Resistance In Vitro to Low-Dose Aspirin Is Associated With Platelet PlA1 (GP IIIa) Polymorphism But Not With C807T (GP Ia/IIa) and C-5T Kozak (GP Ibα) Polymorphisms

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OBJECTIVES We investigated whether three platelet gene polymorphisms, PlA1/A2, C807T, and C-5T Kozak (encoding, respectively, for platelet membrane glycoproteins (GP) IIIa, GP Ia/IIa, GP Ibα), could contribute to the resistance to a low dose of aspirin (160 mg/day).

BACKGROUND Aspirin antiplatelet effect is not uniform in all patients, and the mechanism by which some patients are in vitro resistant to aspirin remains to be determined. However, it has been suggested that polymorphisms of platelet membrane glycoproteins might contribute to aspirin resistance.

METHODS Ninety-eight patients on aspirin (160 mg/day) for at least one month were enrolled. Aspirin resistance was measured by the platelet function analyzer (PFA)-100 analyzer; genotyping of the three polymorphisms was performed using a polymerase chain reaction-based restriction fragment-length polymorphism analysis.

RESULTS Using a collagen/epinephrine–coated cartridge on the PFA-100, the prevalence of aspirin resistance was 29.6% (n = 29). Aspirin-resistant patients were significantly more often PlA1/A1 (86.2%; n = 25) than sensitive patients (59.4%; n = 41; p = 0.01). Of the 29 patients, 25 were reevaluated after having taken 300 mg/day aspirin for at least one month. Only 11 patients still have nonprolonged collagen epinephrine closure time, and these were all PlA1/A1. No relation was found between resistance status and C-5T Kozak or C807T genotypes.

CONCLUSIONS Platelets homozygous for the PlA1 allele appear to be less sensitive to inhibitory action of low-dose aspirin. This differential sensitivity to aspirin may have potential clinical implications whereby specific antiplatelet therapy may be best tailored according to the patient’s PlA genotype. (J Am Coll Cardiol 2003;42:1115–9) © 2003 by the American College of Cardiology Foundation

The use of aspirin for the secondary prevention of vascular events is well established. Recently the Antithrombotic Trials’ Collaboration compiled a meta-analysis of 65 trials using aspirin in high-risk patients and found a 23% odds reduction in vascular events in the aspirin–treated group (1). Aspirin is also a very effective therapy for patients suffering an acute myocardial infarction (MI). As demonstrated by the Second International Study of Infarct Survival (ISIS-2) trial, acute aspirin administration reduced mortality by 23%, a comparable (and importantly additive) effect to thrombolytic therapy (2). Acetylsalicylic acid exerts its antithrombotic effect primarily by interfering with the biosynthesis of thromboxane A2 (TXA2) and inhibition of TXA2-dependent platelet aggregation (3). However, it appears that aspirin’s antiplatelet effect is not uniform in all patients (4). Furthermore the optimal dosage of aspirin for complete inhibition of platelet aggregation is subject of great interindividual variability (5), and the mechanism by which some patients are in vitro resistant to aspirin remains to be determined.

Increasing evidence shows that cell–cell interaction molecules play an important role in cardiovascular pathology, and platelet glycoprotein (GP) polymorphism, as a genetic risk factor for arterial thrombosis, is a new area of human genomics that has been intensively investigated for several years (6). Various allelic variants of key platelet GPs are known to exist within the human gene pool, creating diversity in the expression, function, and immunogenicity of these important adhesion receptor components. The integrin α2β3 is known as the receptor for fibrinogen, or von Willebrand factor, that mediates platelet aggregation. This receptor is also characterized by several heritable dimorphisms (7). The two most common and clinically important β3 alleles encode Leu-33 (PlA1 or HPA-1a) and Pro-33 (PlA2 or HPA-1b), with gene frequency of 0.85 and 0.15, respectively, in the Caucasian population. Since the first report in 1996 on the association of the PlA2 allele as a risk factor for coronary artery disease (8), the impact of PlA2 as
of ticlopidine, clopidogrel, dipyridamole, anti-inflammatory drugs, use of any preparation containing aspirin or non-steroid anti-inflammatory drugs for at least 10 days. For the patients who were resistant to aspirin (160 mg/day), daily intake of aspirin was increased to 300 mg/day, and they were controlled one month later. In parallel, 90 healthy voluntary controls taken from the hospital staff (47 men and 43 women, mean age 41.3 ± 10 years) were studied for genotype prevalence.

**Specimen collection.** Nonfasting blood samples were all obtained between 9 AM and 10 AM (2 to 3 h after aspirin intake). Nine milliliters of blood was collected in 0.129 mol/l buffered sodium citrate tubes (Vacutainer, Becton Dickinson, Rutherford, New Jersey) for analysis by platelet function analyzer (PFA)-100, and 4.5 ml in ethylene diamine tetra acetic acid tube (Vacutainer, Becton Dickinson), for genotype and platelet count determination.

The **PFA-100 system**. The PFA-100 system (Dade-Behring International, Miami, Florida) is a microprocessor-controlled instrument/test cartridge system that simulates platelet-based primary hemostasis in vitro. A syringe aspirates citrated whole blood under steady-flow conditions through a small aperture (150 μm in diameter) cut into a membrane placed in the test cartridge. The membrane is coated with 2 μg of type I collagen and either 10 μg collagen epinephrine (CEPI) bitartrate or 50 μg collagen adenosine diphosphate (CADP) (12). The instrument records the time necessary for the occlusion of the aperture, defined as closure time (CT), which is indicative of platelet function on the whole-blood sample (13). The PFA-100 tests were performed within 2 h after blood sampling.

Normal ranges, which were previously established in our laboratory, are 88 to 186 s for CEPI cartridge, 66 to 121 s for CADP cartridge, and C Ts above normal range were considered abnormal values. If collagen epinephrine closure time (CEPI-CT) was >300 s, the result was reported as 300 s. For all patients, PFA-100 system measurements were performed in duplicate with the same batch of cartridges. Resistance to aspirin was defined as CEPI-CT <186 s (normal range) in samples obtained from patients receiving aspirin as described in the preceding text.

**Genotyping of the GP IIa, GP Iibα, and GP IIIa polymorphisms.** Genomic deoxyribonucleic acid was isolated from peripheral blood mononuclear cells, amplified by polymerase chain reaction (PCR), and digested with the corresponding restriction endonuclease to determine the polymorphism of each GP. Restriction fragments were visualized under ultraviolet light after electrophoresis on polyacrylamide gels and staining with ethidium bromide.

Genomic amplification of the GP IIa exon7/intron7 sequence (accession no. AF035968, nucleotides 2781 to 3023) was performed using the reverse primer described by Kunicki et al. (14) and a mutagenic forward primer used by Corral et al. (15), which allowed the identification of the 807C/T polymorphism by digestion of the PCR product with *Hinf*I (Roche Diagnostics, Meylan, France). The
807C allele of the GP Ia gene displayed a band pattern of 221 bp, whereas the presence of a 243 bp is distinctive of the 807T allele. The GP Ibα was amplified using primers based on the GP Ibα intron1/exon2 sequence (accession no. M22403) from nucleotide 3035 to 3171 using the following forward 5'-GATCCACTCAAGGCTCCCTTG-3' and reverse 5'-TGTCACAGTTCACTTCTAGGT-3' primers (adapted from Afshar-Kharghan et al. [16]). The 137 bp amplified product was digested by AvaI (Roche Diagnostics). In the presence of the PlA2 allele, but not the PlA1 allele, the 109 bp amplified product from T/T homozygotes produced one band of 137 bp, and from heterozygotes, two bands of 109 bp and 28 bp; from C/C homozygotes, no digestion was observed. Indeed, for the 29 patients resistant in vitro to aspirin, the rate of being resistant was 4.4 times higher in PlA1/A1 patients than in patients with at least one PlA2 allele (p = 0.01). Logistic regression showed that platelet gene polymorphism PlA1/A2 and gender were predictive independent factors of aspirin resistance: the odds ratio of being resistant was 4.4 times higher in PlA1/A1 patients than in patients with at least one PlA2 allele (95% confidence interval [CI] = 1.3 to 14.7) and 3.8 times greater in women than in men (95% CI = 1.3 to 10.8). For the C-5T Kozak and C807T genotypes, no relationship was found with the resistance status (Table 2). The prevalence of each genotype did not significantly differ between the control group (n = 90) and the patient group studied (Table 2). Resistance to 300 mg/day aspirin. For the 29 resistant patients, 25 were reevaluated under 300 mg/day aspirin. Eleven patients were still resistant in vitro (CEPI-CT <186 s), whereas the 14 others had prolonged CEPI-CT. All these 11 patients were PlA1/A1.

### RESULTS

**Patients’ characteristics.** By the PFA-100 method, the prevalence of aspirin resistance (defined as CEPI-CT <186 s) was 29.6% (n = 29) (Table 1). Patients who were resistant to aspirin were slightly older (68.5 ± 10.3 years) than those who were sensitive (64.7 ± 10.1 years; p = 0.07) and were more likely women (37.9%; p = 0.01) (Table 1). No difference was found based on demographics or biological data, usual treatment, or number of coronary arteries involved (data not shown) between the two groups.

**Collagen adenosine diphosphate closure time (CADP-CT) in aspirin-resistant patients.** The CADP-CT in patients resistant to 160 mg of aspirin was significantly shorter (73.3 ± 29.8 s, n = 29) than for those in the sensitive group (90.7 ± 21.2 s, n = 69; p = 0.007). No significant difference was found between the aspirin-sensitive group (90.7 ± 21.2 s, n = 69) and the normal range used in the laboratory (91.3 ± 18.5 s; p = 0.09).

**Resistance in vitro to aspirin 160 mg/day related to genotypes.** A significant relationship was found between resistance to 160 mg aspirin and PlA1/A1 genotype (Table 2). Indeed, for the 29 patients resistant in vitro to aspirin, 86.2% (n = 25) were PlA1/A1 and 13.8% (n = 4) had at least one PlA2 allele (p = 0.01). Logistic regression showed that platelet gene polymorphism PlA1/A2 and gender were predictive independent factors of aspirin resistance: the odds ratio of being resistant was 4.4 times higher in PlA1/A1 patients than in patients with at least one PlA2 allele (95% confidence interval [CI] = 1.3 to 14.7) and 3.8 times greater in women than in men (95% CI = 1.3 to 10.8). For the C-5T Kozak and C807T genotypes, no relationship was found with the resistance status (Table 2). The prevalence of each genotype did not significantly differ between the control group (n = 90) and the patient group studied (Table 2).
highlighting the clinical interest in determining aspirin’s inhibitory effects on patients’ platelets. Moreover, Gum et al. (19), following up a cohort of 326 stable cardiovascular patients (mean follow-up = 679 ± 185 days) on aspirin 325 mg/day, demonstrated a greater than threefold increase in the risk of major adverse events associated with aspirin resistance defined on optical platelet aggregation criteria. In our study, the proportion of aspirin-resistant patients was well within the range already described in these types of studies (4,20) as 29 of 98 patients (29.6%) had nonprolonged CEP1-CT despite aspirin treatment (160 mg/day) for more than one month. In accordance to previous reports, resistant patients were more likely to be women and to be older than those having prolonged CEP1-CT (20). Previously, we reported that platelets from aspirin-resistant patients appear to be more sensitive and to be activated by ADP (21). In the present study using a CADP cartridge, we confirmed this sensitivity as the CADP-CT was significantly lower in aspirin-resistant patients compared to sensitive ones. However, even cell-cell interactions have been proposed as a factor modifying the response to various agonists (22); mechanisms by which some patients are resistant to aspirin in vivo are still unknown. Platelet GP polymorphism, which has been extensively studied as a risk factor for cardiovascular disease, has been suggested as a possible mechanism for platelet resistance. Among platelet GP polymorphisms, two have been associated with receptor density at the platelet surface and involved adhesion receptors GP Ia/IIa (C807T polymorphism) (14) and GP Ib-IX-V (C-5T Kozak polymorphism) (16). These initial reports were followed by clinical investigations linking genotype and arterial diseases with contradictory results. In their study, Homoncik et al. (5), studying 10 controls receiving 100 mg of aspirin for 11 days, found that the patient who had the highest GP Ia/IIa at platelet surface exhibited the shortest CEP1-CT. Thus, the investigators hypothesized that the genetically determined collagen receptor density could influence both basal CT and aspirin-induced CT. In our study no relationship was found among C807T, C-5T Kozak polymorphisms, and resistance to low-dose aspirin. However, we showed that \( \text{PI}^{\text{A1A2}} \) positive platelets are less sensitive to classical therapeutic concentrations of aspirin. This relation between \( \text{PI}^{\text{A1A2}} \) polymorphism and resistance to aspirin accords with previous experiments. Indeed, Cooke et al. (10) have shown that aggregation to epinephrine and ADP was identical in \( \text{PI}^{\text{A1A2}} \) and \( \text{PI}^{\text{A1A1}} \) platelets. However, in vitro addition of aspirin more strongly inhibited \( \text{PI}^{\text{A1A2}} \) platelets. Moreover, Michelson et al. (11) clearly demonstrated that \( \text{PI}^{\text{A2}} \)-positive platelets display a lower threshold for activation by ADP in the absence of aspirin than other genotypes.

Conversely, in their in vitro study, Lutomski et al. (23) showed that epinephrine-induced aggregation of \( \text{PI}^{\text{A1A2}} \) platelets was more sensitive to inhibition (compared to \( \text{PI}^{\text{A1A1}} \) and \( \text{PI}^{\text{A2A2}} \) genotypes) by pharmacologically relevant concentrations of 2.5 to 5 \( \mu \)mol/l aspirin, which are typically obtained in vivo. We therefore reevaluated CEP1-CT after increasing aspirin to 300 mg/day. Of the 25 resistant patients to 160 mg/day aspirin, 11 still had nonprolonged CEP1-CT after one month with 300 mg/day aspirin. It can then be suggested that aspirin resistance could be concentration dependent but not for all treated patients. It is noticeable that all the patients resistant to 300 mg aspirin were homozygous for \( \text{PI}^{\text{A1}} \).

In their study, Michelson et al. (11) found that heterozygous platelets (\( \text{PI}^{\text{A1A2}} \)) showed a greater sensitivity to two platelet inhibitors: aspirin and abciximab. The investigators hypothesized that receptor clustering augment GP Ib/IIa signalling (24), and perhaps such clustering may be inhibited in heterozygous platelets such that they are more susceptible to inhibition by aspirin or abciximab. Furthermore, researchers recently found increased adhesion in \( \text{PI}^{\text{A2}} \) GP Ib/IIa-expressing cells, compared to \( \text{PI}^{\text{A1}} \)-expressing cells, which is mediated through differences in outside-in signalling (25). Taken together, these data and ours could be interpreted as the consequence of the inhibition of a still unknown signalling component by the \( \text{PI}^{\text{A1}} \) allele product.

To our knowledge, the present study is the first published clinical trial on coronary artery disease patients addressing the issue of aspirin sensitivity correlation with platelet GP polymorphism upon CEP1-CT. Because our series is rather short, these results require validation in a larger cohort. Indeed, this differential sensitivity to aspirin may have potential clinical implications whereby specific antiplatelet therapy may be best tailored according to a patient’s \( \text{PI}^{\text{A}} \) genotype.

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