Evaluation of Xa/Va activity bound to platelet-rich thrombi in patients with acute coronary syndromes

Luca Olivotti, Giorgio Ghigliotti, Paolo Spallarossa, Pierfranco Rossettin, Rossella Ausilio, Anna Toso, Antonio Barsotti, Claudio Brunelli
Laboratory of Cardiovascular Biology, Department of Internal Medicine, University of Genoa, Genoa, Italy

© 2002 CEPI Srl

Address:
Dr. Luca Olivotti
Laboratorio di Biologia Cardiovascolare
Dipartimento di Medicina Interna
Università degli Studi
Viale Benedetto XV, 6
16132 Genova
E-mail: lucaolivotti@cardio.dimi.unige.it

Introduction
Coronary thrombosis and activation of the coagulation system are the underlying mechanisms in the transition from stable to unstable coronary disease. The presence of a platelet-rich clot inside a major coronary artery is itself a persistent procoagulant stimulus exposed to blood. It has been shown that platelet-rich thrombi reconstructed in vitro from blood or plasma of normal donors have a potent procoagulant activity, which is mainly dependent on the presence of the coagulation complex Xa/Va (formed by activated factor X and V) on the surface of activated platelets. In animal models of arterial injury, the procoagulant activity of Xa/Va complex remains high long after injury, and is resistant to antithrombin III-dependent anticoagulants. Similarly, in patients with acute coronary syndromes, persistent minimal generation of thrombin can be observed for 6 months after the acute event. The present study was undertaken to measure the Xa/Va activity of platelet-rich thrombi reconstructed in vitro from the plasma of patients with acute coronary syndromes, of patients with stable coronary artery disease and of healthy control subjects.

Methods
Population of the study. The study population included 22 consecutive patients with acute coronary syndromes (9 with unstable angina and 13 with non-Q myocardial infarction), 20 age-matched patients with stable coronary artery disease, and 11 age-matched control subjects. Unstable angina was defined as new onset or worsening angina occurring at rest or with minimal exertion, lasting at least 15 min and occurring within 24 hours of hospital admission. Non-Q-wave myocardial infarction was defined by angina together with elevated (> 2 times the upper normal limit) serum levels for creatine kinase-MB fraction and troponin T. Stable coronary disease was defined as the presence of critical coronary lesions at angiography, and stable clinical status in the last 6 months. Control subjects had atypical chest pain and absence of left ventricular wall motion abnormalities, exercise perfusion scan defects, and coronary lesions at angiography.

Blood sampling protocol. All subjects were on aspirin, while none was being administered 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or other lipid-lowering agents. In patients with acute coronary syndromes, blood was sampled at admission, before starting heparin treatment.

Blood samples were obtained without venous stasis using a 21G needle. The first 10 ml of blood were used to measure blood lipid levels. Blood for F1+2 determination was collected in pre-cooled plastic tubes containing a mixture of D-Phe-L-Pro-L-Arg-chloromethylketone (PPACK, Calbiochem, La Jolla, CA, USA), EDTA, and aprotinin. Blood for the other coagulation studies was collected in siliconized glass tubes containing 0.105 M buffered sodium citrate (Becton Dickinson, Franklin Lakes, NJ, USA).
Assessment of platelet-dependent Xa/Va activity in vitro. Platelet clots were prepared from the citrated blood of patients by centrifugation for 15 min at 700 rpm at room temperature to obtain platelet-rich plasma. Platelet count was determined by manual counting with a Burke chamber, and was then normalized to 200 000 platelets/mm³ after dilution with platelet-free plasma, obtained by centrifuging some of the platelet-rich plasma at 3000 rpm for 5 min. Platelet-rich plasma clotting was then started with 5 nM human alpha-thrombin and with 25 mM CaCl₂. Clots were allowed to develop for 1 hour at 37°C, were recovered on steel wires, retracted, and then washed in three 5-ml aliquots of 0.01 M HEPES, 0.15 M NaCl, 0.002 M CaCl₂, pH 7.6 (HEPES saline). The weight of each subject’s clot was recorded at the end of the experiments. It was previously shown that this washing procedure removes procoagulants that are not tightly bound to the thrombus. Clot-associated Xa/Va activity was characterized by incubating these washed clots in 500 µl of HEPES saline containing 0.9 µM purified human prothrombin at 37°C. Aliquots of 90 µl were removed at 0, 5, 10, 20 and 25 min, and added to the same buffer containing 200 µM S-2238, while the hydrolysis of the substrate was monitored at 405 nM by a kinetic plate reader (BioRad, Hercules, CA, USA). Prothrombin activation was characterized by comparing S-2238 hydrolysis rates in the samples to known concentrations of purified human alpha-thrombin. Specificity of this assay for measuring clot-dependent Xa/Va-activity was confirmed in control experiments in which prothrombin was excluded from the HEPES saline buffer, and no S-2238 hydrolysis was induced by the supernatant of clots.

Assessment of in vivo thrombin generation. To measure in vivo thrombin generation, plasma levels of prothrombin fragment F1+2 were measured in duplicate by a commercially available ELISA kit (Enzygnost F1+2 Micro, Behring AG, Marburg, Germany) using blood samples collected in pre-cooled plastic tubes containing a mixture of PPACK, EDTA and aprotinin.

Ex vivo platelet aggregation assays and fibrinogen determination. Ex vivo platelet aggregation assays were performed in platelet-rich plasma adjusted to 200 000 platelets/mm³ obtained as described above. Platelet aggregation was performed according to the Born turbidimetric technique using a DIC PA-3220 Aggregrometer (Kyoto Daiichi Kagatu Co., Kyoto, Japan). Platelet-rich plasma was aggregated at 37°C under continuous stirring with increasing concentrations of collagen (1, 5, and 10 mg/ml) and of ADP (1, 2.5, and 5 nmol/l). Maximal aggregation, recorded for 6 min after adding the agonist, was defined as the ratio of maximal difference between platelet-rich and platelet-poor plasma curves of aggregation.

Statistical analysis. Data are expressed as mean ± SD. Statistical comparisons were made by the χ² test for categorical variables and by the Mann-Whitney U test for continuous variables. A p value of < 0.05 was used to define a statistically significant difference.

Results

The three groups were similar in terms of age, sex, prevalence of hypertension, diabetes, current smoking status, and body mass index (Table I). Serum lipids, ex vivo platelet aggregation, and thrombus weight were al-

Table I. Clinical characteristics of the study patients.

<table>
<thead>
<tr>
<th></th>
<th>Acute coronary syndromes (n = 22)</th>
<th>Stable coronary artery disease (n = 20)</th>
<th>Control subjects (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>70 ± 3</td>
<td>67 ± 2</td>
<td>64 ± 3</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>55</td>
<td>65</td>
<td>55</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>32</td>
<td>20</td>
<td>45</td>
</tr>
<tr>
<td>Hypertensives (%)</td>
<td>68</td>
<td>75</td>
<td>63</td>
</tr>
<tr>
<td>Diabetics (%)</td>
<td>14</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.8 ± 0.7</td>
<td>25.8 ± 0.7</td>
<td>26.3 ± 1.6</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>205 ± 9</td>
<td>212 ± 8</td>
<td>189 ± 15</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>43 ± 2</td>
<td>49 ± 3</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>152 ± 9</td>
<td>156 ± 23</td>
<td>139 ± 25</td>
</tr>
<tr>
<td>Platelet aggregation (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen 5 µg/ml</td>
<td>46 ± 5</td>
<td>40 ± 5</td>
<td>42 ± 8</td>
</tr>
<tr>
<td>ADP 2.5 mmol/l</td>
<td>48 ± 5</td>
<td>45 ± 7</td>
<td>46 ± 9</td>
</tr>
<tr>
<td>Thrombus weight (mg)</td>
<td>16.3 ± 1.4</td>
<td>13.1 ± 0.9</td>
<td>13.3 ± 1.7</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>391 ± 14                        *</td>
<td>347 ± 14</td>
<td>338 ± 17</td>
</tr>
<tr>
<td>White blood cell count (/mm³)</td>
<td>7832 ± 608§§</td>
<td>6110 ± 362</td>
<td>6488 ± 410</td>
</tr>
</tbody>
</table>

* = p < 0.05 vs controls; † = p < 0.05 vs stable coronary artery disease.
so similar among the three groups (Table I). Fibrinogen levels and white blood cell counts were significantly higher in patients with acute coronary syndromes than in stable coronary artery disease patients and in control subjects.

Patients with acute coronary syndromes showed significantly higher levels of \textit{in vitro} thrombus-associated Xa/Va activity than controls, and higher levels of \textit{in vivo} thrombin generation (F1+2) than both patients with stable coronary artery disease and control subjects (Fig. 1).

The experimental setting for the measurement of clot-associated Xa/Va activity (platelet-rich clot + buffer + prothrombin) was compared with three incomplete conditions, that were buffer + prothrombin (no clot), buffer + clot (no prothrombin), and buffer + prothrombin + platelet-free clot (no platelets). These incomplete conditions were tested on plasma from 5 different healthy donors, and the results confirmed that only the simultaneous presence of platelet-rich clot + prothrombin allowed significant generation of thrombin (Fig. 2).

Discussion

Our study shows that the quantification of Xa/Va activity of platelet-rich clots reconstructed \textit{in vitro} from the plasma of patients is a feasible and specific technique. Clot-associated Xa/Va activity and plasma levels of F1+2 are increased in patients with acute coronary syndromes, despite treatment with a platelet inhibitor like aspirin. In addition to F1+2, clot-associated Xa/Va activity can be used as a measure to test the efficacy of antithrombotic/anti-ischemic treatments given to patients with acute coronary syndromes.

References