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Cultured normal human hepatocytes do not synthesize lipoprotein-associated coagulation inhibitor: Evidence that endothelium is the principal site of its synthesis

(extrinsic pathway inhibitor/megakaryocytes/vascular cells/circulating blood cells/polymerase chain reaction)

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ABSTRACT **Human plasma contains a factor Xa-dependent inhibitor of tissue factor/factor VIIa complex termed lipoprotein-associated coagulation inhibitor (LACI). The present study examines the site(s) of LACI synthesis. In this study, cultured hepatocytes isolated from normal human liver were found to be essentially negative in LACI mRNA as revealed by Northern blot analysis using a full-length LACI cDNA as probe. The conditioned media from these cultures were also essentially negative for LACI activity. Similarly, poly(A)$^+$ RNA obtained from normal human liver did not contain detectable LACI mRNA. In contrast, cultured human umbilical vein endothelial cells and human lung tissue (rich in endothelium) both contained abundant amounts of LACI mRNA. Moreover, erythrocyte lysates and culture media from normal monocytes, lymphocytes, or neutrophils did not contain measurable LACI activity; these cells were also negative for LACI mRNA. Platelets, however, contained LACI activity. The likely source of platelet LACI is the megakaryocyte cell since a megakaryocyte cell line (MEG-01) was found to contain LACI mRNA and to secrete small amounts of LACI activity. Additionally, human vascular smooth muscle cells and lung fibroblasts were also found to synthesize only small amounts of LACI. From these observations, we conclude that normal liver does not synthesize LACI and that endothelium is the principal source of plasma LACI. The undegraded LACI synthesized by endothelial cells had a molecular weight of ≈41,000.**

During normal hemostasis, the extrinsic pathway of coagulation begins by disruption of the vasculature at a site of injury and exposure of the blood to tissue factor (TF). As a result, the plasma factor VII/VIIa complexes with TF and initiates clotting through the activation of both factors IX and X (1, 2). Recently, a factor Xa-dependent plasma inhibitor of TF/VIIa complex was rediscovered and firmly identified (2, 3). This inhibitor has been variously termed tissue factor inhibitor (4), TF/VIIa inhibitor (5), extrinsic pathway inhibitor (2), and lipoprotein-associated coagulation inhibitor (LACI) (6). The inhibitor (henceforth referred to as LACI) has been purified from the conditioned medium of a hepatoma cell line HepG2 (4) and recently from human plasma where essentially all of it is associated with lipoproteins (7). LACI cDNAs have been identified in a human fetal liver cDNA library (6) and have been isolated from placenta (6) and endothelial cell (8) cDNA libraries. The translated amino acid sequence revealed that LACI consists of three tandem Kunitz-type inhibitory domains with three N-linked potential glycosylation sites. Each domain has a molecular weight of ≈13,000–15,000 (6). The mechanism of inhibition by LACI involves its binding first to factor Xa and then to factor VIIa in the TF/VIIa complex (9, 10). The amino-terminal domain of LACI binds to factor VIIa, the middle domain binds to factor Xa, but the function of its carboxyl-terminal domain is not clear (11).

LACI has been reported to be synthesized and secreted by cultured human umbilical vein endothelial (HUVE) cells (5, 12) and by bovine pulmonary artery endothelial cells (5). An *in vitro* transformed human liver cell line, Chang liver (6), and two hepatoma cell lines, SK (6) and HepG2 (4–6), have also been reported to synthesize and secrete LACI. Whether or not normal hepatocytes synthesize LACI is not known. Nonetheless, from studies employing liver-derived cell lines, it has been assumed that normal hepatocytes represent one physiologic site of LACI synthesis (4–6, 12, 13). However, in three studies employing functional assays (5, 13, 14) and in one study employing antigen assays (15), it has been determined that plasma LACI levels in general are not reduced in patients with hepatocellular disease. These observations thus raise the possibility that normal liver may not be a site of LACI synthesis. The present studies were undertaken to examine this question.

EXPERIMENTAL PROCEDURES

Materials. HUVE cells and human vascular smooth muscle (HVSM) cells were harvested by the method of Jaffe *et al.* (16). A normal human fetal lung fibroblast (HFLF) cell culture (GM-1380) was obtained from the Coriell Institute for Medical Research (Camden, NJ). A normal human intestinal smooth muscle (HISM) cell culture (17) and the HepG2 cell culture (18) were obtained from the American Type Culture Collection. All tissue culture media and supplements were obtained from Sigma. ^{125}I-labeled goat anti-rabbit IgG was obtained from ICN, and [α-^{32}P]dCTP (≈3000 Ci/mmol; 1 Ci = 37 GBq) was obtained from DuPont/NEN. NaB^3H_4 and [^{35}S]methionine were obtained from Amersham. Protein molecular weight standards and rabbit anti-bovine whole serum were obtained from Sigma, and Ficoll-Paque and dextran T-500 were purchased from Pharmacia.

Cell Culture. HUVE cells were grown as described (5, 16), and confluent cells of passage 2 were used for all experi-

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Abbreviations: TF, tissue factor; LACI, lipoprotein-associated coagulation inhibitor; HUVE, human umbilical vein endothelial; HVSM, human vascular smooth muscle; HFLF, human fetal lung fibroblast; HISM, human intestinal smooth muscle; FCS, fetal calf serum; BSA, bovine serum albumin.

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ments. HVSM cells were cultured by a slight modification of the HUVE cell isolation and culture method (16). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% heat-inactivated fetal calf serum (FCS) and 1% nonessential amino acids. The smooth muscle cells overgrew the contaminating endothelial cells within 2–3 weeks in DMEM and appeared to be a pure population of spindle-shaped cells growing in multiple layers. These cells were used without further subculturing. HISM cells, HFLF cells, and HepG2 cells were grown in DMEM with 10% FCS and 1% nonessential amino acids. The establishment and several properties of a human megakaryoblastic cell line, MEG-01, have been described (19, 20). The MEG-01 cells were maintained in RPMI 1640 medium supplemented with 10% FCS as outlined (19, 20).

Normal human hepatocytes were isolated and cultured in A. P. Li's laboratory as described (21). Cells were isolated from normal liver weighing 40–50 g by the basic two-step collagenase perfusion procedure (22). After isolation, the cells were washed twice in Waymouth medium 752/1 by centrifugation at 50 × *g*. The hepatocytes were then suspended in culture medium consisting of Waymouth 752/1 and supplements given earlier (21, 23). The hepatocytes were plated on 35-mm diameter plastic dishes coated with either a thin layer of collagen (24) or basement membrane Matrigel (Collaborative Research) (21). Greater than 80% of the cells attached to the matrix within 2 hr, and the viability of the attached cells as determined by the trypan blue exclusion method (21) was >85%.

Peripheral blood cells and platelets were isolated from normal blood obtained from healthy donors with informed consent. Erythrocytes and neutrophils were isolated according to Böyum (25) as outlined (26). Mononuclear cells were isolated by a minor modification of the procedure outlined by Böyum (25). A unit (≈450 ml) of heparinized blood was centrifuged at 120 × *g* for 15 min. Platelet-rich plasma was discarded, and the cells were suspended in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS) to a final volume of 450 ml. Thirty-five-milliliter aliquots were layered onto 15 ml of Ficoll-Paque and centrifuged at 400 × *g* for 40 min. The mononuclear cell layer from each tube was removed and washed with DPBS containing 5 mM EDTA (DPBS/EDTA); the cells were pelleted by centrifugation at 400 × *g* for 20 min. The pelleted cells were washed three times with DPBS/EDTA, centrifuging each time at 120 × *g* for 12 min to further reduce the number of contaminating platelets. The cells were then suspended to a density of ≈10^6 cells per ml in RPMI 1640 medium supplemented with 15% FCS and plated on fibronectin-coated (3 μg/cm^2) dishes. A 15-ml cell suspension was used for each 100-mm dish, and the cells were allowed to adhere for 45 min at 37°C. The nonadherent cells were washed twice with DPBS/EDTA and were used to isolate lymphocytes by three successive 45-min incubations on plastic dishes. The adherent cells were washed gently with RPMI medium followed by incubation with a 1:1 mixture of RPMI medium and DPBS/EDTA for 15 min at 37°C. The monocytes were detached by gentle tapping and centrifuged at 400 × *g* for 10 min prior to culturing. Neutrophils, lymphocytes, and monocytes were identified using Wright's stain. Monocytes were also identified by the cytoplasmic nonspecific esterase activity (27). Purity by the above criteria was >95%, and the viability by the trypan blue exclusion method (21) was >93% for each cell type. Washed human platelets were prepared by the method of Mustard *et al.* (28).

Immunological Dot Blots and Metabolic Labeling. Antisera specific for the control trpE protein and trpE–LACI fusion protein were obtained by immunizing rabbits with the purified proteins obtained from *Escherichia coli* RR1 cells. Production and characterization of these antibodies will be published separately. Details of the immunoblotting and metabolic labeling experiments are given in the legends to Figs. 1 and 3.

Assays. LACI activity was measured by its ability to inhibit TF/VIIa-catalyzed activation of ^{3}H-labeled factor IX in the presence but not in the absence of factor X. Details of our procedure have been described (5). Pooled plasma from 20 healthy donors was assumed to contain 1000 milliunits of LACI per ml. Metabolic activity of normal hepatocytes was determined by measuring P450 enzymes activity (7-ethoxycoumarin diethylase activity). The method employed was that of Edwards *et al.* (29) as outlined (21).

LACI cDNA Cloning and Northern Blotting. A full-length LACI cDNA was cloned by using the polymerase chain reaction (30); the details are given below. The primers used had the following sequence: Primer A, 5′-TA*CTGCAG*CG (CCAAGAACTTTCATCAGAGA); primer B, 5′-GC*GAATTC*GC(TAATGTTACATTGCTATAAC). Primer A contains a *Pst* I site and primer B contains an *Eco*RI site. The restriction site sequences are in boldface italic. The sequences in parentheses are the sequences complementary to LACI cDNA regions (nucleotides 103–122 for primer A and 1052–1071 for primer B, see ref. 6). Northern blot analysis was carried out as described by Sambrook *et al.* (31). Total RNA was isolated by the method of Chomczynski and Sacchi (32), and normal human liver and lung poly(A)$^+$ RNAs were obtained from Clontech.

Gel Filtration. The apparent molecular weights of the LACI species present in the conditioned media of HepG2 and HUVE cells were determined by gel filtration using the Pharmacia FPLC system. Details are provided in the legend to Fig. 3.

RESULTS

Secretion of LACI Activity by HUVE, HepG2, and Normal Liver Cells. Accumulation of LACI activity as a function of time in the conditioned medium of HUVE cells and HepG2 cells is presented in Fig. 1. It is apparent from the data that under these experimental conditions, a hepatoma cell line (HepG2) synthesizes and secretes much greater amounts of LACI than that produced and secreted by cultured endothelial cells (HUVE). From these data and from the knowledge that other hepatoma cells also synthesize LACI (6), it is reasonable to presume that normal liver parenchymal cells synthesize LACI as well (4–6, 12, 13). To test this assumption, we assayed LACI activity in 24-hr supernatants of normal hepatocyte cultures. These data presented in Table 1 reveal that LACI activity secreted by normal human hepatocytes in culture is ≈1% of that produced by the cultured hepatoma cell line HepG2. Thus, the data of Table 1 suggest that LACI may not be synthesized by liver parenchymal cells *in vivo*.

LACI cDNA Cloning. To determine whether or not LACI mRNA is present in normal liver or lung (rich in endothelium) tissue, we initially cloned a full-length LACI cDNA for use as a probe in Northern blotting experiments described below. A human placenta λgt11 Clontech library was amplified in *E. coli* Y1090, and polymerase chain reaction amplification (30, 33) of the LACI cDNA was achieved by using 1 μg of phage DNA isolated from the above library. The amplified DNA (≈1 kilobase pair) was isolated (33), digested with *Pst* I/*Eco*RI endonucleases, cloned into pUC18 and M13mp18 or M13mp19 vectors, and sequenced by the dideoxynucleotide chain-termination method (34). The insert contained the entire LACI open reading frame (nucleotides 103–1071, ref. 6) and the *Pst* I/*Eco*RI restriction sites.

Northern Blotting. Results of the Northern blotting experiments are shown in Fig. 2. HepG2 cells (Fig. 2, lane 1) contained the two described LACI mRNA species of 4.0 and

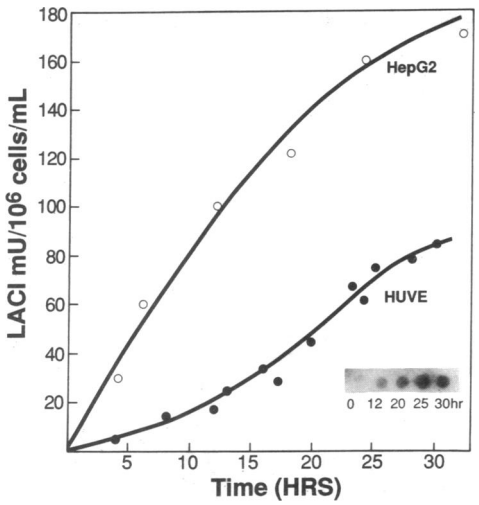

FIG. 1. Time course of secretion of LACI activity by HepG2 and HUVE cells in culture. Confluent cell monolayers (35-mm diameter Petri dishes) were incubated with the specific growth media containing 2% FCS for a given period at which time conditioned media were collected, centrifuged at 10,000 × *g* for 10 min, and lyophilized. The lyophilized samples were dissolved in 0.05 M Tris/0.15 M NaCl, pH 7.5 (Tris/NaCl) and dialyzed against the same buffer at 4°C prior to LACI assays. The activity represented is the mean of two experiments performed in duplicate. (*Inset*) LACI immunological dot blots of HUVE conditioned medium collected without FCS at 0, 12, 20, 25, and 30 hr. The sample volume applied to the nitrocellulose filter was 0.5 ml, and the blocking buffer was 5% nonfat dry milk in Tris/NaCl buffer. The rabbit anti-LACI fusion protein antiserum was used at a 100-fold dilution, and ^{125}I-labeled goat anti-rabbit IgG was diluted to 10^6 cpm per 30 ml prior to use. The filter was air dried and exposed to Kodak X-AR5 film at −70°C with one intensifying screen for 15 hr.

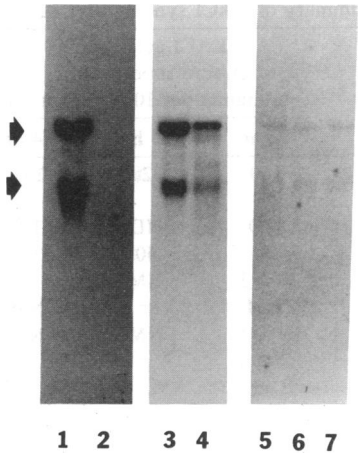

FIG. 2. Northern blot analysis of LACI mRNAs in cultured cells and in normal liver and lung tissue. Lane 1, 30 μg of total RNA from HepG2 cells; lane 2, 10 μg of poly(A)$^+$ RNA from normal human liver tissue; lane 3, 30 μg of total RNA from HUVE cells; lane 4, 10 μg of poly(A)$^+$ RNA from normal human lung tissue; lane 5, 30 μg of total RNA from MEG-01 cells; lane 6, 30 μg of total RNA from HISM cells; lane 7, 30 μg of total RNA from HFLF cells. RNA was denatured and electrophoresed on a 1.2% agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized with a full-length LACI cDNA probe (≈1.5 × 10^8 cpm/μg) (31). The filters were exposed to Kodak X-AR5 film at −70°C with one intensifying screen for 3 days. The arrows on the left indicate the two bands (4.0 kb and 1.4 kb) of LACI mRNAs (6). The difference between the two mRNA sizes has been primarily attributed to the presence of ≈2.6 kb of additional 3′ untranslated sequence in the 4-kb band (8). The BRL standard containing RNA fragments of 0.24, 1.35, 2.4, 4.4, 7.5, and 9.5 kb was used for sizing the LACI mRNAs.

1.4 kilobases (kb) (for description of these mRNA species, see the legend to Fig. 2). In contrast, LACI mRNA was not present at a detectable level in normal human liver tissue (Fig. 2, lane 2). In a control experiment when 0.2 μg of liver poly(A)$^+$ RNA was electrophoresed and probed with ^{32}P-labeled factor X cDNA, a discrete mRNA band of ≈1.4 kb was observed; this rules out the possibility of RNA degradation during isolation. Moreover, when three preparations of total RNA (30 μg each) from cultured normal hepatocytes were analyzed, they were found to contain only small amounts of LACI mRNA (3–7% of HepG2 cells), presumably derived from contaminating sinusoidal endothelial cells. The analysis of RNA from cultured HUVE cells and from the lung tissue is shown in lanes 3 and 4, respectively, of Fig. 2. Clearly the cultured endothelial cells and the lung tissue rich in endothelium contained the LACI mRNAs. These data therefore provide further evidence that normal liver is not the site of LACI synthesis and that endothelium may represent the primary site of its synthesis *in vivo*.

Table 1. LACI activity in 24-hr supernatants of normal human hepatocyte cultures

Day of culture	Activity	
	LACI	ECOD
1	1 ± 1	26 ± 5
3	2 ± 1	20 ± 8
5	1 ± 1	37 ± 13
7	2 ± 2	52 ± 7
9	1 ± 1	65 ± 9
11	2 ± 1	47 ± 19

The activity is the mean of three analyses. For LACI it is adjusted to the number of milliunits per 10^6 cells per ml, and for 7-ethoxycoumarin diethylase (ECOD) it represents the percent activity of that obtained with cells 2 hr after plating. The cells were cultured on Matrigel-coated dishes, and the LACI activity was measured on 10-fold concentrated culture supernatants. Similar results were obtained when cells were cultured on collagen matrix.

Secretion of LACI by Other Cells Associated with the Vascular System. The conditioned media obtained from cultured monocytes, lymphocytes, and neutrophils were found to be essentially negative in LACI activity. These cells also did not contain detectable LACI mRNA (Table 2). Erythrocyte lysates were also found not to contain LACI activity. HVSM, HISM, and HFLF cells also synthesized and secreted only small amounts of LACI (Fig. 2 and Table 2). As reported earlier (35), platelets were found to contain LACI. Consistent with the previous report, the amount of LACI released from 3 × 10^8 platelets (average number present in 1 ml of whole blood) by three freeze–thaw cycles (−70°C/37°C) was ≈6% of that present in 1 ml of plasma. MEG-01 cells were also found to contain LACI mRNA and to secrete small amounts of LACI activity (Table 2 and Fig. 2), suggesting that LACI in platelets is derived from megakaryocytes.

Gel-Filtration of Conditioned Media (Plus or Minus FCS) from HUVE and HepG2 Cells. In these experiments, conditioned media from HUVE and HepG2 cells collected in the presence of 2% FCS or 0.5% bovine serum albumin (BSA) were concentrated and run on a Superose 12 column (Fig. 3). For each cell type, in the absence of FCS in the culture medium, LACI activity was eluted in two distinct peaks—one corresponding to a molecular weight of 41,000 ± 2000 and the second corresponding to a molecular weight of 33,000 ± 2000 (average of three runs). When FCS was included in the culture media, the LACI activity was eluted essentially in the higher molecular weight form (41,000 ± 2000) for both cell types (Fig. 3).

Evidence of *de Novo* Synthesis of LACI by Cultured HUVE Cells. To confirm that endothelial cells synthesize LACI in

Table 2. A summary of LACI synthesis by various cells

Cell	LACI activity in culture supernatants, milliunits per 10^6 cells per ml			% LACI mRNA*
	12 hr	18 hr	24 hr	
HepG2	100	120	152	≈200
Normal hepatocytes	ND	ND	1.5	≈4
HUVE	20	40	65	100
Monocytes	1 ± 1	NM	1 ± 1	NM
Lymphocytes	NM	1 ± 1	NM	NM
Neutrophils	1 ± 1	NM	NM	NM
HVSM	2 ± 1	4 ± 2	5 ± 2	≈7
HISM	3 ± 2	5 ± 1	6 ± 2	≈6
HFLF	2 ± 1	5 ± 2	7 ± 2	≈6
MEG-01	1 ± 0	3 ± 1	5 ± 1	≈6

To test for LACI activity, cells were washed and then cultured in media containing 2% FCS for different lengths of time. Conditioned media were collected and centrifuged, and the supernatants were assayed for LACI activity after a 5-fold concentration. Results presented are the average of two determinations except for the normal hepatocytes where the average of all determinations (from Table 1) is given. LACI mRNA levels for each cell type were quantitated by using an LKB UltroScan XL laser densitometer. ND, not determined; NM, not measurable.
*The percentages are relative to the HUVE value.

culture, confluent cell monolayers were incubated for 20 hr with growth medium (plus 2% FCS) in the absence or presence of cycloheximide (1 μg/ml), an inhibitor of protein synthesis. As noted earlier (5), the conditioned medium in the presence of cycloheximide contained only 10% of the LACI activity as compared to that obtained in the absence of cycloheximide. Moreover, progressive accumulation of immunoreactive LACI was also observed in the conditioned medium of HUVE cells cultured in the absence of cycloheximide (Fig. 1 *Inset*). *De novo* synthesis of LACI by endothelial cells was also observed in an amino acid ([^{35}S]methionine) incorporation assay (Fig. 3 *Inset*).

DISCUSSION

The objective of the present studies was to identify the physiologic site(s) of LACI synthesis. The studies presented in this report establish that normal liver is not the site of LACI synthesis and that endothelium, in all probability, represents the primary site of its synthesis. The significance of small amounts of LACI secreted by other cells including vascular smooth muscle cells and fibroblasts is not readily evident since, to initiate extrinsic clotting, factor VII from the plasma must be made available at the site of injury. If so, then plasma LACI would be available at the injury site to regulate the clotting process, thus obscuring the importance of LACI secreted by other cells. The importance of platelet LACI has been discussed by Novotny *et al.* (35). The studies presented in this report further establish that platelet LACI is derived from megakaryocytes. Peripheral blood cells—monocytes, lymphocytes, and neutrophils—neither contained LACI mRNA nor secreted LACI in culture. This observation is consistent with our earlier observation that two monocytoid cell lines (U937 and HL-60) secreted from none to very small amounts of LACI (5, 37).

Normal vascular endothelium is primarily nonthrombogenic in nature. In addition to producing clot-promoting factors such as von Willebrand factor and plasminogen activator inhibitor 1, it produces many antithrombotic factors, including prostaglandin I_2, thrombomodulin, haparan sulfate, and tissue-type plasminogen activator (38). We now are establishing that endothelium also represents the primary

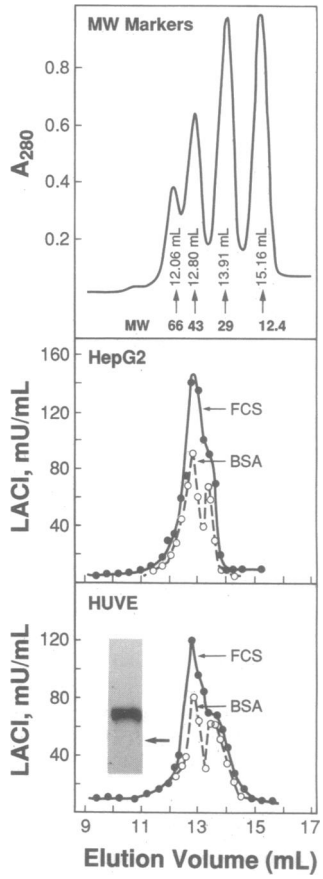

FIG. 3. Elution profile of LACI activity on Superose 12 gel-filtration chromatography using a Pharmacia FPLC system. Conditioned media from confluent monolayers (±2% FCS) cultured for 48 hr were collected, and the proteins present in 40–70% ammonium sulfate precipitates were dissolved in Tris/NaCl buffer and dialyzed against the same buffer. Each sample was centrifuged at 100,000 × *g* for 15 min and a 200-μl sample containing ≈2 units of LACI per ml was loaded onto the column at room temperature. The column was developed with Tris/NaCl buffer (0.2 ml/min) and fractions of 100 μl were collected in 10 μl of BSA (1 mg/ml). (*Bottom*) LACI activity from HUVE cell medium collected in the presence of 2% FCS (●) or 0.5% BSA (○). (*Middle*) LACI activity from HepG2 cell medium collected in the presence of 2% FCS (●) or 0.5% BSA (○). (*Top*) Absorbance elution profile of molecular weight (MW) markers—namely, BSA (66,000), ovalbumin (43,000), carbonic anhydrase (29,000), and cytochrome *c* (12,400). (*Bottom Inset*) Biosynthetically ^{35}S-labeled LACI from 20-hr medium of HUVE cells. The cells (two experiments) were cultured in methionine-free RPMI 1640 medium (plus 2% dialyzed FCS) supplemented with 50 μCi of [^{35}S]methionine per ml. The proteins present in the 40–70% ammonium sulfate precipitates of conditioned medium were dialyzed against Tris/NaCl buffer and incubated overnight at 4°C with one-tenth volume each of rabbit anti-bovine whole serum and rabbit anti-trpE protein. The samples were centrifuged and protein A-Sepharose (30% by volume) was added to the supernatants for 1 hr at room temperature. The suspensions were again centrifuged, and rabbit anti-LACI fusion protein (10% by volume) was added to the supernatants for 2 hr at room temperature. The protein A-Sepharose (15% by volume) was then added for 1 hr. The samples were centrifuged, and the pellets washed three times with Tris/NaCl buffer. The pellets were suspended in 2% SDS/5% 2-mercaptoethanol and analyzed by SDS gel electrophoresis according to Laemmli (36). The autoradiogram shown depicts the results of one experiment. It was developed from a gel to which 60 μl of a sample containing 700 cpm was applied. The exposure time was 10 days at −70°C with one intensifying screen. The major band had a molecular weight of 40,000 and the minor band (indicated by arrow) had a molecular weight of 34,000. In the second experiment (gel not shown), a band corresponding to a molecular weight of ≈70,000 was also observed. Its intensity was estimated to be ≈15–20% of the 40,000 molecular weight band. It may represent an aggregated form (dimer) of LACI. A similar molecular weight LACI moiety has also been observed in HepG2 cell cultures (4).

synthesis site of LACI, an important inhibitor of extrinsic pathway of coagulation. These properties of normal endothelium presumably serve to promote the right balance between hemostasis and recovery.

Three transformed liver-derived cell lines and presumably fetal liver synthesize LACI (6), whereas normal hepatocytes do not (present study). Since the regulation of LACI gene expression has not been investigated as yet, it is difficult to state under what conditions this gene may be turned on in the hepatocytes. Moreover, a small group of patients with fatal hepatic dysfunction has been described to have low plasma LACI levels (13). There are also data suggesting that abetalipoproteinemia may be associated with low levels of LACI in plasma (15). Since in abetalipoproteinemia due to liver dysfunction levels of low density lipoproteins may be low and since a significant amount of LACI is associated with low density lipoproteins in plasma (7), it is possible that in the few fatal hepatic dysfunction patients low levels of LACI reflect accelerated clearance of free LACI rather than decreased synthesis by the liver.

In an earlier study (5) and in the present study, two different molecular weight species of LACI (≈41,000 and 33,000) were noted in the conditioned media collected in the *absence* of FCS from HepG2 and HUVE cell cultures. Two similar molecular weight species were also noted in the serum-free media of stimulated U937 cell cultures (37). However, only the higher molecular weight species (≈41,000) of LACI was observed either in the FCS- or in the protease inhibitor-containing medium obtained from HepG2 or HUVE cell cultures (refs. 4 and 12; present study). Since FCS is known to contain protease inhibitors, it would appear that the ≈33,000 molecular weight form of LACI is a proteolytic product of the ≈41,000 molecular weight form and the presence of the protease inhibitors in the conditioned medium prevent the degradation of the 41,000 molecular weight form to the 33,000 molecular weight form. Since the carboxyl-terminal domain of LACI does not appear to have an apparent function in *in vitro* assays, we speculate that the ≈33,000 molecular weight active form of LACI lacks a portion of the carboxyl segment of the inhibitor. Human plasma also contains two predominant forms of LACI with molecular weights of ≈40,000 and ≈34,000 (5, 7, 12).¶ Recent data of Novotny *et al.* (7) provide conclusive evidence that the plasma 34,000 molecular weight species represents the native undegraded form of LACI and the 40,000 molecular weight species represents native LACI associated with the apolipoprotein A-II in mixed disulfide linkages. Thus, the differences in size of plasma LACI (≈34,000) and those of HepG2, HUVE, and U937 LACI (≈41,000) may be related to different degrees of glycosylation.

¶The molecular weight of free LACI and apolipoprotein A-II disulfide-linked LACI for ref. 7 was calculated by using the information provided in the "Note Added in Proof" section of that article.

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