Increased Platelet Sensitivity to Collagen in Individuals Resistant to Low-Dose Aspirin

Tomio Kawasaki, MD, PhD; Yasushi Ozeki, PhD; Takehiro Igawa, PhD; Jun-ichi Kambayashi, MD, PhD

Background and Purpose—The purpose of this study was to assess individual differences in the pharmacological effects of acetylsalicylic acid (ASA) on bleeding time as measured by in vitro platelet aggregation and to examine the consistency of responses over time.

Methods—We measured template IIR bleeding time and platelet aggregation in 8 healthy male volunteers before and 2 hours after ingestion of 324 mg of ASA. An individual was considered a nonresponder if his post-ASA bleeding time was not 2 SDs above his baseline bleeding time, where SD was estimated from the baseline bleeding times of the 8 volunteers. The same experiment was done after a 30-month interval.

Results—Five volunteers were identified as ASA responders, and 3 were identified as nonresponders. Bleeding time before and after ingestion of ASA was 408 ± 121 seconds (mean ± SD) and 720 ± 225 seconds, respectively, in ASA responders and 330 ± 30 seconds and 330 ± 52 seconds, respectively, in ASA nonresponders. The mean ED50 for collagen-induced platelet aggregation, that is, the mean concentration of collagen that caused a response at 50% of maximum, was 0.91 μg/mL (95% CI, 0.73 to 1.14) in ASA responders and 0.48 μg/mL (95% CI, 0.38 to 0.60) in nonresponders. When optimum concentrations of collagen, ie, concentrations that yielded 90% maximum aggregation, were used as stimuli, the mean IC50 for ASA, that is, the mean concentration that yielded 50% inhibition, was 322.5 μmol/L (95% CI, 264.8 to 392.6) in ASA responders and 336.1 μmol/L (95% CI, 261.0 to 432.8) in nonresponders. The variability in individual responsiveness in the second experiment remained consistent with that in the first experiment.

Conclusions—ASA resistance may be caused by an increased sensitivity of platelets to collagen. A platelet aggregation study specific for collagen dose response may be useful for strict selection of ASA responders for low-dose ASA therapy and for identifying ASA nonresponders for high-dose ASA therapy. (Stroke. 2000;31:591-595.)

Key Words: aspirin ■ bleeding time ■ collagen ■ platelets

Acetylsalicylic acid (ASA) is one of the most commonly used pharmacological substances. Its effectiveness as an antiplatelet agent in preventing cardiovascular events has been clearly demonstrated.1–7 Low ASA doses of 300 mg are often recommended because of relatively few associated bleeding and gastrointestinal side effects.8 However, some patients still suffer a thromboembolic event despite low-dose ASA therapy. The fact remains that low-dose ASA exerts a beneficial effect without side effects in some patients but not in others,9–11 ie, different patients require different ASA dosages to achieve complete inhibition of platelet function. Since Duke first described the bleeding time test in 1910,12 bleeding time has been used to identify disorders of primary hemostasis, indicating risks for bleeding with ASA therapy. Buchanan and Brister13 reported that an ASA dose of 325 mg prolonged the bleeding time in 60% of volunteers (ASA responders) but not in others (ASA nonresponders). Although there has been no definite correlation reported between in vivo bleeding time and in vitro platelet aggregation tests, the efficacy of ASA with respect to platelet function also has been evaluated by platelet aggregation tests.14–16

We evaluated the possible correlation between in vivo ASA responsiveness in terms of bleeding time and in vitro collagen dose dependency in a platelet aggregation study. We hoped to elucidate the mechanism by which ASA affects platelet function differently in different individuals.

Subjects and Methods

The study comprised 2 experiments, one done in September 1996 (experiment 1) and the other in March 1999 (experiment 2). Eight healthy Japanese volunteers (all men between the ages of 23 and 42 years; mean ± SD age, 28.4 ± 6.2 years) were recruited for this study. The inclusion criteria were apparent good health and no cardiovascular disease signs or symptoms. The exclusion criteria were a history of bleeding, known hypersensitivity to ASA, or any psychiatric problem. Smoking was not an exclusion criterion in this study, but participants abstained from smoking on the day of the study. Two

Received October 29, 1999; accepted November 30, 1999.

From the Department of Surgery 2, Osaka University Medical School, Osaka, Japan (T.K.); Thrombosis and Vascular Research Laboratory, Otsuka Pharmaceutical Co, Ltd, Tokushima, Japan (Y.O.); and Maryland Research Laboratories, Otsuka America Pharmaceutical, Inc, Rockville (T.I., J-i.K.).

E-mail kawasaki@surg2.med.osaka-u.ac.jp

© 2000 American Heart Association, Inc.

Stroke is available at http://www.strokeaha.org

591
subjects were identified as habitual smokers. Written informed consent to participate in this study was obtained from all volunteers according to the Declaration of Helsinki. Volunteers were instructed to take no nonsteroidal anti-inflammatory drugs for at least 14 days before the study. They were allowed clear liquids but no food for breakfast on the morning of the test day. Volunteers were also required to fast beginning at 8 PM the night before the test.

Blood sampling was performed with a double-syringe technique in resting volunteers via 19- or 21-gauge scalp vein needles; samples were collected into a syringe containing 1/10 volume of 0.13 mol/L sodium citrate. Subsequently, platelet-rich plasma (PRP) was obtained by centrifugation of the citrated blood at 1200 rpm at room temperature, and platelet-poor plasma (PPP) was obtained from the samples by centrifugation at 1500 rpm for 15 minutes.

Platelets were counted electronically with an automatic blood cell counter (LC-114, Horiba), and accuracy was confirmed with a hemocytometer under phase-contrast microscopy. PRP platelet count was adjusted to 2.0 × 10^9 cells per milliliter with autologous PPP. Platelet activation was registered, and an aggregation curve was drawn via aggregometer (NKK Hema Tracer 1; SSR Engineering Co, Ltd; 6 channels) by the change in light transmission. PPP was used as a control for 100% light transmission. Aliquots of PRP were incubated for 7 minutes at 37°C with particulate collagen from bovine Achilles tendon (Collagenreagent Horm, Nycomed Arzneimittel). All studies were performed between 1 and 2 hours after the platelets were harvested. The amplitude of aggregation was measured 5 minutes after the addition of collagen; when aggregation was reversible, the maximal amplitude reached during a 5-minute observation period was measured. The reaction was terminated by putting the samples in an ice bath, and a 100-μL aliquot of each aggregating sample was centrifuged at 10,000g for 15 seconds. The supernatants were stored at −70°C until assay for thromboxane B2 (TXB2). Eicosanoids were extracted with a C18-minicolumn, and TXB2, measured as thromboxane A2 (TXA2), was determined via specific enzyme immunoassay.17

The bleeding time was measured in duplicate simultaneously with blood collection by the automatic Simplette IIIR template device (Orkanon Teknika) with duplicate horizontal cuts at a venous pressure of 40 mm Hg before administration of ASA. The average of 2 bleeding times for each test was obtained and used for study. A baseline bleeding time was established for each volunteer before ASA ingestion. Each volunteer was given a 324-mg oral dose of aspirin. Two hours later, a second Simplette IIIR template bleeding time test was performed, and the blood was collected. All tests were performed by a single technician. An individual was considered an ASA nonresponder if his post-ASA bleeding time was not 2 SD above his baseline time, where the SD was estimated from the baseline bleeding times of the subjects who participated. Any individual whose post-ASA bleeding time was prolonged >2 SD above his own baseline bleeding time was defined as an ASA responder. In experiment 1, the bleeding time and the platelet aggregation study was performed in 7 of the 8 volunteers who had participated in experiment 1. One ASA responder had taken an anti-inflammatory drug and was unable to participate in experiment 2.

Collagen was administered in vitro at 0.125 to 4.0 μg to clarify the susceptibility of platelets to collagen in responders and nonresponders. Agonist potency was expressed as the concentration of collagen that caused a response at 50% of maximum (ED50). When indicated, concentration-response curves were analyzed by linear regression analysis with the Statistical Analysis System software program (SAS, release 6.12, SAS Institute Inc) and are reported together with 95% CIs. Half-inhibition concentrations (IC50) of ASA were calculated by the median-effect equation with the SAS software program. Significance was assigned by Student’s 2-tailed t test; a value of P < 0.05 was considered significant. Bleeding time data were compared with the Mann-Whitney nonparametric U test. Repeated-measures ANOVA was used to compare levels of TXA2 between ASA responders and ASA nonresponders after platelet aggregation without ASA treatment.

Results

In experiment 1, the mean ± SD bleeding time before ASA intake in the 8 volunteers was 379 ± 101 seconds, and it increased to 574 ± 266 seconds after ASA ingestion. Five volunteers were identified as ASA responders, and 3 were identified as ASA nonresponders. There were no significant differences between responders and nonresponders in age (29.8 ± 7.83 versus 28.7 ± 3.2 years), body weight (71.6 ± 5.9 versus 71.0 ± 1.7 kg), or height (173.8 ± 4.5 versus 173.7 ± 6.4 cm). One ASA responder and 1 nonresponder were determined to be habitual smokers. The bleeding time (mean ± SD) before administration of ASA did not differ between ASA responders and nonresponders: 408 ± 121 and 330 ± 30 seconds, respectively (P > 0.05). Bleeding times (mean ± SD) determined 2 hours after ingestion of 324 mg ASA were 720 ± 225 seconds in ASA responders and 330 ± 52 seconds in nonresponders (Figure 1).

Seven volunteers were involved in experiment 2: 4 ASA responders and 3 nonresponders. None of the study subjects changed from a responder in experiment 1 to a nonresponder in experiment 2. There were no significant differences between responders and nonresponders in age, body weight, or height. The bleeding time (mean ± SD) before ASA intake in the 7 volunteers was 384 ± 48 seconds, and it increased to 504 ± 202 seconds after ASA ingestion. The bleeding time (mean ± SD) before administration of ASA did not differ between ASA responders and nonresponders: 405 ± 52 and 357 ± 31 seconds, respectively (P > 0.05). Bleeding time (mean ± SD) 2 hours after ingestion of 324 mg ASA was 623 ± 189 seconds in ASA responders and 345 ± 54 seconds in nonresponders (Figure 1).

![Figure 1. Changes in bleeding time after ASA administration in ASA responders and nonresponders in experiment 1 (n = 8) and experiment 2 (n = 7). Solid square represents the ASA responder who did not participate in experiment 2.](http://stroke.ahajournals.org/doi/figure/1)
The plasma levels of TXA$_2$ measured in experiment 1 before intake of ASA did not differ between ASA responders and nonresponders ($4.13\pm 1.12$ pmol TXA$_2$/2$\times 10^8$ platelets versus $4.24\pm 1.62$ pmol TXA$_2$/2$\times 10^8$ platelets; $P>0.05$), nor did they differ after intake of the total 324 mg ASA ($1.59\pm 0.54$ pmol TXA$_2$/2$\times 10^8$ platelets versus $1.00\pm 0.08$ pmol TXA$_2$/2$\times 10^8$ platelets; $P>0.05$). There were no significant differences between responders and nonresponders in the percent inhibition of platelet aggregation or synthesis of TXA$_2$ stimulated by 2 mg/mL collagen after intake of 324 mg ASA: $86.1\pm 6.5\%$ inhibition versus $75.2\pm 6.2\%$ inhibition, $0.141\pm 0.050$ nmol TXA$_2$/2$\times 10^8$ platelets versus $0.112\pm 0.072$ nmol TXA$_2$/2$\times 10^8$ platelets, respectively.

To better clarify the difference in susceptibility of platelets to collagen between ASA responders and nonresponders, platelet aggregation was determined in vitro with collagen (0.125 to 4.0 mg/mL) used as an inducer. The dose-response curves of collagen in ASA responders and nonresponders are shown in Figure 2. There was a significant difference in the light transmission at lower collagen doses of 0.375 to 0.75 mg/mL between ASA responders and nonresponders, whereas there was little difference at higher collagen concentrations of 2 to 4 mg/mL. In experiment 1, the ED$_{50}$ values for collagen in ASA responders and nonresponders were 0.91 µg/mL (95% CI, 0.73 to 1.14) and 0.48 µg/mL (95% CI, 0.38 to 0.60), suggesting that platelets from ASA nonresponders were more sensitive to collagen than platelets from ASA responders (Figure 3). The curves demonstrated a responsiveness in the ASA nonresponders parcelling or equaling that of the responders at the higher dosage end of the graph. Collagen in the range of 0.25 to 0.75 µg/mL seemed to show quite a different stimulatory effect on platelet aggregation between ASA responders and nonresponders (Figure 3). In experiment 2, the ED$_{50}$ value for collagen in ASA responders and nonresponders was 0.80 µg/mL (95% CI, 0.50 to 1.10) and 0.33 µg/mL (95% CI, 0.26 to 0.40), respectively, suggesting that platelets from ASA nonresponders were more sensitive to collagen than platelets from ASA responders (Figure 4). ED$_{50}$ values for collagen in ASA responders and nonresponders were quite different, and this was confirmed by the 2 experiments done at an interval of 30 months.

In experiment 1, production of TXA$_2$ from collagen-induced platelet aggregates increased dose dependently at increasing doses of 0.5 to 4 µg/mL collagen, although there was no difference between ASA responders and nonresponders (data not shown; $P>0.05$ in ANOVA analysis). In experiment 1, the optimum concentration of collagen used for IC$_{50}$ of ASA was defined to give 90% amplitude of the maximum aggregation obtained by 4 µg/mL collagen. In vitro addition of ASA to PRP led to a decrease in collagen-induced platelet aggregation but without a significant difference between IC$_{50}$ in ASA responders (322.5 µmol/L; 95% CI, 264.8 to 392.6) and IC$_{50}$ in ASA nonresponders (336.1 µmol/L; 95% CI, 261.0 to 432.8) (Figure 5).

**Discussion**

While debate has centered on the potential efficacy of low-dose ASA for the prevention of ischemic events, it must be noted that the protective effect of ASA is variable in individuals despite complete inhibition of cyclooxygenase.$^{13,18,19}$ Bleeding time has been advocated as a simple test
of platelet function, sufficiently sensitive to permit diagnosis or exclusion of platelet dysfunction, and template bleeding times have been quite accurate under both normal conditions and ASA administration.20,21 Although the bleeding time test is accurate and useful, it should be noted that prolonged bleeding time can cause keloid formation and repeated bleeding time tests can leave scars. To assess the effectiveness of ASA for preventing vascular events, an easy-to-use, reliable, and repeatable test is mandatory. Fiore et al22 noted that ASA responders could be separated from ASA nonresponders by bleeding time studies for the purpose of preventing hemorrhagic side effects and that neither baseline bleeding time nor other tests of hemostatic function, such as platelet aggregation in response to arachidonic acid, epinephrine, or ristocetin or measurement of serum TXA2, discriminated ASA responders from ASA nonresponders. In addition, administration of 325 mg ASA has been reported to effect no difference in the inhibition of fixed-dose collagen-induced platelet aggregation between ASA responders and nonresponders or in the inhibition of platelet TXA2.13 In our study similar results were obtained in an ex vivo study, demonstrating after ingestion of 324 mg ASA no difference in the extent of inhibition of fixed-dose (2 μg/mL) collagen-induced platelet aggregation and inhibition of TXA2 between ASA responders and nonresponders. We speculated that studies using lower fixed-dose collagen may be needed to identify ASA responders and ASA nonresponders because the collagen concentration-response curves of ASA responders and nonresponders were equal at 2 μg/mL, as shown in Figures 3 and 4. We also failed to differentiate ASA nonresponders from ASA responders in the production of TXA2 at various doses of collagen or in the IC50 values of ASA. Accordingly, the difference in platelet function between ASA responders and nonresponders is expected to be small. The inverse effect of doses of ASA on prolongation of bleeding time, ie, shortening of the bleeding time at higher ASA doses, has been attributed to the relative insensitivity of endothelial cyclooxygenase to ASA.22,23 Since lower ASA doses were used in this study, the effect of endothelial cyclooxygenase on ASA may have been eliminated.

The present study focused on individual responsiveness to ASA in terms of bleeding time and the sensitivity of platelets to collagen as measured by platelet aggregation, collagen being a physiologically important activating agent. The results obtained for bleeding time in ASA nonresponders suggest 2 possible mechanisms: either susceptibility of platelets to ASA was decreased without changing the platelet responsiveness to the agonist, or an increase in platelet sensitivity to the agonist decreased sensitivity to ASA. In the present study ASA inhibited platelet aggregation in a dose-dependent manner, with similar IC50 values of approximately 300 μmol/L in ASA responders and nonresponders, when the optimum concentration of collagen that gave 90% amplitude of the maximum aggregation was selected per individual. This means that there was no difference in the susceptibility of platelets to ASA when the optimum doses of agonist were selected to yield the same intensity of platelet aggregation. The result is in disagreement with speculation that susceptibility of platelets to ASA decreases in ASA nonresponders without a change in the platelet responsiveness to agonist. We have shown that platelets without ASA treatment show significantly increased sensitivity to collagen in ASA nonresponders compared with that in ASA responders, especially when relatively low concentrations of collagen are used. The results suggest platelet reactivity to be increased and that platelet sensitivity to ASA may thereby be decreased in ASA nonresponders.

In our study ASA resistance was studied by bleeding time in 8 healthy volunteers. The proportion of ASA nonresponders was 38%, which is about the same as the 40% reported by Buchanan and Brister.13 Since the proportion of ASA nonresponders was quite high, we consider it very important to screen ASA nonresponders for high-dose ASA therapy. Buchanan and Brister13 also reported that most ASA nonresponders tested with low doses of ASA were responders at high doses (1300 mg ASA) and that the difference between ASA responders and nonresponders is relative. Each of the ASA responders and nonresponders was classified in the same group according to bleeding time study after the 30-month interval, and the difference in ED50 values between ASA responders and nonresponders should be quite valid in 2 experiments done at an interval of 30 months. Thus, it could well be speculated that the difference between ASA responders and ASA nonresponders is a distinct variation in platelet responsiveness to the agonist.

We selected healthy volunteers as subjects in this study to assess individual differences more clearly by avoiding the potential effect of atherosclerosis or possible influence of antiplatelet drugs. Because of our limitations in sample size and the type of volunteers, further evaluation is needed based on a large study population comprising both healthy controls and atherosclerosis patients.

In conclusion, the aforementioned results indicate that the difference between ASA responders and ASA nonresponders is due to variations in platelet reactivity to collagen. Therefore, a classic platelet aggregation study based on agonist
dose response may be useful in both strictly selecting ASA responders for low-dose ASA therapy and identifying ASA nonresponders as possible candidates for high-dose ASA therapy. In addition, because the difference in ASA responders and ASA nonresponders depends on platelet responsiveness to the agonist, a dose-response platelet aggregation study may well be useful in fixing the dosage for patients receiving not only ASA but other antiplatelet therapies as well.

Acknowledgments
This study was supported in part by grant-in-aid for scientific research 09671228 from the Ministry of Education, Science, and Culture, Japan. Yoshie Nagamura, Fumiko Unemi, and Hideki Ito of the Thrombosis and Vascular Research Laboratory, Otsuka Pharmaceutical Co, Ltd, and Etsuji Suehisa of the Central Laboratory for the Thrombosis and Vascular Research Laboratory, Otsuka Pharmaceutical Co, Ltd, and Etsuji Suehisa of the Central Laboratory for Clinical Investigation, Osaka University Medical School, are gratefully acknowledged for their excellent technical assistance.

References
17. Raghunath M. Extraction of various arachidonic acid metabolites from minimal amount of biological fluid with a new generation of miniaturized solid phase extraction. J Liquid Chromatogr. 1990;13:969–980.
Increased Platelet Sensitivity to Collagen in Individuals Resistant to Low-Dose Aspirin
Tomio Kawasaki, Yasushi Ozeki, Takehiro Igawa and Jun-ichi Kambayashi

Stroke. 2000;31:591-595
doi: 10.1161/01.STR.31.3.591
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/31/3/591

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/