Plasma Antigen Levels of the Lipoprotein-Associated Coagulation Inhibitor in Patient Samples

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Human plasma contains an inhibitor of tissue factor-initiated coagulation known as the lipoprotein-associated coagulation inhibitor (LACI) or also known as the extrinsic pathway inhibitor (EPI). A competitive fluorescent immunoassay was developed to measure the plasma concentration of LACI in samples from normal individuals and patients with a variety of diseases. The LACI concentration in an adult control population varied from 60% to 160% of the mean with a mean value corresponding to 89 ng/mL or 2.25 nmol/L. Plasma LACI levels were not decreased in patients with severe chronic hepatic failure, warfarin therapy, primary pulmonary hypertension, thrombosis, or the lupus anticoagulant. Plasma LACI antigen was decreased in some, but not all patients with gram-negative bacteremia and evidence for disseminated intravascular coagulation. Plasma LACI levels were elevated in women undergoing the early stages of labor (29%), in patients receiving intravenous tissue-type plasminogen activator (45%), and in patients receiving intravenous heparin (37%). A radioligand blot of the pre- and post-heparin plasma samples shows the increase to be in a 40-Kd form of LACI. Very low levels of plasma LACI antigen were found in patients with homozygous abetalipoproteinemia and hypobetalipoproteinemia, diseases associated with low plasma levels of apolipoprotein B containing lipoproteins. Following the injection of heparin into one patient with homozygous abetalipoproteinemia, the plasma LACI antigen level increased to a level comparable with that in normal individuals after heparin treatment.

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Red Cross Blood donors, St Louis Bi-State Chapter of the American Red Cross; patients with thrombosis, Dr William Haire (University of Nebraska); patients with primary pulmonary hypertension, Dr Stewart Rich (University of Chicago); patients receiving tissue-type plasminogen activator (tPA), Dr Paul Eisenberg (Washington University); patients with homozgyous abetalipoproteinemia, hypobetalipoproteinemia, apolipoprotein E deficiency, and Tangier disease, Dr Stephen M. Prescott (one abeta, one Tangier) (University of Utah), Dr James Keating (one abeta) (Children's Hospital, St Louis), and Drs Daniel J. Rader and H. Bryan Brewer, Jr (three Tangier, one Tangier with heparin, four abetas, one abeta with heparin, two hypobetas, one apo E deficiency before and after heparin) (National Institutes of Health). All other samples were obtained from the chemistry or hematology laboratories at the Jewish Hospital of St Louis or Barnes Hospital, St Louis.

Assays. LACI functional assays (tissue factor-inhibition assay) were performed as described. The assays for fibrin degradation products (FDP) were performed in the clinical coagulation laboratory at Barnes Hospital and used latex particles coated with antibody to human fibrinogen. A positive result is seen as agglutination of the particles in the presence of diluted serum samples. Samples were considered positive at an FDP concentration of greater than 8 μg/mL.

LACI immunoassay. A competitive florescent immunoassay was constructed in which an anti-LACI rabbit serum is used to bind LACI, goat antirabbit IgG covalently linked to latex particles is used to capture the rabbit IgG, and FITC-LACI is added to quantitate the number of free anti-LACI IgG sites remaining. To an Eppendorf tube is added 200 μL of the plasma sample to be tested (diluted 1/4 or greater in TBSA [50 mmol/L tris-HCl, pH 7.5/100 mmol/L NaCl/O.1% BSA]) and 200 μL of anti-LACI rabbit serum (diluted 1/350 in TBSA). After an overnight incubation, four 40-μL aliquots of the mix are added to wells of a 96-well assay plate (Baxter-Pandex Laboratories, Inc, Mundelein, IL) that already contain 20 μL of goat antirabbit IgG linked to latex beads (resuspended at 0.5% wt/vol in TBSA). After 30 minutes of incubation at room temperature, 20 μL of FITC-LACI (5 μg/mL in TBSA) are added, and 6 minutes later a vacuum applied to the assay plate separating the solution from the latex particles. The particles are washed once for 2 minutes with TS (50 mmol/L tris-HCl, pH 7.5/100 mmol/L NaCl) and the amount of bound florescence quantitated with a Screen Machine (Baxter-Pandex). Standard curves were constructed using dilutions of pooled human plasma (George King Biomedical) in TBSA.

Immunoprecipitations, immunoblots, and radioligand blots were performed as previously described.

Covalent linkage of antirabbit IgG to latex particles. Five milligrams of affinity-purified goat antirabbit IgG were added to 6 mL carboxylated latex particles (4.2% wt/vol) and 0.1 mol/L sodium citrate, pH 5.0, was added to a final volume of 50 mL. After gentle
rocking at room temperature for 1 hour, 50 mg solid EDAC [l-ethyl-3-(2-dimethylaminopropyl)carbodiimide hydrochloride] was added and the mixture rocked gently at room temperature for 1 more hour. The particles were pelleted by centrifugation at 3,000 g and resuspended in a final volume of 50 mL TBSA. Following one more TBSA wash, the particles were resuspended in a final volume of 50 mL TBSA and stored at 4°C.

Flowrescin labeling HepG2 LACI. HepG2 LACI (0.1 mg) was dialyzed overnight into HS (0.05 mol/L HEPES, pH 7.4/0.1 mol/L NaCl/0.05% p-octylglucoside) and diluted to a final volume of 800 μL. To the LACI was added 200 μL of FITC at 1 mg/mL in 0.5 mol/L sodium carbonate, pH 9.5. This mixture was rocked at room temperature for 1 hour and free fluorescein was separated from FITC-LAC1 by chromatography on a Biogel P-2 column (Pharmacia). Fractions were assayed for protein and FITC by absorbance at 280 and 495 nm. The FITC-LAC1 fractions were pooled and stored at 4°C in HS/0.05% p-octylglucoside.

RESULTS

Standardization of immunoassay. An example of a standard curve for the immunoassay is shown in Fig 1. The coefficient of variation for a control plasma, assayed in quadruplicate, on a single plate on a single day was 2.3%. Six standard curves, using serial dilutions of different control plasmas, were parallel to a standard curve using dilutions of pooled human plasma (data not shown). Using serum samples from 43 adult Red Cross blood donors, LACI levels as determined by immunoassay were shown to correlate with LACI levels as determined by functional assay (tissue factor-inhibition assay), with an r value of .89 (Fig 2).

Population distribution. The concentration of LACI antigen in a control population is shown plotted on a frequency histogram in Fig 3. The control population consisted of Red Cross blood donors, visitors to a health fair, and medical students. The 95% range of plasma LACI antigen extended from 60% to 160% of the mean, with the
mean level corresponding to 89 ng/mL of LACI or 2.25 nmol/L.

Patient samples. The plasma LACI antigen levels in a variety of patient diagnostic groups are shown in Fig 4. Plasma LACI antigen was increased 29% in women undergoing the early stages of labor compared with sex and age matched controls (Fig 4). Plasma LACI antigen was not decreased in patients with severe chronic hepatocellular disease or in patients on warfarin therapy (Fig 4). Plasma LACI antigen was elevated in patients with gram-negative bacteremia, without FDP. However, plasma LACI antigen was decreased in some patients with gram-negative bacteremia and FDP (Figs 4 and 5). A comparison of the individual values shows that the ranges of values in each group are quite wide and overlap considerably (Fig 5). There was no correlation between LACI levels and levels of FDP (not shown).

Plasma LACI antigen decreased postoperatively in patients undergoing total hip and knee replacement surgery (Fig 6); however, the degree of LACI decrease appears to correspond to the degree of postoperative blood loss. Plasma LACI levels were not decreased in patients with the lupus anticoagulant or primary pulmonary hypertension (Fig 4), including several patients with thrombogenic primary pulmonary hypertension.23

Plasma LACI antigen was not decreased in a group of 90 patients with thrombosis (Fig 4). This group included 65 patients with deep venous thrombosis and/or pulmonary emboli, 10 patients with peripheral arterial occlusion, five patients with stroke, and 10 patients with coronary artery disease. Venooclusion increased LACI levels 15% in these patients. The highest levels of plasma LACI antigen were found in patients receiving intravenous heparin. Twenty-three patients undergoing cardiac catheterization received 2,500 U of intravenous heparin during the procedure and had plasma samples drawn before and then 10 to 15 minutes after injection. The increase in plasma LACI following intravenous heparin appears to be quite variable in this group, ranging from 150% to 650% of baseline levels. This finding was confirmed in a more controlled fashion by injecting increasing doses of heparin into seven healthy male volunteers and taking plasma samples 6 minutes after injection (Fig 7). The time course of LACI increase in a normal volunteer following intravenous dose of 7,500 U of heparin (100 U/kg) is shown in Fig 8. In this volunteer, LACI levels peak at 6 to 8 minutes at 950% of the baseline level. A radioligand blot of these plasma samples, probed with 115-factor Xa, shows the increase to be in a 40-Kd form of LACI (Fig 8, insert). When the same volunteer was
injected with dermatan sulfate (2 mg/kg), plasma LACI antigen did not increase significantly (data not shown). Plasma LACI levels also increased significantly (45%) in patients infused with tPA for acute myocardial infarction (Fig 4). These patients had not received heparin before these samples were taken.

Plasma LACI antigen was elevated a mean of 12% in patients with increased levels of very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) from a variety of causes, including familial hypercholesterolemia (Fig 4). There was no correlation between the level of plasma LACI antigen and the levels of LDL cholesterol, high-density lipoprotein (HDL) cholesterol, or triglycerides in 50 hospitalized patients (data not shown). The lowest levels of plasma LACI antigen were found in six patients with homozygous abetalipoproteinemia and two patients with hypobetalipoproteinemia (Fig 4), distinct diseases associated with low plasma levels of apolipoprotein B-containing lipoproteins. These patients do not suffer from abnormal thrombosis. Four patients with Tangier disease, lacking normal HDL, have normal plasma LACI levels. An immunoblot of a Tangier patients’ lipoproteins show that they have both major forms of plasma LACI (34 and 40 Kd) present in LDL (Fig 9). One patient with homozygous abetalipoproteinemia and one patient with Tangier disease were injected with 60 U/kg of heparin and bled 6 minutes later showing normal increases in plasma LACI antigen (Fig 4). One patient with complete apolipoprotein E deficiency had an elevated baseline level of plasma LACI antigen (221% of pooled plasma) and a supranormal response to intravenous heparin (1412% of pooled plasma) (Fig 4). An immunoblot of this patient’s lipoproteins show a pattern similar to the normal pattern (Fig 9).

DISCUSSION

Several groups have studied plasma LACI levels using functional assays. We describe here the first immunoassay used to measure LACI antigen in patient plasma samples. The data in this study confirm results from other groups studying patients with liver disease, on warfarin therapy, with the lupus anticoagulant, and with pregnant women.

Is LACI consumed in DIC? As opposed to groups who found uniformly low, or normal to high levels of LACI in DIC, we found tremendous variation in LACI levels in patients with gram-negative bacteremia and FDP. Although it is tempting to attribute the lowest LACI levels in this group to consumption of LACI, we believe that cannot be done. These patients were clinically very complicated, often hypotensive, receiving multiple blood products, and with multi-organ failure. How LACI levels would be affected by these clinical variables, in the presence or absence of DIC, is not known.

Is LACI synthesized by the liver in vivo? That LACI levels are normal in patients with severe chronic hepatic failure does not conclusively answer this question. Patients with hepatic failure both synthesize and clear lipoproteins at a rate that is lower than normal. Abnormal LACI levels in some patients with lipid disorders suggest that plasma LACI levels are related to lipoprotein metabolism. One recent study found low levels of LACI activity and messenger RNA (mRNA) in primary hepatocyte cultures; however, it is not clear that this accurately reflects in vivo activity.

Is LACI a physiologically important natural anticoagulant? We had hoped to answer this question by finding a patient or family in which LACI deficiency was associated with thrombosis. None of the 90 individuals with thrombosis tested in this study had a low level of plasma LACI antigen. We are now searching for plasma samples from patients with strong family histories of thrombosis, which were not present in the patients tested thus far.

Fig 9. Immunoblots of lipoproteins from a control patient and patients with abetalipoproteinemia, Tangier disease, and apolipoprotein E deficiency. Plasma samples were gel filtered to separate LDL, HDL, and free LACI fractions. Fractions were immunoprecipitated with anti-LACI monoclonal antibody 2B12-latex particles, run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with a rabbit antibody to the amino terminus of LACI.
Sandset et al, using a functional assay, had previously reported that LACI levels increase twofold following an intravenous dose of 7,500 U of heparin. We have found significantly greater increases in plasma LACI antigen following heparin infusion. Although the source of HRL is not known, Sandset et al hypothesized that a pool of LACI exists in vivo associated with vascular endothelial glycosaminoglycans. We have recently purified heparin-releasable LACI from post-heparin plasma and found that it is structurally different from lipoprotein LACI, it is not tightly associated with lipoproteins, and it binds heparin-agarose with high affinity.

The source and mechanism of LACI increase following intravenous tPA is also not known. Sakela and Rifkin have reported that plasmin proteolytically cleaves endothelial cell heparan sulfate glycosaminoglycans, thereby releasing complexes of partly degraded heparan sulfate and basic fibroblast growth factor. If LACI were anchored to endothelial cell heparan sulfate proteoglycans in vivo, then LACI could theoretically be released by a similar mechanism.

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