A Thrombelastograph whole blood assay for clinical monitoring of NSAID-insensitive transcellular platelet activation by arachidonic acid

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Optical platelet aggregation (OPA) with platelet-rich plasma (PRP) was compared with a Thrombelastograph (TEG) whole blood assay for monitoring arachidonic acid (AA)-induced platelet activation. Assays were performed on 47 interventional cardiology and 24 general surgery patients receiving aspirin therapy for cardiovascular disease, as well as 48 volunteers asked to take nonsteroidal anti-inflammatory drugs (NSAIDs) or 12 volunteers on chronic NSAID therapy unrelated to diagnosed cardiovascular disease. Whole blood TEG monitoring of NSAID inhibition detected NSAID-insensitive AA activation of platelets in a significantly higher number of cardiology (23%) and surgery (25%) patients and normal volunteers on chronic NSAID (25%) therapy relative to normal subjects not on chronic NSAID therapy (0%). Whole blood NSAID insensitivity was observed with cyclooxygenase-I inhibitors, such as aspirin and ibuprofen; was not affected by Celebrex, a cyclooxygenase-II inhibitor; but was completely inhibited by thromboxane-receptor antagonists. This was not due to platelet NSAID insensitivity, because complete inhibition of AA-activation responses in PRP was observed with either TEG or OPA assays. We confirmed that thromboxane B_2 formation in PRP from NSAID-insensitive subjects was completely inhibited by NSAIDs. However, significant amounts were formed in whole blood from NSAID-insensitive subjects, but not in whole blood from NSAID-sensitive subjects. Thromboxane formation after AA addition was not found in washed blood cells with 90% reduced platelet counts or in leukocyte-rich buffy coat fractions, but could be restored by addition of PRP. NSAID-insensitive activation was inhibited by nordihydroguaiaretic acid, with an IC_{50} of 30 μmol, implicating 12- and/or 15-lipoxygenases in this transcellular pathway. (J Lab Clin Med 2005;146: 30–35)

Abbreviations: AA = arachidonic acid; ADP = adenosine diphosphate; KH = kaolin and heparinase; MA = maximum amplitude; NSAID = nonsteroidal anti-inflammatory drug; NDGA = nordihydroguaiaretic acid; OPA = optical platelet aggregation; PRP = platelet-rich plasma; PPP = platelet-poor plasma; SD = standard deviation; TEG = Thrombelastograph®; TxB_2 = thromboxane B_2

Several platelet-independent, transcellular pathways for nonsteroidal anti-inflammatory drug (NSAID)-insensitive platelet activation by arachidonic acid (AA) have been suggested.[1,2] In 1 study, 40% of patients undergoing coronary artery bypass did not have prolonged bleeding times in response to aspirin therapy.[3] This was associated with 12-hydroxyeicosatetraenoic acid

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synthesis and increased platelet adhesion, possibly through a transcellular aspirin-insensitive 12- and/or 15-lipoxygenase pathway.[4] Aspirin-resistant conversion of AA into platelet-activating thromboxane has also been linked to a monocyte prostaglandin H synthase insensitive to cyclooxygenase inhibitors and inducible by inflammatory stimuli.[5,6] Transcellular activation by platelet–leukocyte cross-talk in whole blood with physiological calcium has also been suggested.[7]

We have recently tested the Thrombelastograph (TEG) heparin-anticoagulated whole blood assay (TEG Haemostasis Analyzer 5000; Haemoscope, Niles, Ill), which measures platelet interaction with a fibrin network analogous to platelet aggregation under physiological calcium conditions.[8] This TEG assay correlates with optical platelet aggregation (OPA). Similar to OPA, the TEG assay is sensitive to both clopidogrel adenosine diphosphate (ADP) inhibition and NSAID inhibition of AA-mediated platelet activation.

In this study, we correlated and compared the TEG whole blood assays to OPA for the detection of NSAID inhibition in 2 different patient populations, as well as in normal volunteers. In addition, we report on the use of TEG to distinguish NSAID-insensitive from NSAID-sensitive transcellular platelet activation in whole blood.

METHODS

Materials. AA and ADP were obtained from Chrono-Log Corporation (Havertown, Penn) and made up and stored as concentrated stock solutions in accordance with the manufacturer's instructions. Specific thromboxane A_2 receptor (TxA_2-R) antagonists, SQ29548 and ICI 192605, were obtained from BIOMOL Research Laboratories (Plymouth Meeting, Penn). Nordihydroguaiaretic acid (NDGA) was obtained from Oxford Biomedical Research (Oxford, Miss). Stock 1000-fold concentrated solutions were composed in ethanol.

Human subjects. Our Institutional Review Board reviewed the protocol used to obtain informed consent from 47 candidates for interventional cardiology and 24 elective general surgery patients on aspirin therapy for cardiovascular disease. Patients were excluded from the study if they had thrombocytopenia, diagnosed hemophilia or chronic bleeding problems (eg, peptic ulcer), or decreased liver function. For studies on the affect of NSAIDs unrelated to diagnosed cardiovascular disease, 60 normal volunteers were recruited, 12 already taking a continuous regimen of NSAIDs at various dosages and types and 48 taking a 325-mg uncoated aspirin dose and/or standard doses of other cyclooxygenase I or II inhibitors for a period equivalent to peak pharmacokinetic effect (1–2 hours with uncoated aspirin or ibuprofen, 3–5 hours with Celebrex) before giving a blood sample.

Thrombelastograph assays. Blood was drawn into Vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ), anticoagulated with 14.7 U/mL heparin, and assayed within 2 hours for platelet functional responses to 1 mmol AA. The platelet response, which is analogous to OPA-measured aggregation, was used as an assay for NSAID inhibition as described previously.[8] The TEG assay with heparin-anticoagulated blood was used to determine the maximum amplitude (MA) of clot shear elasticity. The MA is proportionate to the extent of platelet activation elicited by 1 mmol AA and represents the interaction of the activated platelet glycoprotein IIb/IIIa complex with a fibrin network formed by a reptilase–Factor XIIIa mixture (provided by Haemoscope). Responses were compared with the maximal MA of the same blood clotted by thrombin with the addition of heparinase and kaolin (MA_{KH}), in accordance with the manufacturer's instructions. A percent MA response (TEG %MA) was calculated by subtracting the MA without platelet activator (MA_0) from the MA with AA (MA_{AA}), dividing by MA_{KH} minus MA_0, and then multiplying the result by 100%. This %MA value has been shown to correlate well with percent aggregation as determined by OPA.[8]

Optical platelet aggregometry. Heparin-anticoagulated blood was also used to isolate platelet-rich plasma (PRP) and platelet-poor plasma (PPP) by differential centrifugation for assay of OPA using a Chrono-Log Aggregometer with AggroLink software (Chrono-Log). OPA assays of maximum aggregation for 10 minutes after the addition of 1 mmol AA and calculation of percent aggregation were carried out as described previously.[9] OPA was assayed without platelet count adjustment.

Cell fractionation. Further cell fractionation was carried out after removing the PRP layer, as described earlier. For some experiments, the leukocyte-rich buffy coat was also collected separately. The blood cell fractions were diluted 1:1 with a balanced salt solution (Normosol; Abbott Laboratories, North Chicago, Ill) containing 6 U/mL heparin. The samples were centrifuged at $100 \times g$ for 20 minutes at room temperature, and the top layer containing platelets was discarded. This procedure was repeated twice more, until platelet counts were reduced to $<10\%$ of the original whole blood sample. The top of the red blood cell fraction minus the buffy coat was also removed, to further reduce white blood cell count to $<15\%$ of the original whole blood counts. Blood cell counts in various fractions were determined on an Ichor/Plateletworks analyzer (Helena Laboratories, Beaumont, Tex).

Thromboxane B_2 (TxB_2) assays. TEG samples with reaction conditions as described in Results were run in quadruplicate. After MA values were obtained, the samples were collected into Microfuge tubes (Beckman Coulter, Fullerton, Calif). The samples were centrifuged at $10{,}000 \times g$ for 5 minutes at room temperature, and the sera was collected and frozen at $-70°C$. TxB_2 concentrations in serum samples were assayed with an enzyme immunoassay kit (Oxford Biomedical Research) following the manufacturer's directions.

Statistics. Paired *t*-tests were used to establish significant changes from individuals' baseline platelet function. The significance of group differences was tested using unpaired *t*-tests for parametric data, Mann-Whitney *U*-tests and Kruskal-Wallis tests for nonparametric data, and χ^2 tests for categorical data. Statistical tests were done with StatView 4.0 software (SAS Institute, Cary, NC).

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