It is well established that platelets and inflammation play a central role in coronary atherosclerotic heart disease and its consequences (i.e., unstable angina, myocardial infarction, and sudden cardiac death) (1,2), with numerous large clinical trials demonstrating significant benefit of platelet inhibition with various agents (3–7). Clopidogrel is an inhibitor of adenosine diphosphate (ADP)-induced platelet aggregation by reducing ADP binding to its receptor (8,9).

Clopidogrel’s clinical efficacy was demonstrated in the Clopidogrel in Unstable angina to Prevent Recurrent Events (CAPRIE) trial, in which its long-term administration to patients with acute coronary syndromes compared with patients receiving only ASA. We have shown previously that the effect of ASA on platelets is modified by the glycoprotein IIIa single nucleotide polymorphism PlA2. Hence, an important pharmacogenetic question remains whether the antiplatelet effect of clopidogrel is uniform for all patients, or, like acetylsalicylic acid, more selective.

The combination of ASA and clopidogrel appears superior to either agent alone in inhibiting platelet function. PlA2 functions as an important modifier for platelet responsiveness to ASA but not to clopidogrel. These findings could have significant impact on the future design of pharmacogenetic antithrombotic strategies for patients with coronary heart disease.

We hypothesized that the efficiency and safety of antithrombotic strategies might be impacted by specific gene variants implicated in coronary thrombosis under various clinical circumstances (15,16). This pilot trial was designed to assess the relative efficacy of aspirin, clopidogrel, or their combination in inhibiting ex vivo platelet function in a cohort of patients with established coronary heart disease (CHD). We demonstrate that platelet function is markedly dependent upon the type of agonist used, agonist concentration, antiplatelet strategy, and the platelet antigen polymorphism (PlA) genotype. The latter finding could have important pharmacogenetic consequences for the design of antithrombotic strategies for patients with CHD.

**METHODS**

**Study subjects.** To study the effects of the PlA phenotype on in vivo antiplatelet therapy, we genotyped a cohort of patients with established and stable coronary artery disease, verified by cardiac catheterization, who require long-term antiplatelet therapy. We studied the modifier effect of platelet antigen polymorphism (PlA2) on platelet inhibition by acetylsalicylic acid (ASA, i.e., aspirin) and clopidogrel, or their combination in patients with coronary heart disease.

**OBJECTIVES**

We studied the modifier effect of platelet antigen polymorphism (PlA2) on platelet inhibition by acetylsalicylic acid (ASA, i.e., aspirin), clopidogrel, or their combination in patients with coronary heart disease.

**BACKGROUND**

Clopidogrel, when administered with ASA, was shown to significantly improve the outcome of patients with acute coronary syndromes compared with patients receiving only ASA. We have shown previously that the effect of ASA on platelets is modified by the glycoprotein IIIa single nucleotide polymorphism PlA2. Hence, an important pharmacogenetic question remains whether the antiplatelet effect of clopidogrel is uniform for all patients, or, like acetylsalicylic acid, more selective.

**METHODS**

Thirty PlA1/A1 and 30 PlA1/A2 patients were assigned randomly to ASA 325 mg/day, clopidogrel 75 mg/day, or both. After 10 days, platelet function was studied.

**RESULTS**

Clopidogrel provided stronger platelet inhibition than ASA with adenosine diphosphate as the agonist, and combination therapy resulted in greater inhibition than either inhibitor used alone (p < 0.0001). The use of ASA resulted in greater inhibition compared with clopidogrel with epinephrine (p < 0.0001) and collagen as agonists (p < 0.0001). With collagen as the agonist, platelets from PlA1/A2 donors were markedly and significantly less inhibited by ASA (p = 0.005). In contrast, with clopidogrel, no significant difference could be detected between inhibition of PlA1/A1 and PlA1/A2 platelets.

**CONCLUSIONS**

The combination of ASA and clopidogrel appears superior to either agent alone in inhibiting platelet function. PlA2 functions as an important modifier for platelet responsiveness to ASA but not to clopidogrel. These findings could have significant impact on the future design of pharmacogenetic antithrombotic strategies for patients with coronary heart disease.
antiplatelet therapy for secondary prevention. All study subjects provided written informed consent, and the protocol was approved by the Institutional Review Board for Human Subjects of the Ohio State University. We compared two groups of subjects that were matched for age, gender, race, medical history, and blood pressure. The first group consisted of 30 PlA1/A1 homozygotes, and 30 PlA1/A2 subjects comprised the second group. Ten subjects from each group were assigned randomly to receive one of three treatment regimens for 10 days. The regimens were ASA (325 mg, daily) alone (10 PlA1/A1 and 10 PlA1/A2), clopidogrel (75 mg, daily) alone (10 PlA1/A1 and 10 PlA1/A2), or ASA (325 mg, daily) and clopidogrel (75 mg daily) in combination (10 PlA1/A1 and 10 PlA1/A2). Platelet assays were performed after 10 days of therapy (enough time for both drugs to reach steady state). All patient data were coded to maintain a blinded evaluation.

**Platelet preparation.** Phlebotomy was performed in the morning within 4 h of the patients ingesting study medication, under fasting conditions, and without caffeine intake for at least 12 h. Platelet-rich plasma (PRP) was obtained from citrated blood as previously reported (17). Five concentrations of three conventional agonists were used in separate assays, including epinephrine (0, 0.5, 1, 2, 5, and 10 μmol/l; Sigma Chemical Co., St. Louis, Missouri), ADP (0.5, 1, 2, 5, and 10 μmol/l; Sigma Chemical Co.), and collagen (type 1; 0, 0.5, 1, 2, 5, and 10 μg/ml; Chrono-log, Havertown, Pennsylvania).

**Platelet aggregation.** A standardized platelet concentration of 300,000 platelets/μl for a 250-μl reaction was prepared by dilution of PRP with autologous plasma depleted of platelets after a platelet count was determined with a Z1 Coulter counter (Coulter Corp., Miami, Florida). Samples were incubated at 37° for 30 min before the aggregation assay using the method of Born (18). Separate platelet aggregation assays were performed with various concentrations of agonists under constant shear (1,000 rpm) and temperature (37°) for 15 min in a Chrono-log 470-VS 4-channel aggregometer (Chrono-log).

**Platelet glycoprotein (GP) IIb/IIIa activation and α-granule release.** Platelets from PRP were diluted to 1,000 platelets/μl with Tyrode’s buffer in polypropylene tubes. To assess GP IIb/IIIa receptor activation, resting platelets were supplemented with fluorescein isothiocyanate-fibrinogen (4.7 μg/ml final concentration, Molecular Probes, Eugene, Oregon) before agonist stimulation. Ten minutes after exposure to agonist, the reaction was stopped, and the platelets were fixed in formaldehyde (1.5% final concentration, Fisher Scientific, Pittsburgh, Pennsylvania). Fixed platelets were then incubated with phycoerythrin-conjugated monoclonal antibody CD62P (0.09 μg/ml final concentration, BD PharMingen, San Diego, California) and analyzed by two-color flow cytometry (FACS Calibur, Becton Dickinson, San Jose, California). Ten thousand gated events were collected and analyzed using Cellquest software. All data were stored in listmode fashion for archiving.

**Figure 1.** Aggregation (mean percent ± SEM) with adenosine diphosphate (A), collagen (B), or epinephrine (C) in study subjects by treatment strategy. Significant differences under different conditions of blockade were noted. Multiple comparisons (six included in adjustment per plot) at each agonist’s concentrations revealed significant differences (p < 0.05) between treatment strategies as indicated. *Applies for differences between treatment with acetylsalicylic acid (ASA) and with clopidogrel. †Applies for differences between treatment with clopidogrel and with ASA plus clopidogrel in combination. ‡Applies for differences between treatment with ASA and with ASA plus clopidogrel in combination.
Data analysis. All patient data were coded to maintain a blinded evaluation. For baseline clinical data, a chi-square analysis was used for categorical data and a Student t test for continuous variables. For platelet aggregation and GP IIb/IIIa activation data, a three-factor (PlA genotype, treatment strategy, and agonist concentration) analysis of variance (ANOVA) with one repeated factor (agonist concentration) was performed separately for each agonist. Modeling was performed by PROC Mixed procedure in SAS Version 8.1 (SAS Institute, Cary, North Carolina) (19,20). Multiple comparisons were made (if significant differences were found) with least square means analysis with the Bonferroni adjustment (21). Data were transformed if normality and equal variances of the residuals were not met. In all ANOVA models on the raw data, neither the equal variances nor the normality assumptions on the error terms were met. The normality and equal variances assumptions were examined on the residuals from the ANOVA with the Shapiro-Wilkes test and the plots, respectively. Therefore, the data were transformed using a square-root transformation, and all statistical analyses are based on the transformed data. The assumptions were again checked after the square-root transformation, and they appeared to be met.

RESULTS

Clopidogrel provided significantly greater inhibition of platelet aggregation induced with ADP, and even more inhibition was observed when clopidogrel was added to ASA as compared with treatment with ASA only (Fig. 1A; p < 0.0001). With collagen, significant differences were found between treatments (Fig. 1B; p < 0.0001), with ASA plus clopidogrel and also ASA-only treatments, providing significantly greater inhibition when compared with clopidogrel. With epinephrine, ASA significantly inhibited aggregation when compared with clopidogrel (Fig. 1C; p < 0.0001), with no added benefit of clopidogrel in combination therapy with ASA. Similar to aggregation, fluorescein isothiocyanate-fibrinogen binding was significantly reduced by clopidogrel and the clopidogrel-plus-ASA combination therapy compared with ASA only with ADP stimulation (Fig. 2; p < 0.0001). However, this treatment effect in ADP-stimulated platelets was not observed between treatment strategies for α-granule release as assessed by CD62P binding (p = 0.89).

We then investigated the effect of the PlA polymorphism. The PlA genotype effect on platelet inhibition was markedly stronger when collagen was used as the agonist compared with epinephrine and less strong with ADP. An interaction was noted between ADP concentration and genotype (Fig. 3A; p < 0.005) in terms of aggregation, but this genotype effect was not statistically significant in GP IIb/IIIa activation (p = 0.21) or in α-granule release (p = 0.31). With collagen, PlA1/A1 platelets were significantly more inhibited by ASA than PlA1/A2 platelets in terms of aggregation (Fig. 3B; p = 0.005) and GP IIb/IIIa activation (Fig. 4; p < 0.0001) as assessed by fibrinogen binding. The difference between PlA1/A1 and PlA1/A2 platelets was significant, whether with ASA alone or combination therapy (Fig. 3B). No statistically significant interaction was noted between epinephrine concentration and genotype with the dosage(s) of antiplatelet drug(s) used.

Finally, alpha-granule release in collagen-stimulated platelets was dependent on PlA genotype, treatment strategy, and agonist concentration (Fig. 5; p = 0.005). PlA1/A2 platelets had significantly higher alpha-granule release regardless of treatment strategy at higher concentrations of collagen, whereas in PlA1/A1 platelets, the addition of clopidogrel to ASA therapy yielded further decreases in CD62P expression.

DISCUSSION

In this report, we demonstrate that the PlA2 polymorphism significantly modifies the inhibition of platelet function brought about by ASA and clopidogrel and is dependent on the agonist used. When collagen stimulates aggregation, clopidogrel behaves as a weak inhibitor relative to ASA. However, when added to ASA, the combination of the two drugs is marginally more efficient than ASA alone, an incremental effect that is not observed with epinephrine as the agonist. These interactions between agonists and inhibitors are highly instructive and, in the case of collagen, associated with strong concurrent interaction with the platelet SNP (T1565C), PlA.
their PI\textsubscript{A1/A1} counterparts (Figs. 3B, 4, and 5). When clopidogrel is added to ASA, the inhibition of PI\textsubscript{A1/A2} platelets is still less than that of their PI\textsubscript{A1/A1} counterpart; although PI\textsubscript{A1/A1} platelets benefit incrementally by the addition of clopidogrel to ASA, PI\textsubscript{A1/A2} platelets do not. Considering that most patients display the PI\textsubscript{A1/A1} genotype, this mild effect of clopidogrel inhibition also could account for some of the relative clinical superiority of the ASA-plus-clopidogrel combination compared with the use of only ASA. In a study of ADP-stimulated platelet reactivity in patients with stents, Angiolillo et al. (22) reported that a 300-mg clopidogrel loading dose had less of an effect in PI\textsubscript{A1/A2} subjects. One main difference between this study and our report is that we treated subjects for 10 days, during which time steady state clearly is achieved. It would appear that the 300-mg loading dose is not enough to induce rapid inhibition for patients who need it (22). This finding is consistent with our previous statement that PI\textsubscript{A1/A2} individuals treated with 250 mg of ticlopidine had five times the rate of stent thrombosis as that observed with 500 mg of ticlopidine (16).

Since our initial report on the differential sensitivity of platelets to aspirin as a function of the PI\textsuperscript{A} polymorphism (15), it appears that there have been discordant results either substantiating (23) or conflicting our results (24–26). In their initial study, Undas et al. (24) found that PI\textsuperscript{A1/A1} individuals had significantly greater platelet inhibition with ASA than PI\textsuperscript{A1/A2} individuals. They reported that carriers of the PI\textsuperscript{A2} polymorphism appeared to be more resistant to ASA than noncarriers, as measured by bleeding times (25). In this setting, collagen is likely to function as a key agonist for platelets that escape disrupted vessels in the skin, and we have shown in this study that inhibition of collagen-induced aggregation with ASA is more efficient for PI\textsuperscript{A1/A1} patients than for PI\textsuperscript{A1/A2} patients. However, we also show the PI\textsuperscript{A} polymorphism has modifier effects on platelet inhibition by...
ASA that can vary with the agonist (epinephrine vs. collagen). Macchi et al. (26) further substantiate this concept in a study that demonstrated ASA-resistant patients were more likely to be of the PlA1/A1 genotype. In this study, resistance to ASA was determined with the platelet function analyzer (PFA-100), utilizing a collagen/epinephrine-coated cartridge. It is possible that in stimulating platelets in this system, epinephrine may yield the predominant effect. These results may help to understand and reconcile the divergent results reported.

In their interesting study of post-MI patients, Moshfegh et al. (27) reported greater inhibition with clopidogrel and combination of ASA plus clopidogrel compared with ASA alone in ADP-stimulated aggregation in vitro. We were able to confirm their findings with our study of patients with stable CHD. Thus, it is possible that the clinical benefit of clopidogrel, when added to ASA, is mediated by the incremental inhibition of ADP-stimulated platelet response. In the CAPRIE trial, the use of clopidogrel alone was shown superior to ASA, although the difference between the two inhibitors was marginal (10). In the CURE trial, the first primary outcome difference between ASA/placebo versus ASA/clopidogrel combination (20% relative risk reduction) was two to three times greater than the outcome difference between ASA and clopidogrel (CAPRIE: ~9% relative risk reduction) (10,14). Synergy between receptor pathways is plausibly responsible for the additive effect of ASA on platelet inhibition (Tx-receptor pathway) in the presence of ADP as an agonist and clopidogrel as an inhibitor of the purinergic receptor pathway.

**Figure 4.** Collagen-stimulated PlA1/A1 platelets were significantly more inhibited by acetylsalicylic acid (aspirin) than PlA1/A2 platelets (p < 0.0001) as assessed by fluorescein isothiocyanate-fibrinogen binding (mean fluorescence units ± SEM). Multiple comparisons (six included in adjustment per plot) at each concentration of collagen revealed significant differences (p < 0.05) between PlA1/A1 and PlA1/A2 platelets as indicated. *Applies for differences between PlA1/A1 and PlA1/A2.

**Figure 5.** In collagen-stimulated platelets, α-granule release (as measured by CD62P; P-selectin expression) was dependent on PlA genotype, treatment strategy, and agonist concentration (mean fluorescence units ± SEM; p = 0.005).
Our study provides the rationale for additional large pharmacogenetic trials, trials that represent a true opportunity to learn how to target therapeutic strategies to patients whose benefit would be greatest, thereby providing an opportunity to limit side effects and cost for large populations of patients with CHD.

**REFERENCES**

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