Platelets Secrete a Coagulation Inhibitor Functionally and Antigenically Similar to the Lipoprotein Associated Coagulation Inhibitor

By William F. Novotny, Thomas J. Girard, Joseph P. Miletich, and George J. Broze, Jr.

Stimulation with thrombin or the calcium ionophore, A23187 caused human platelets to release a coagulation inhibitor similar to the Lipoprotein Associated Coagulation Inhibitor (LACI). This was documented functionally, with clotting assays measuring tissue factor inhibition and factor Xa inhibition, as well as immunologically, in a competitive immunoassay. The total amount of LACI released by 3 x 10^9 platelets after two hours stimulation was 7% to 8% of the amount found in 1 ml of serum. Half of the LACI was released by five minutes. The LACI was present in the platelet supernatant and was not associated with the platelet membrane or shed vesicles. The tissue factor and factor Xa inhibitory activities that were released were neutralized by preincubating the platelet supernatants with specific rabbit polyclonal anti-LACI IgG. On Western blot, platelet LACI appeared to run as a doublet with a molecular weight (mol wt) 45,000 to 47,000. Blood samples obtained from the site of a wound (template bleeding time) demonstrated a progressive increase in LACI concentration. A cDNA probe, derived from endothelial cell LACI cDNA, hybridized selectively to 4.0 and 1.4 kb transcripts in a preparation of platelet mRNA.

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Materials and Methods

Materials. Factor X deficient plasma and pooled human plasma were obtained from George King Biomedical (Overland Park, KA). SDS and pretained molecular weight (mol wt) standards were received from BioRad (Richmond, CA). Bovine serum albumin (BSA), fraction V was obtained from Miles Scientific (Naperville, IL). Aplexon A oil was obtained from Aplexon Products, London, England. Platelet factor 4 measurements were performed by radioimmunoassay using a kit from Abbott (Chicago). Lactic dehydrogenase (LDH) measurements were performed using a kit from Sigma (St Louis) according to the method of Wrableseski and LaDue. All other reagents were from Sigma or Fisher.

Methods. Human factor IIa, factor VIIa, antithrombin III, and a crude preparation of tissue factor were prepared as previously described. Protein S was separated from other vitamin K dependent clotting factors by chromatography on blue agarose. After barium citrate precipitation of human plasma and DEAE-Sephase chromatography, the leading edge of the prothrombin peak was loaded onto a blue agarose column. Under the conditions used (100 mmol/L NaCl and 20 mmol/L Na citrate, pH 6.0) prothrombin, factor X and Protein C were retained on the column while protein S passed through. Bovine factor X was purified, labeled with 125I, and activated as previously described and LACI was purified from conditioned, serum free medium of cultured HepG2 cells. LACI (HepG2) concentrations were determined from the absorbance of 280 nm using an extinction coefficient of 5.3 as estimated from its amino acid composition.

Labeling LACI with Fluorescein Isothiocyanate. 500 µL of LACI (HepG2) (152 µg/mL in .1 mol/L MOPS, pH 7.5/0.1% β-octylglucoside) was added to 125 µL of FITC (1 mg/mL in .5 mol/L sodium carbonate, pH 9.0) and the mixture rocked at room temperature for two hours. The FITC-LACI was separated from free FITC by chromatography on Sephadex G-25, and stored in .02 mol/L MOPS, pH 7.5/0.1% β-octylglucoside at 4°C.

Polyclonal anti-LACI antibodies. The anti-LACI rabbit polyclonal IgG and anti-LACI N-terminal peptide (residues three to 26) IgG were obtained as previously described. The anti-LACI C-terminal peptide was obtained as follows: A 23 amino acid peptide (residues 253 to 276) based on the predicted C-terminal sequence of LACI was synthesized and used to immunize a New Zealand white

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rabbit using standard techniques. The IgG was purified by chromatography on Protein A sepharose and the anti-peptide IgG was isolated by affinity chromatography on a column of the C-terminal peptide linked to Affigel-15 (Biorad).

Isolation of platelets. The platelets were isolated by differential centrifugation from healthy donors who had not taken aspirin for over 10 days. Before use, the platelets were pelleted by centrifugation at 2,000 x g for ten minutes and resuspended in the reaction buffer (0.1 mol/L NaCl, 0.05 mol/L tris-HCl, pH 7.4, and glucose 5.5 mmol/L) at a concentration of 10^7 platelets per milliliter.

Stimulation of platelets. 490 microliter- aliquots of platelets were pipetted into 1.5 mL polypropylene tubes and stimulated by adding 10 μL of thrombin at 50 U/mL (final concentration 1 U/mL) or 10 μL of A23187 at 50 μmol/L (final concentration 1 μmol/L) followed by mixing. All incubations were at room temperature.

Isolation of platelets. The platelets were isolated by differential centrifugation through a water insoluble, low viscosity oil mixture with a density between that of platelets and the reaction buffer. The platelet pellet was cut out of the bottom of the tube with a scalpel and the platelets resuspended in 500 μL reaction buffer. In some experiments the resuspended platelets were frozen and thawed before being assayed.

Tissue factor inhibition assay. A three stage assay was used according to previous descriptions. In the first stage, the sample to be tested was incubated with tissue factor, factor Vila, factor X, and calcium. After a half hour incubation, the sample was diluted 100-fold and added to a mixture of TBSA and CaCl2 in a fibrometer cup at 37°C. Factor X was added to the mixture and following a one-minute incubation, a mixture of factor X deficient plasma and rabbit brain cephalin was added and the time to clot formation was measured with a fibrometer (Becton-Dickinson, Cockeysville, MD).

Factor Xa inhibition assay. A two-stage assay was used. In a fibrometer cup at 37°C, 60 μL of the sample to be tested was added to 60 μL of TBSA (14 mol/L NaCl/50 mol/L tris base/pH 7.4/BSA 1%) with 1 mmol/L EDTA and 60 μL of bovine Xa at 25 ng/mL (diluted in TBSA). After five minutes incubation, 60 μL of 25 mmol/L CaCl2 and 60 μL of a mixture containing ten parts factor X deficient plasma and one part rabbit brain cephalin were added and the time to clot formation was measured with a fibrometer.

Substituting for samples, TBSA served as controls in both clotting assays. The concentration of tissue factor in the tissue factor inhibitor assay (TFI assay) was chosen to produce control clotting times in the range of 35 to 40 seconds. Standard curves were constructed by plotting the prolongation of clotting times over control v the concentration of LACI. Sources of LACI used for the standard curves were purified LACI (HepG2), serum from the platelet donors, and the platelet supernatants. All three sources gave parallel standard curves in both assays.

Immunologic assay for LACI. A competitive immunoassay was constructed where an anti-LACI rabbit serum was used to bind LACI, anti-rabbit IgG linked to latex beads was used to capture the rabbit IgG, and FITC-LACI was added to quantitate the number of free anti-LACI IgG sites remaining. To a well in a 96-well assay plate (Pandex Laboratories, Inc, Mundelein, IL) was added 25 μL of the sample to be tested and 20 μL of anti-LACI rabbit serum (diluted 1:350 in TBSA). After one hour incubation at room temperature, 20 μL of goat anti-rabbit IgG linked to latex beads (Pandex) (resuspended at 5% w/v) was added. After 15 minutes additional incubation at room temperature, 20 μL of FITC-LACI (5 μg/mL in TBSA) was added and six minutes later a vacuum was applied to the assay plate separating the solution from the latex beads. The beads were washed twice for two minutes with TSB (50 mmol/L tris base, pH 7.4/14 mol/L NaCl) and the amount of bound fluorescence was quantitated with a Screen Machine (Pandex). Standard curves constructed using pure LACI (HepG2) and human plasma or serum were parallel.

Protein S assay. A two site monoclonal antibody (MoAb) assay, similar to those previously described for protein C24 and protein Z,25 was used to measure protein S antigen (full details will be published elsewhere). The two MoAbs used are non-cross reactive, not calcium dependent, recognized protein S bound to C4B binding protein and free protein S, and recognized both thrombin treated and native protein S. Standard curves constructed using purified protein S and pooled human plasma were parallel.

Western blots. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 1 mmol/L thick slab gels using a 15% separating gel and the buffer of Laemml. Electrophoretic transfer to nitrocellulose paper was performed in the buffer system of Towbin using a Mini-Protein II apparatus (Biorad) at 100 V for one hour. The nitrocellulose paper was then blocked with 2.5% nonfat dry milk in TS for one hour at room temperature and incubated overnight with either 121-hXa (at 5 x 10^4 CPM/50 mL TBSA) or the various anti-LACI IgGs (diluted in TBSA). The anti-LACI polyclonal IgG was diluted to 25 μg/mL, the anti-LACI N, and C-terminal peptide IgGs were diluted to 2.5 μg/mL. The paper was then washed three times with 2.5% nonfat dry milk in TS for five minutes. The 121-hXa blot was air dried and exposed to Kodak XAR-5 film (Kodak, Rochester, NY). The immunologic blots were incubated with a goat anti-rabbit IgG linked to alkaline phosphatase (diluted 1:2,000 in TBSA) at room temperature for one hour. After washing the paper again three times with 2.5% nonfat dry milk in TS for five minutes, the color was developed by incubating the paper in 50 mL of buffer (100 mmol/L tris-HCl, pH 9.5/100 mmol/L MgCl2) to which had been added 5 mg of nitro blue tetrazolium and 2.5 mg of 5-bromo-4-chloro-3 indolyl phosphate.

Bleeding time samples. Bleeding time blood samples were obtained from healthy volunteers using the method described by Weiss et al26 with the following modification: the heparinized microhematocrit tubes were spun in a hematocrit centrifuge, the tubes were nicked at the plasma-white cell interface with an ampule file, the tubes were broken in half, and the plasma removed for assay. Plasma LACI concentration was measured by immunoassay. For the samples used for platelet factor 4 measurements, the tubes were prewashed with a platelet inhibitor solution included in the platelet factor 4 kit by the manufacturer, Abbott (contains EDTA, 2-chloroadenosine, procaine, and heparin).

Northern blots. Platelet and neutrophil RNAs were a gift from Dr G. Roth.30 HepG2 RNA was prepared from cultured cells as previously described. Thirty micrograms of platelets and HepG2 RNA were electrophoresed in a 0.8% agarose gel containing 2% formaldehyde and transferred to nitrocellulose. The RNA blot was hybridized with a 32P-labeled XEci cDNA insert that contained the entire LACI coding sequence. The blot was washed with a final stringency of 0.1% SSC, 0.1% SDS, at 68°C.

RESULTS

Tissue factor inhibition. Incubation of platelets at 10^9/mL with either 1 U/mL thrombin or 1 μmol/L of the calcium ionophore, A23187 led to the release of tissue factor inhibitory (TFI) activity with time (Fig 1). Approximately 50% of the TFI activity present at two hours after stimulation was released at five minutes. The total TFI activity released after two hours of stimulation with thrombin corresponded to a LACI concentration of 10 ng/10^9 platelets and assuming a normal blood platelet concentration of 3 x 10^12/mL, this would represent 3% of the serum LACI concentra-

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Assuming normal platelet concentration of 3 x 10^8/mL.

**Table 1.** Comparison of LACI Assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>LACI/10^9 Platelets (ng)</th>
<th>LACI/ml Serum* (ng)</th>
<th>Platelet LACI/ Serum LACI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue factor inhibitor assay</td>
<td>10</td>
<td>100</td>
<td>3.0</td>
</tr>
<tr>
<td>Xa inhibitor assay</td>
<td>30</td>
<td>115</td>
<td>7.8</td>
</tr>
<tr>
<td>Immunoassay</td>
<td>28</td>
<td>118</td>
<td>7.1</td>
</tr>
</tbody>
</table>

*Platelet donor serum.
†Assuming normal platelet concentration of 3 x 10^8/mL.
Table 2. Tissue Factor Inhibition Assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time to Clot (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBSA</td>
<td>35.5</td>
</tr>
<tr>
<td>Platelets, unstimulated*</td>
<td>36.5</td>
</tr>
<tr>
<td>Platelets, stimulated with thrombin, EDTA washed</td>
<td>36.7</td>
</tr>
<tr>
<td>Platelets, stimulated with thrombin 2 h</td>
<td>64.0</td>
</tr>
<tr>
<td>Platelets, F/T</td>
<td>59.0</td>
</tr>
<tr>
<td>Platelets, F/T, excluding factor X in first stage of assay</td>
<td>35.5</td>
</tr>
<tr>
<td>Platelets, F/T, excluding Ca²⁺ in first stage of assay</td>
<td>42.3</td>
</tr>
<tr>
<td>Platelets, F/T, preincubated with rabbit α LACI IgG</td>
<td>34.1</td>
</tr>
<tr>
<td>Serum diluted 1:10 with TBSA</td>
<td>62.2</td>
</tr>
<tr>
<td>Serum diluted 1:10, preincubated with α LACI rabbit IgG</td>
<td>39.3</td>
</tr>
</tbody>
</table>

Abbreviation: F/T, frozen/thawed.

*Platelet concentration 10⁹/mL.

Western blot. Western blot analysis, using ¹²⁵I-Xa as a probe, showed platelet LACI to run as a doublet with an estimated mol wt of 45,000 to 47,000 (Fig 4) and to run at a slightly higher mol wt than purified LACI (HepG2). The same result was found when anti-LACI N-terminal peptide IgG (Fig 4), anti-LACI C-terminal peptide IgG and anti-LACI polyclonal IgG (not shown) were used as probes for LACI.

Platelet LACI mRNA: A 1.3 kb fragment of endothelial cell LACI cDNA, which contained the entire protein coding region, was radiolabeled by nick-translation and used to identify LACI message. Initial dot blot experiments showed that the probe hybridized with platelet, but not neutrophil mRNA. Northern blot analysis of platelet mRNA revealed hybridization to two RNAs (a discrete band at 4.0 kb and a broad band at 1.4 kb) which are the same sizes as LACI mRNA in HepG2 cells (Fig 5).

Plasma LACI levels in bleeding time samples. To determine the possible contribution of platelet LACI to total plasma LACI concentrations at the site of a wound, the template bleeding time model of Weiss et al was used.²⁸ Average sequential values (± SEM) for blood volumes and plasma LACI concentrations obtained from normal volunteers are shown in Fig 6 along with a representative curve for platelet factor 4. LACI levels in the plasma escaping from the incision site increased two- to threefold from an average baseline concentration of 120 ng/mL.

Discussion

Our results demonstrate that a coagulation inhibitor functionally and antigenically similar to LACI is released by stimulated or lysed platelets. The amount of LACI released excludes plasma contamination or loose adsorption of LACI to the platelet membrane as explanations for the LACI present in platelet supernatants. Rather, LACI appears to be specifically stored and released by platelets.

The total amount of LACI released by 3 x 10⁸ platelets is about 7% to 8% of the amount found in 1 mL human serum but the concentration of LACI at a developing platelet thrombus could be much higher. It is not clear why the tissue...
factor inhibition assay gave lower levels of LACI than the factor Xa inhibition assay and the immunologic assay. One likely explanation, however, was that in the presence of calcium and phospholipid, platelet factor V interferes with the tissue factor inhibition assay by competing with LACI for factor Xa (unpublished observations March 1987).

Comparison of the time course of LACI release to that of two α-granule proteins showed that LACI release was similar to that of Protein S (shown to colocalize with fibrinogen in unstimulated platelets) but significantly slower than that of platelet factor 4. The reason for this discrepancy is not clear and attempts to further localize platelet LACI by immunofluorescent techniques are under way.

Platelet LACI is released as a soluble protein and is not associated with the platelet membrane or shed vesicles. Whether it is associated with lipoproteins, as is plasma LACI, remains to be determined. Bajaj et al have described two mol wt forms of tissue factor/VIIa inhibitor present in human plasma, at 40 Kd and 33 Kd. Seen on western blot, platelet LACI appears to run as a doublet with a mol wt around 45,000 to 47,000. Ours was not an accurate mol wt estimate, however, as the samples were unreduced and prestained mol wt standards were used. Nevertheless, the platelet LACI migrated on SDS-PAGE with an apparent mol wt slightly greater than that of LACI (HepG2).

Northern blot analysis of platelet mRNA showed the LACI cDNA insert hybridizing to two RNAs, a discrete band of 4.0 kb RNA, and a broad band centered at 1.4 kb RNA. This pattern had been seen previously with HepG2, endothelial cell, Chang liver, and SK hepatoma cell RNA. That LACI mRNA was found in platelets suggests that the source of platelet LACI is intrinsic production by megakaryocytes/platelets and not the sequestration of LACI from plasma.

The concentration of LACI progressively increased in blood samples obtained at the site of a bleeding time wound, reaching two- to threefold the initial plasma concentration by the time bleeding had stopped. It is most likely that this increase was related to the release of LACI from platelets accumulating at the hemostatic plug. The possible contribution of LACI from other cells, for example, endothelial cells and monocytes, cannot be excluded at present, but thus far, platelets are the only cell in which the rapid, inducible release of LACI had been demonstrated. The clinical significance of platelet LACI is a matter of speculation at this point but it might play a role in limiting the extent of tissue factor initiated coagulation at the site of a wound by inhibiting tissue factor/VIIa and fluid phase factor Xa.

REFERENCES


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