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 10^6 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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BLOOD COAGULATION is initiated via the extrinsic pathway when plasma factor VII or VIIa binds to its membrane cofactor, tissue factor, in a Ca^2+ dependent fashion. The factor VII(a)/tissue factor complex proteolytically activates both factors X and IX. When factor Xa binds to activated factor V on the platelet membrane, the resulting complex is a potent prothrombin activator.

Human serum or plasma contains an inhibitor of tissue factor initiated coagulation. Initial characterization of this inhibitor has shown it to be associated with plasma lipoproteins, to require the presence of catalytically active factor Xa, and to be directed against the VII(a)/tissue factor complex. We refer to this inhibitor as the Lipoprotein Associated Coagulation Inhibitor (LACI), while other laboratories call it EPI for the Extrinsic Pathway Inhibitor or the tissue factor/VIIa inhibitor. A protein having identical functional and antigenic properties as LACI has been isolated from the conditioned, serum free medium of a cultured human hepatoma cell line (HepG2). Recently, LACI cDNAs obtained from placental and endothelial cell libraries have shown to encode identical proteins and, as expected, LACI mRNA has been found in HepG2 cells.

The delineation of the properties of purified LACI (HepG2) led to a proposed mechanism for LACI's inhibition of the VII(a)/tissue factor complex. LACI has been shown to bind at or near the active site of factor Xa and to inhibit factor Xa directly. The LACI-factor Xa complex is noncovalent and is able to inhibit the VII(a)/tissue factor complex in a reversible manner that involves the formation of a quaternary complex.

As human platelets are known to contain a number of coagulant and anticoagulant proteins, we initiated a series of experiments to determine if platelets contained LACI.

MATERIALS AND METHODS

Materials. Factor X deficient plasma and pooled human plasma were obtained from George King Biomedical (Overland Park, KA). SDS and precasted molecular weight (mol wt) standards were received from BioRad (Richmond, CA). Bovine serum albumin (BSA), fraction V was obtained from Miles Scientific (Naperville, IL). Platelet factor 4 was obtained from American Red Cross, New York, NY. 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 Wraggleswki 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.

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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\(^2\) from healthy donors who had not taken aspirin for over 10 days. Before use, the platelets were pelleted by centrifugation at 2,000 \( \times \) 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 5.5 mmol/L) at a concentration of 10\(^6\) platelets per milliliter.

**Stimulation of platelets.** 490 microliter-aliquots of platelets were pipetted into 1.5 mL polypropylene tubes and stimulated by adding 10 \( \mu \)L of thrombin at 50 U/mL (final concentration 1 U/mL) or 10 \( \mu \)L of A23187 at 50 \( \mu \)mol/L (final concentration 1 \( \mu \)mol/L) followed by mixing. All incubations were at room temperature. At the end of the timed stimulation periods, platelets were rapidly separated from the reaction mixture by high speed centrifugation through a water insoluble, low viscosity oil mixture with a density between that of platelets and the reaction buffer\(^2\) and 400 \( \mu \)L of the supernatant was removed. The platelet pellet was cut out from the bottom of the tube with a scalpel and the platelets resuspended in 500 \( \mu \)L 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.\(^3,4\) 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 CaCl\(_2\) in a fibrometer cup at 37\(^\circ\)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\(^\circ\)C, 60 \( \mu \)L of the sample to be tested was added to 60 \( \mu \)L of TBSA (.14 mol/L NaCl/50 mmol/L tris base/pH 7.4/BSA 1%) with 1 mmol/L EDTA and 60 \( \mu \)L of bovine Xa at 25 ng/mL (diluted in TBSA). After five minutes incubation, 60 \( \mu \)L of 25 mmol/L CaCl\(_2\) and 60 \( \mu \)L 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 plotted 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 \( \mu \)L of the sample to be tested and 20 \( \mu \)L of anti-LACI rabbit serum (diluted 1:350 in TBSA). After one hour incubation at room temperature, 20 \( \mu \)L 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 \( \mu \)L of FITC-LACI (5 \( \mu \)g/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 C\(^2\) and protein Z\(^3\) 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 Laemmli.\(^5\) Electrophoretic transfer to nitrocellulose paper was performed in the buffer system of Towbin\(^6\) 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 \(^{125}\)I-Xa (at 5 \( \times \) 10\(^6\) CPM/50 mL TBSA) or the various anti-LACI IgGs (diluted in TBSA). The anti-LACI polyclonal IgG was diluted to 25 \( \mu \)g/mL, the anti-LACI N, and C-terminal peptide IgGs were diluted to 2.5 \( \mu \)g/mL. The paper was then washed three times with 2.5% nonfat dry milk in TS for five minutes. The \(^{125}\)I-Xa 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 NACI/5 mmol/L MgCl\(_2\)) 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 al\(^7\) with the following modification: the heparinized microhematocrit tubes were spun in a hemocentrifuge, 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 NaCl).

**Northern blots.** Platelet and neutrophil RNAs were a gift from Dr. G. Roth.\(^8\) HepG2 RNA was prepared from cultured cells as previously described.\(^9\) 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 \(^{32}\)P-labeled \(\alpha\)ECI cDNA insert that contained the entire LACI coding sequence.\(^10\) The blot was washed with a final stringency of 0.1% SSC, 0.1% SDS, at 68\(^\circ\)C.

**RESULTS**

**Tissue factor inhibition.** Incubation of platelets at 10\(^8\)/mL with either 1 U/mL thrombin or 1 \( \mu \)mol/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\(^8\) platelets and assuming a normal blood platelet concentration of 3 \( \times \) 10\(^8\)/mL, this would represent 3% of the serum LACI concen-
Fig 1. Release of tissue factor inhibitory (TFI) activity from platelets. Platelets (10⁹/mL) were stimulated with 1 U/mL thrombin, 1 μmol/L A23187, or TBSA, as a control. Maximum TFI activity is defined as that present after two hours stimulation with thrombin. ⊛, thrombin; ⊜, A23187; ⊝, control.

Comparison of the TFI activity in the platelet supernatant with that in the frozen/thawed platelet pellet demonstrated that at two hours <5% of the TFI activity remained with the platelet pellet (Fig 2). When platelets were stimulated with thrombin for two hours in the presence of EDTA, washed twice with TBSA/1 mmol/L EDTA, resuspended and assayed, they did not show TFI activity (Table 1). Further, when the platelet supernatant was ultracentrifuged at 100,000 × g for one and one-half hours, a small pellet of platelet “dust” was obtained but the TFI activity was found in the supernatant and not with this pellet. The TFI activity in the platelet supernatant was similar to plasma and HepG2 LACI in that it required the presence of factor Xa and Ca²⁺, and was neutralized by preincubation with rabbit anti-LACI IgG (Table 2).

The thrombin induced release of other platelets’ constituents was also studied. As previously described by Schwartz et al.,³³ platelets released protein S upon stimulation with thrombin (data not shown). One half of the maximal protein S was released by four minutes. Less than 8% of the total platelet protein S was left in the platelet pellet after two hours stimulation and the quantity of protein S released after two hours of stimulation was equal to 350 ng/3 × 10⁸ platelets. This corresponded to 1.8% of the level found in pooled human plasma (N = 50). After two hours of stimulation with thrombin, 12 μg of platelet factor 4 was released by 10⁹ platelets, with 50% of the platelet factor 4 released by 30 seconds.

Factor Xa inhibition. Incubation of platelets of 10⁹/mL with 1 μmol/L A23187 led to the release of factor Xa inhibitory (XaI) activity with time (Fig 3A). This experiment could not be performed with thrombin-stimulated platelets as thrombin interferes with the XaI assay. One half of the maximum (two hours) XaI activity was released by five minutes and 10% of the maximum XaI activity remained with the platelet pellet following two hours of stimulation. The XaI activity released after two hours stimulation corresponded to a LACI concentration of 30 ng/10⁹ platelets or 7.8% of the amount found in this donor’s serum (Table 1). The platelet XaI activity was neutralized upon preincubation of the platelet supernatant with rabbit anti-LACI IgG (data not shown).

Immonoassay. Since the tissue factor inhibition assay and factor Xa inhibition assays gave different results on the total amount of LACI released by stimulated platelets, an immunoassay was developed to measure total LACI mass. Platelets (10⁹) stimulated with 1 U/mL thrombin for two hours released 28 ng of LACI or 7.1% of the amount found in this donor’s serum (Table 1).

Table 1. Comparison of LACI Assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>LACI/10⁹ 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>Immuno assay</td>
<td>28</td>
<td>118</td>
<td>7.1</td>
</tr>
</tbody>
</table>

*Platelet donor serum.
†Assuming normal platelet concentration of 3 × 10⁹/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 αLACI 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 αLACI 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 × 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, has not been 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.

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Platelets secrete a coagulation inhibitor functionally and antigenically similar to the lipoprotein associated coagulation inhibitor

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