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.

MATERIALS AND METHODS

Materials. Factor X deficient plasma and pooled human plasma were obtained from George King Biomedical (Overland Park, KA). SDS and precast molecular weight (mol wt) standards were received from BioRad (Richmond, CA). Bovine serum albumin (BSA), fraction V was obtained from Miles Scientific (Naperville, IL). Aepzon A oil was obtained from Aepzon 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 Wurlaubwski and LaDue. All other reagents were from Sigma or Fisher.

Methods. Human factor IIa, factor VIIa, antithrombin IIIa, and a crude preparation of tissue factor19 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 DEA-Sepharose 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,1 and activated23 as previously described and LACI was purified from conditioned, serum free medium of cultured HepG2 cells.2 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.23,13

Labeling LACI with Fluorescein Isothiocyanate. 500 μL of LACI (HepG2) (152 μg/mL in .1 mol/L MOPS, pH 7.5) 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 0.2 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) were obtained as described.2 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 LACI14 was synthesized and used to immunize a New Zealand white...
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 10^9 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. At the end of the timed stimulation periods, platelets were rapidly separated from the reaction mixture by high speed centrifugation. The supernatant was removed. The platelet pellet was purified by chroma-
tination of this volunteer by TFI assay (Table 1). Platelets incubated with buffer alone released no TFI activity. Measurements of lactic dehydrogenase (LDH) in the platelet supernatants revealed <1% of the total LDH levels available following lysis of the platelets by sonication or freeze/thawing.

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 1). 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 x 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 Ca++, 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., 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 x 10^8 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 10^8 platelets, with 50% of the platelet factor 4 released by 30 seconds.

**Factor Xa inhibition.** Incubation of platelets of 10^9/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/10^9 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).

**Immunoassay.** 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 (10^9) 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).

![Fig 1](image1.png)  ![Fig 2](image2.png)

**Fig 1.** Release of tissue factor inhibitory (TFI) activity from platelets. Platelets (10^9/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.

**Fig 2.** Segregation of TFI activity between the supernatant and platelet pellet. Platelets (10^9/mL) were stimulated with 1 U/mL thrombin and the TFI activity released into the supernatant and that remaining in the platelets was determined as described in the Materials and Methods section. •, Supernant; ◇, PLT pellet.

<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>
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</tbody>
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*Platelet donor serum.
†Assuming normal platelet concentration of 3 x 10^9/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 Ca 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>
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Abbreviation: F/T, frozen/thawed.
*Platelet concentration 10^9/mL.

Western blot analysis, using ^125^I-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. 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 10^8 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$^{15}$ (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$^{31}$) 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.$^{19}$ 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.$^{14,31,35}$ 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$^{34}$ 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/VII(a) and fluid phase factor Xa.

REFERENCES

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