ERD/A resulted in substantial time-dependent inhibition of platelet activity biomarkers as reflected by conventional aggregometry, rapid cartridge-based platelet analyzer, and whole blood flow cytometry.

Treatment with 81 mg daily aspirin in healthy Japanese volunteers resulted in inhibition of collagen-, arachidonic acid-, and adenosine-diphosphate- induced platelet aggregation. Rapid platelet analyzer revealed consistently prolonged closure time by the PFA-100 instrument. We observed the diminished expression of all studied receptors even two weeks after randomization. The intensity of platelet-leukocyte interaction (CD151+14) was significantly lower in the aspirin group after two weeks that in the ERD/A study arm.

Treatment with ERD/A reduced platelet aggregation and expression of surface receptors in a similar manner to aspirin. After two weeks of therapy with ERD/A, we observed a more prominent and statistically significant inhibition of platelet aggregation induced by collagen, even after 30 days this difference was smoothed. The expression of platelet receptors CD41a, CD62p, PAC-1, and both of PAR-1 thrombin receptors, were significantly lower in ERD/A group after two and four weeks of drug administration, while additional inhibitory effect of ERD/A on CD31 was delayed, and observed after 30 days of treatment.

Table 2: Platelet data.

Variable	Baseline	2 weeks	4 weeks
Aggregation: ADP5μM (%)			
Aspirin	74±8.0	56.0±4.3*	49.7±3.6*
ERD/A	75.6±9.1	54.4±5.2*	54.2±4.2*
p-value	NS	NS	NS
Collagen 5 mg/ml (%)			
Aspirin	77.5±10.3	57.0±4.8*	53.1±6.1*
ERD/A	70.8±9.6	51.1±6.2*	50.9±5.8*
p-value	NS	0.008	NS
Arachidonic acid (%)			
Aspirin	83.4±5.6	13.7±6.8*	12.2±6.5*
ERD/A	83.8±6.4	14.6±10.3*	14.6±6.9*
p-value	NS	NS	NS
PFA-100 (EPI/Coll, s)			
Aspirin	174.6±12.2	263.5±19.6*	254.5±23.4*
ERD/A	166.1±7.9	270.4±29.5*	278.3±24.5*
p-value	NS	NS	0.0011
CD31, log MFI			
Aspirin	81.9±11.0	59.2±6.8*	59.8±7.9*
ERD/A	79.1±11.1	57.1±8.8*	52.3±9.2*
p-value	NS	NS	0.024
CD41 log MFI			
Aspirin	456.1±72.0	398.1±67.8*	377.1±73.9*
ERD/A	468.5±72.7	334.8±60.1*	313.9±54.9*
p-value	NS	0.011	0.019
PAC-1 log MFI			
Aspirin	11.6±1.7	8.7±1.5*	8.5±1.7*
ERD/A	10.9±1.8	7.5±1.2*	7.3±1.0*
p-value	NS	0.014	0.032
CD51/61 log MFI			
Aspirin	8.8±1.3	6.5±0.9*	6.9±0.8*
ERD/A	8.3±1.4	6.9±0.9*	6.7±0.9*
p-value	NS	NS	NS
CD62p (% +)			
Aspirin	11.8±1.6	9.0±1.2*	7.2±1.0*
ERD/A	11.1±1.3	8.1±1.1*	6.3±1.1*
p-value	NS	0.032	0.032
CD151+14 log MFI			
Aspirin	117.8±23.9	84.6±21.9*	115.3±20.4
ERD/A	117.2±22.0	118.9±20.9	113.0±22.3
p-value	NS	0.001	NS
PAR-1 (WEDE-15) log MFI			
Aspirin	48.1±3.5	41.4±3.5*	36.1±5.3*
ERD/A	49.3±5.2	34.6±6.6*	30.7±3.7*
p-value	NS	0.002	0.003
PAR-1 (SPAN-12) log MFI			
Aspirin	30.0±4.1	22.3±2.7*	23.3±3.1*
ERD/A	30.4±3.6	19.9±3.2*	19.7±2.9*
p-value	NS	0.002	0.037

*-*p-value <0.05* vs. own baseline; NS, not significant; MFI, mean fluorescence intensity; %+ , percent of positive cells.

Discussion

This study provides the first randomized evidence of the magnitude, durability, and duration of the changes of platelet biomarkers activity after 30 days of therapy with aspirin and ERD/A in a healthy cohort of Japanese patients. The antiplatelet properties of ERD/A were stronger, and broader than those of aspirin. Applying a wide panel of techniques minimizes the error by measuring different parameters indicative of various platelet characteristics. In the present trial, the antiplatelet activity of both agents was documented by conventional optical aggregometry induced by several agonists, and by the PFA-100 platelet analyzer assessing shear-induced activation. In addition, we utilized whole blood flow cytometry techniques measuring expression of multiple receptors located on the platelet surface. Considering the marked heterogeneity of platelet activity among and within groups, we used multiple tests to comprehensively assess platelet function to ensure adequate evaluation of platelets. In subgroup analyses the apparent superiority of ERD/A over aspirin has been observed from day 15 to day 30 after treatment assignment. The expression of almost all platelet receptors (CD31, CD41, PAC-1, CD62p, and both thrombin PAR-1 receptors), collagen-induced aggregation have been significantly reduced to 15 and/or 30 days after therapy with ERD/A compared to aspirin. In general, the index findings are similar to the results of another previously published AGATE study in which we demonstrated that two weeks of therapy may be necessary to achieve sustained mild platelet inhibition, but that the most benefit is observed later at Day 30 (4). The similarity between AGATE and AGATE-Japan trials is presented in Table 3. Contrary to patients after ischemic stroke enrolled in AGATE study, healthy Japanese volunteers demonstrated less profound inhibition of thrombin PAR-1 receptors and absence of effect on platelet-leukocyte interaction. The heterogeneity between study groups could be responsible for these differences.

The major pathway of antiplatelet efficacy of aspirin is well described, while the data on the effect of dipyridamole on platelet function are numerous, but less clear. The differences in the results could be attributable to vasodilatation (15), antithrombotic effects (16), antioxidant effects (17, 18), or prostacyclin (19, 20) and nitric oxide (21, 22) stimulating properties of dipyridamole, rather than to any direct antiplatelet efficacy. This is especially evident when platelet function is assessed exclusively by conventional plasma aggregometry. Dipyridamole may yield additional benefit by suppressing thrombus formation (1), and inhibiting smooth muscle cell proliferation (23, 24) as demonstrated from in-vitro and animal models. The findings of AGATE-Japan study suggest in a perfect unison with previously published AGATE study that adjunctive benefits of dipyridamole and low-dose aspirin constituting Aggrenox (ERD/A) may extend beyond simply diminishing platelet aggregation. Moreover, this mechanism is universal for all study (treatment) groups, including healthy volunteers and patients after stroke regardless of their ethnic identity.

Despite the achievement of an impressive 60% reduction in stroke-related deaths over the last three decades in Japan (25), stroke remains one of the major causes of disability and death in this country with an annual budget for cerebrovascular disease of about JPY 1.7 trillion (13billion) (26). The estimated annual incidence of stroke in Japan is 4,755 per 100,000 persons (27), which far exceeds the incidence of strokes in the USA (the age-adjusted incidence of first ischemic stroke per 100 000 was 88 in whites, 191 in blacks, and 149 in Hispanics) (28). Surprisingly, the age-adjusted annual incidence rate (per 1000) for total stroke in Japanese-American men has declined markedly from 5.1 to 2.4; for thromboembolic stroke, from 3.5 to 1.9; and almost reached the average rate (28). The decline in stroke mortality in the Honolulu Heart Program (HHP) target population was similar to that reported for US white males 60 to 69 years of age during the same period (during the 1969–1988 follow-up period of the HHP) (29). Therefore, a big potential for stroke prevention and treatment in Japan still exists. Due to several reasons including inter-racial difference in drug response uncertainty, the most up-to-date and widespread guidelines are not reflected in Japanese regulatory documents.

The inter-racial differences in drug response variability are a well-known phenomena. Ethnic or racial differences in pharmacokinetics and pharmacodynamics have been attributed to distinctions in the genetic, physiological and pathological factors between ethnic/racial groups. The pharmacokinetic/pharmacodynamic profile is also known to be influenced by several extrinsic factors, such as socioeconomic background, culture, diet and environment (30). Saturation of enzymes (31), transporters (32) or receptors (32) at high drug concentrations are also possible reasons for ethnic or/and racial discrepancies between single- and multiple-dose regimens, or between low- and high-dose drug administrations.

Ethnic differences in plasma protein-binding of drugs to alpha1-acid glycoprotein appear to be very common, with a consistent trend towards Caucasians having higher binding (lower plasma free fractions) than other ethnic groups (33). This consideration may require a careful dose reduction and monitoring of drugs with high protein-binding capacity like dipyridamole. For example, in one experiment, Chinese patients demonstrated a

Table 3: Platelet biomarkers in AGATE-Japan and AGATE trials.

	% of biomarker inhibition vs. baseline			
Biomarker	**AGATE-Japan**		**AGATE**	
	15 days	**30 days**	**15 days**	**30 days**
ADP 5μM	28.0	28.3	28.5	26.0
Collagen mg/ml	27.8	28.1	NA	NA
Arachidonic Acid	82.6	82.6	NA	NA
PFA-100	62.8	67.5	48.9	52.3
CD31	27.8	33.9	31.5	44.0
CD41	28.5	33.0	16.8	22.8
Pac-1	31.2	33.0	48.9	61.3
CD51/61	20.5	19.3	NA	NA
CD62p	27.0	43.2	50.0	51.0
CD151+14	1.5	3.6	23.9	34.1
WEDE-15	29.8	37.7	57.7	57.7
SPAN-12	34.5	35.2	56.9	62.3

smaller volume of distribution of hypotensive drug fosinoprilat (that is also highly bound to plasma proteins), and those individuals required lower doses of this drug then Caucasians (34). However, Japanese males, in contrast to Japanese females, exhibited higher alpha1-acid glycoprotein binding capacity than patients of Chinese descent (35). Interestingly, the majority of volunteers enrolled in our study from the ERD/A group were males accounting for the minimal number of adverse events in this study arm.

Considering anticipations of possible deviant pharmacodynamics/pharmacokinetics response in Japanese people, as well as their genetic and cultural singularity, it is still hard to expect that this population would benefit less from anti-thrombotic therapy (including aspirin, clopidogrel or Aggrenox) that the rest of the world. Moreover, the most exciting results of anti-thrombotic therapy were obtained from the COMMIT (36) study yielding absolute mortality benefit in Chinese patients after myocardial infarction.

We realize that our small observational study did not enable us to predict all of the possible risks and benefits of ERD/A in the Japanese population, therefore, the initiation of a larger study with the clinical endpoints is warranted.

Study limitations

The major limitation of our study was the inability to present or investigate platelet response to ERD/A in Japanese patients after recent ischemic stroke. However, the existing racial representation in the Baltimore metropolitan area makes such, even pilot, clinical study practically impossible or could postpone study enrolment for years.

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