Thrombosis Research xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Multi-parameter assessment of platelet inhibition and its stability during aspirin and clopidogrel therapy ☆

A. Anil Timur [a], Gurunathan Murugesan [a], Li Zhang [b], John Barnard [b], Deepak L. Bhatt [c], Kandice Kottke-Marchant [a,*]

[a] Robert J. Tomsich Pathology and Laboratory Medicine Institute, Cleveland Clinic, Cleveland, OH, USA
[b] Quantitative Health Sciences, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA
[c] Brigham and Women's Hospital Heart & Vascular Center, and Harvard Medical School, Boston, MA, USA

ARTICLE INFO

Article history:
Received 21 January 2014
Received in revised form 4 April 2014
Accepted 15 April 2014
Available online xxxx

Keywords:
Aspirin
cardiovascular diseases
clopidogrel
drug resistance
percutaneous coronary intervention
platelet function assay

ABSTRACT

Introduction: Poor response to antiplatelet drugs is associated with adverse outcomes. We assessed platelet inhibition and its stability and tested correlation and agreement between platelet function assays.
Methods: Peripheral blood from 58 patients on both aspirin and clopidogrel who underwent percutaneous coronary intervention (PCI) was collected at hospital discharge (visit-1) and at 30-90 days (visit-2). Platelet function was measured using light transmission aggregometry (LTA-AA and LTA-ADP), Verify*Now*® (Aspirin; ARU and P2Y12; PRU), *ex vivo* TxB_2, urinary 11dhTxB_2, and VASP (PRI) assays. Data were analyzed as continuous, quartiles and binary. Patients were defined as aspirin poor responder (PR) with ARU ≥550, LTA-AA maximum ≥20%, TxB_2 ≥1 ng/mL or 11dhTxB_2 ≥1,500 pg/mg of creatinine and as clopidogrel PR with PRU ≥240, PRU ≥208, LTA-ADP maximum ≥40%, PRI ≥50%, or PRI ≥66%.
Results: Aspirin PR was 3-33% and clopidogrel PR was 10-35% in visit-1. LTA-AA, 11dhTxB_2, and all clopidogrel-response measures showed correlation and agreement between visit-1 and visit-2. The highest agreement between two visits was revealed by PRU ≥240 and PRI ≥66% (PRU-κ = 0.7, 95% CI = 0.47, 0.93; PRI-κ = 0.69, 95% CI = 0.42, 0.95, p-values < 0.001). Comparison of platelet function assays in a single visit (visit-1) revealed a poor correlation between LTA-AA and 11dhTxB_2 assays and no agreement among aspirin-response assays. The highest correlation and agreement were obtained between Verify*Now*® P2Y12 and VASP assays (rho = 0.7, p-value < 0.001 and PRU ≥208-PRI-κ = 0.41-0.42, 95% CI = 0.13, 0.69, p-values < 0.001).
Conclusions: Platelet inhibition is stable during aspirin and clopidogrel treatment. Clopidogrel-response assays correlate and agree with each other better than aspirin-response assays.

Introduction

Patients with stable or acute coronary syndromes and those undergoing percutaneous coronary intervention (PCI) receive pharmacotherapy targeting the inhibition of platelet activation and coagulation cascade [1–4]. The established and commonly used antiplatelet medications include aspirin and the thienopyridine clopidogrel. Aspirin irreversibly inhibits cyclooxygenase-1 (COX-1) and thereby prevents the conversion of arachidonic acid (AA) to thromboxane A_2 (TxA_2), while clopidogrel irreversibly blocks adenosine diphosphate (ADP) receptor P2RY12 [5]. However, platelet inhibition is still inadequate in 5.5-60% of the patients receiving aspirin and in 4-30% of the patients on clopidogrel [6]. High platelet reactivity against both drugs, dual non-responsiveness, was reported in 6% of the patients of whom one-half were not responsive to aspirin and were also non-responsive to clopidogrel [7,8].

Poor drug response has been associated with adverse clinical outcomes. Patients who were poor responders to aspirin were reported to have 4-fold risk of fatal and non-fatal cardiovascular, cerebrovascular, or vascular events compared to aspirin responsive patients [9]. Similarly, clopidogrel poor responders were shown to have 3-5-fold risk of myocardial infarction (MI), stent thrombosis, recurrent ischemic events, and cardiovascular death [10]. Therefore, to assess platelet inhibition and its stability is crucial for evaluation of antiplatelet treatment. On

Abbreviations: (AA), arachidonic acid; (ACE), angiotensin converting enzyme; (ADP), adenosine diphosphate; (ARU), aspirin reaction unit; (CABG), coronary artery bypass graft; (CBC), complete blood count; (COX-1), cyclooxygenase-1; (11dhTxB_2), 11-dehydro thromboxane B_2; (HDL), high density lipoprotein; (κ), kappa statistic; (LDL), low density lipoprotein; (LTA), light transmission aggregometry; (MI), myocardial infarction; (PAD), peripheral arterial disease; (PCI), percutaneous coronary intervention; (PGE1), prostaglandin E1; (PPP), platelet poor plasma; (PPR), persistent poor responder; (PR), poor responder; (PRI), platelet reactivity index; (PRP), platelet rich plasma; (PRU), P2Y12 reaction unit; (SD), standard deviation; (TxA_2), thromboxane A_2; (TxB_2), thromboxane B_2; (VASP), vasodilator stimulated phosphoprotein; (WBC), white blood cell.

☆ The study was partly presented at the XXIV Congress of the International Society on Thrombosis and Haemostasis, Amsterdam, Netherlands on July 4, 2013.

* Corresponding author at: Robert J. Tomsich Pathology and Laboratory Medicine Institute, L21, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA. Tel.: +1 216 444 2484; fax: +1 216 445 9535.
E-mail address: marchak@ccf.org (K. Kottke-Marchant).

http://dx.doi.org/10.1016/j.thromres.2014.04.023
0049-3848/

the other hand, identification of inadequate platelet inhibition is challenging due to the lack of correlation between currently available platelet function assays [11]. In this study, we aimed to assess the variability and durability of platelet inhibition in patients on combined aspirin and clopidogrel therapy over a 30-90-day period following PCI. Our secondary aim was to test the correlation and agreement between various platelet function assays.

Materials and methods

Patient population, study design, and sampling

ASCLOGEN (ASpirin and CLOpidogrel: Genotype vs. Platelet Function Phenotype in Clinical Response) is a prospective observational study which enrolled male and female patients undergoing PCI at the Cleveland Clinic (Cleveland, Ohio). Dual antiplatelet therapy with aspirin and clopidogrel was administered to these patients, with dosages determined by standard clinical practice. All patients originating from Northeast Ohio were sequentially approached to enroll in the substudy to determine the stability of platelet function with time. The first 62 patients that met the geographic requirements and consented were enrolled in the substudy. Exclusion criteria for the ASCLOGEN enrollment included the use of ticlopidine, dipyridamole, steroidal drugs, and COX-2 inhibitors during two weeks prior to enrolment, GPIIb/IIIa inhibitor within 7 days prior to enrolment or during PCI, pregnancy, known history of platelet disorders, thrombocytopenia ($<125 \times 10^3/\mu l$), severe anemia (hemoglobin <10 g/dL and hematocrit <30%), allergy to aspirin or clopidogrel, major surgical procedures in the past week, interruption in aspirin and/or clopidogrel therapy for more than two weeks for any event in the 30-90 days following the catheterization. The study was approved by the Institutional Review Board.

Two visits were arranged for each patient to collect samples. Visit-1 was at patient discharge from the hospital following PCI and visit-2 was at 30-90 days after PCI. Daily dosage of aspirin was 81 mg, 162 mg, and 325 mg in 47%, 5%, and 48% of the patients, respectively. Almost half of the patients were on 75 mg/day clopidogrel. The rest was clopidogrel naive and received a 150-600 mg clopidogrel loading dose before PCI. Twenty patients also received 150-325 mg periprocedural clopidogrel. At visit-2, 53% of the patients were found switched from the initial dose of aspirin to one of three doses administered. All patients but two were taking 75 mg clopidogrel daily; two patients were taking 150 mg/day clopidogrel.

For each visit, peripheral venous blood was drawn into 1.8 ml and 3.15 ml vacutainer tubes containing 3.2% trisodium citrate and processed to measure platelet function within two hours of sampling. A urine sample >20 ml was also collected from each patient and stored at -80 ºC. Complete blood count (CBC) was carried on Beckman Coulter A^C •T diff™ Analyzer (Beckman Coulter, Miami, Florida).

Table 1
Characteristics of the study cohort.

Characteristic	n (%)[1] or Mean ± SD
Male	47 (81)
Female	11 (19)
Age	63 ± 9
Current Smoking	8 (14)
Past Smoking	37 (64)
Hypertension	43 (74)
Diabetes mellitus	15 (26)
Peripheral artery disease (PAD)	7 (12)
Myocardial infarction (MI)	25 (43)
Stroke	2 (3)
Previous percutaneous coronary intervention (PCI)	27 (47)
Previous coronary artery bypass graft (CABG)	17 (29)
Total cholesterol (mg/dL)	160 ± 46
HDL (mg/dL)	42 ± 13
LDL (mg/dL)	90 ± 40
Medications	
Aspirin	58 (100)
Clopidogrel	58 (100)
Statins	53 (91)
Thrombin inhibitors	48 (83)
β-blocker	47 (81)
ACE inhibitors	26 (46)
Proton pump inhibitors	19 (35)
Angiotensin receptor blockers	6 (10)

HDL = high density lipoprotein, LDL = low density lipoprotein, ACE = angiotensin converting enzyme, SD = standard deviation.
[1] Total number of subjects may vary depending on the availability of data.

Platelet function assays

VerifyNow® assay

Verify*Now*® assay (Accumetrics, Inc., San Diego, California) measures platelet-induced aggregation in whole blood. The assay cartridges Verify*Now*® Aspirin and Verify*Now*® P2Y12 use AA and ADP as the platelet agonists, respectively, and contain a lyophilized preparation of human fibrinogen-coated beads. Two 1.8 ml-citrated whole blood samples were used for the assay. The instrument measures light transmittance as aggregation occurs and data are converted into Aspirin Reaction Unit (ARU) or P2Y12 Reaction Unit (PRU).

Light transmission aggregometry (LTA)

Platelet rich plasma (PRP) was prepared by centrifuging the citrated whole blood at 180 g for 15 minutes at room temperature. The platelet count was adjusted to 200-300 $\times 10^3/\mu l$ with platelet poor plasma (PPP) obtained by centrifuging the remaining blood at 2,880 g for 15 minutes. Platelet aggregation was stimulated with 0.5 mg/mL (1.6 mmol/L) AA and 10 μM ADP and measured for 10 minutes using BioData PAP-4 platelet aggregometer (BioData Corp., Horsham, Pennsylvania). A 50 μl of Indomethacin/EDTA solution (1:20 mixture of 0.04 M Indomethacin and 0.05 M EDTA) was then added to prevent further platelet aggregation and samples were centrifuged at 2,880 g for 15 minutes. Obtained supernatants were kept frozen at -80 ºC for TxB_2 assay. Aggregation was expressed as the maximal per cent change in light transmittance from baseline which was set with PPP. Two measures of aggregation, maximum and late aggregation at 10 minutes, were analyzed.

Thromboxane B_2 (TxB_2) assay

TxB_2, a stable metabolite of thromboxane A_2, was measured in plasma following *ex vivo* activation of platelets for LTA assay. The supernatants of platelet aggregates were first purified using Bond Elute C18 column (96-well plate, Varian, Inc., Walnut Creek, California). TxB_2 in these purified samples was then measured using TxB_2 ELISA Kit (Enzo® Life Sciences, Inc., Plymouth Meeting, Pennsylvania) according to the manufacturer's protocol.

Urinary 11-dehydro thromboxane B_2 ($11dhTxB_2$) assay

Urinary $11dhTxB_2$ levels were measured using AspirinWorks® Test Kit (Corgenix, Inc., Broomfield, Colorado), normalized with urine creatinine level of the same sample and reported as pg/mg of creatinine.

Vasodilator stimulated phosphoprotein (VASP) phosphorylation assay

Phosphorylation of VASP was assessed by flow cytometry using PLT VASP/P2Y12 kit (Biocytex, Marseille, France). Briefly, citrated blood was incubated with phosphorylation activator prostaglandin E1 (PGE1) alone or PGE1 and phosphorylation inhibitor ADP simultaneously. Phosphorylated VASP was then labeled fluorescently using specific primary and secondary antibodies and analyzed according to the manufacturer's protocol. Results were converted to platelet reactivity index (PRI; %) which is calculated using the corrected mean fluorescence intensities (MFIc) and the formula PRI = $[(MFIc_{PGE1}-MFIc_{PGE1\ +\ ADP})/MFIc_{PGE1}] \times 100$. Negative values were imputed to zero.

Please cite this article as: Timur AA, et al, Multi-parameter assessment of platelet inhibition and its stability during aspirin and clopidogrel therapy, Thromb Res (2014), http://dx.doi.org/10.1016/j.thromres.2014.04.023