Hedgehog Lipoprotein(a) Is a Modulator of Activation of Plasminogen at the Fibrin Surface

An In Vitro Study

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Lipoprotein(a) (Lp[a]), a highly atherogenic lipoprotein particle, is the prominent apolipoprotein B-containing lipoprotein in the hedgehog (Laplaud PM et al, J Lipid Res 1988;29:1157-1170). In the present work, we studied the consequences of the structural homology between the specific Lp(a) glycoprotein, apoprotein(a), and plasminogen on the generation of plasmin by fibrin-bound tissue-type plasminogen activator. The activation of plasminogen was initiated by adding either native plasma or Lp(a)-free plasma supplemented with the equivalent of 0.25 mg/ml of either purified Lp(a) or albumin to a surface of fibrin prepared on microtitration plates and to which human tissue-type plasminogen activator was specifically bound. With the Lp(a)-free plasma, an increase in the binding and activation of plasminogen as a function of time was observed. In contrast, in the presence of Lp(a) (i.e., native plasma or the reconstituted system), a significant decrease in the binding of plasmin(ogen) (~60%) was obtained. These data indicate that hedgehog Lp(a) interferes with the binding and activation of plasminogen at the fibrin surface and may thereby behave as a factor regulating the extent of fibrin deposition. These results support our previous data indicating that high levels of Lp(a) may have antifibrinolytic effects in humans (Rouy D et al, Arterioscler Thromb 1991;11:629-638), are in agreement with the observation that Lp(a) is a risk factor for atherosclerotic disease, and provide further support to the view of Lp(a) as a link between atherosclerosis and thrombosis. (Arteriosclerosis and Thrombosis 1992;12:146-154)

Lipoprotein(a) (Lp[a]) is a genetically determined lipoprotein particle that contains a specific apolipoprotein, apoprotein(a) (apo[a]), that is highly homologous to plasminogen, the precursor of the fibrinolytic enzyme plasmin. However, apo(a), unlike plasminogen, is not a substrate for plasminogen activators, and high levels of the Lp(a) particle in human plasma are considered an independent risk factor for atherosclerotic cardiovascular disease. A number of studies have therefore been recently devoted to search for a possible role for Lp(a) in both atherosclerosis and thrombosis. Recent reports have indicated that Lp(a) may bind to and displace plasminogen from the surface of endothelial cells and monocytes and from immobilized fibrinogen and fibrin and, in addition, may inhibit plasminogen activation in purified systems and in plasma. In subjects with high levels of Lp(a), decreased generation of plasmin at the plasma-fibrin interface was found to be associated with the binding of Lp(a) to lysine residues of fibrin.

Lp(a) is the prominent apolipoprotein B-containing lipoprotein in a typical hibernator, the European hedgehog (Erinaceus europaeus). As described recently, apo(a) in the hedgehog shares some physicochemical and immunologic properties with its human counterpart. The hedgehog may therefore constitute a useful natural animal model for the in vivo investigation of the interactions between this highly atherogenic particle and the fibrinolytic system. In the present study, we have characterized the fibrinolytic system of the hedgehog and have investigated the effect of plasma Lp(a) on the binding and activation of plasminogen at the fibrin surface by tissue-type plasminogen.
activator (t-PA). We show that during ongoing plasminogen activation, both plasminogen and apo(a) (in the form of Lp[a]) bind to the fibrin surface and that the generation of plasmin depends on the concentration of these proteins. These results clearly indicate that the apo(a)-like glycoprotein of the hedgehog interacts with fibrin and may constitute a regulatory component of the fibrinolytic system of this hibernator.

Methods

Chemicals and Reagents

All the chemicals used were of the best reagent grade that were commercially available. Other products were purchased from the following sources: EDTA, bovine serum albumin, Tween 20, and polypethylene glycol (PEG) from Serva, Heidelberg, FRG; Ultrogel AcA 44 and DEAE-Trisacryl from IBB, Villeneuve-La-Garenne, France; chromogenic substrate methylmalonylhydroxyprolylarginine-p-nitroanilide (CBS 1065) from Diagnostica Stago, Asnières, France; lysine- and gelatin-Sepharose 4B and PD-10 Sephadex G-25 M columns from Pharmacia Fine Chemicals, Uppsala, Sweden; polyvinyl chloride U-shaped microtitration plates and plate sealers from Dynatech, Marnes-La-Coquette, France; glutaraldehyde, 25% (vol/vol) aqueous solution from TAAB Laboratories, Reading, UK; [125I]NaI from Amersham International, Amersham, Buckinghamshire, UK; and nitrocellulose from OSI, Paris, France. Benzamidine and 6-aminohexanoic acid were purchased from Aldrich, Strasbourg, France. Dansyl-l-glutamyl-glycyl-l-arginine chloromethyl ketone (GGACK) was obtained from France Biochem, Meudon, France; nonfat dry milk from Gloria, Courbevoie, France; and M-Par tubing (Spectrum Medical Industries, Los Angeles, Calif.; exclusion limit, 6,000-8,000) for three times centrifugation and brought on ice to the laboratory. Materials containing no Lp(a), aliquots of hedgehog plasma were adjusted to a density of 1.21 g/ml with solid potassium bromide. Sodium azide (0.02%, vol/vol) was added, and the resulting solution was ultracentrifuged in an MSE Prepspin 50 ultracentrifuge (MSE Scientific Instruments, Crawley, UK), equipped with an 8x14-ml aluminum fixed-angle rotor, for 36 hours at 100,000g at 17°C. The bottom fraction was then recovered by aspiration and dialyzed in Spectra-Multrion tubing (PerSeptive Biosystems, Framingham, Mass.; exclusion limit, 6,000-8,000) for three times 12 hours at 4°C against a solution containing 1.0 M NaCl, 1 mM EDTA, and 0.02% (vol/vol) Na3[NaH2PO4]

Buffers

Buffer A was 0.05 M NaH2PO4 buffer, pH 7.4, containing 0.08 M NaCl. Buffer B was 0.05 M NaH2PO4 buffer, pH 6.8, containing 0.08 M NaCl. Assay buffer was buffer A containing 0.2% (wt/vol) bovine serum albumin and 0.01% (vol/vol) Tween 20. Binding buffer was buffer B containing 0.4% (wt/vol) bovine serum albumin, 0.01% (vol/vol) Tween 20, and 2 mM EDTA. All other buffers were prepared as described in the text.

Animals and Diets

Male adult hedgehogs (Erinaceus europaeus) were bred in the Centre d'Etudes Biologiques des Animaux Sauvages located in western central France. They were kept individually in 6-m2 parks under natural conditions of light, temperature, and rainfall. The animals were fed daily with a mixture of crushed chicken meat and commercial food for dogs (Canina Duquesne-Purina, Paris, France), containing the following proportions by weight of the major constituents: protein, 20%; animal fat, 6%; carbohydrate, 5%; vitamin A, 15,000 IU/kg; and vitamin D, 1,500 IU/kg. Water was provided ad libitum.

Blood Specimens

Blood was taken from hedgehogs that had been fasted overnight for approximately 18 hours. The animals were slightly anesthetized with halothane (Fluothane, Coopers Veterinaire, Meaux, France) in oxygen, and blood was withdrawn from the jugular vein and collected on 3 mM (final concentration) EDTA. Plasma was then separated by low-speed centrifugation and brought on ice to the laboratory.

Preparation of Lipoprotein(a)-Free Hedgehog Plasma (d>1.21 g/ml Density Fraction)

We have previously demonstrated that the Lp(a) particle in the plasma of hedgehogs is contained in the 1.040–1.100 g/ml density range. Thus, to obtain material containing no Lp(a), aliquots of hedgehog plasma were adjusted to a density of 1.21 g/ml with solid potassium bromide. Sodium azide (0.02%, vol/vol) was added, and the resulting solution was ultracentrifuged in an MSE Prepspin 50 ultracentrifuge (MSE Scientific Instruments, Crawley, UK), equipped with an 8x14-ml aluminum fixed-angle rotor, for 36 hours at 100,000g at 17°C. The bottom fraction was then recovered by aspiration and dialyzed in Spectrapor tubing (Spectrum Medical Industries, Los Angeles, Calif.; exclusion limit, 6,000-8,000) for three times 12 hours at 4°C against a solution containing 0.15 M NaCl, 1 mM EDTA, and 0.02% (vol/vol) Na3[NaH2PO4]

Isolation of Hedgehog Lipoprotein(a)

To isolate hedgehog Lp(a), the methodology reported in Reference 10 was used. In brief, aliquots from hedgehog plasma pools were first brought to a density of 1.055 g/ml by addition of potassium bromide, layered with sodium chloride (d=1.055 g/ml), and centrifuged for 24 hours at 100,000g at 17°C in the same machine and rotor as reported above. The top 3 ml was discarded, and the bottom fraction was collected, placed in similar new centrifuge tubes, and mixed with sodium chloride (d=1.190 g/ml) to obtain a final density of 1.100 g/ml. Such samples were subsequently ultracentrifuged for 26 hours at 100,000g at 17°C. The top fraction (1.5 ml) was then collected and dialyzed for three times 12 hours at 4°C against 0.15 M NaCl, pH 7.0, containing 1 mM EDTA and 0.02% (vol/vol) Na3[NaH2PO4]

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tained a mixture of Lp(a) and LDL-like particles. After concentration, these two types of lipoprotein particles were separated by means of gel-filtration chromatography on a column of Sepharose CL-4B (12x1,000 mm) operated at 4°C. Elution was performed with 0.15 M NaCl, pH 7.0, at a rate of 12 ml/hour, and 2-ml fractions were collected. Elution was monitored at 280 nm. This type of chromatography led to incomplete separation of the two lipoprotein populations; therefore, the fractions corresponding to the first peak were pooled and rechromatographed in the same system. The resulting peak was approximately gaussian. Fractions corresponding to the trailing edge of this peak were discarded, and the remainder of the peak was shown to contain pure Lp(a).  

**Purification of Apoprotein(a)**

Apo(a) was purified according to a modification of the methodology of Armstrong et al.  

In brief, native Lp(a) was brought to a concentration of ~1 mg/ml total apoprotein in 20 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.4, 0.15 M NaCl, 1 mM EDTA, and 0.02% (vol/vol) NaN₃. Dithiothreitol was freshly added to a final concentration of 10 mM, and the solution was incubated at 37°C for 3 hours. The solution was then adjusted to 1.100 g/ml with solid potassium bromide and centrifuged in the MSE 6x14-ml titanium swing-out rotor at 150,000gₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑἐωσё argv for 24 hours at 10°C. After completion of centrifugation, apo(a) was a pellet that had sedimented at the base of the tube. It was solubilized in 20 mM Tris, pH 8.0, 1 mM EDTA, and 10 mM dithiothreitol, containing 85 mM sodium dodecyl sulfate (SDS).  

**Preparation of Hedgehog Plasminogen and Fibrinogen**

Plasminogen was purified from fresh hedgehog plasma by the method of Deutsch and Mertz with some modifications. The plasma was supplemented with protease inhibitors (100 kIU/ml aprotinin, 2 mM PMSF, 10 μM p-nitrophenyl-p'-guanidinobenzoate, and 1 μM PPACK), and all steps were performed at 4°C. The plasma was passed over a column of lysine-Sepharose 4B. The gel was washed with buffer A containing 10 kIU/ml aprotinin until the absorbance at 280 nm equaled that of buffer. The lysine-adsorbed proteins were eluted with a linear gradient in 6-aminohexanoic acid (0–50 mM), concentrated in a dialysis bag against solid PEG (Mw ~20,000), and applied to a column of Ultrogel AcA 44 equilibrated with buffer A. Three peaks were typically observed in the profile. Fractions corresponding to each peak were pooled separately, concentrated, dialyzed against buffer A, and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and fibrin autography. One of the proteins present in the third peak was sensitive to cleavage by plasminogen activators and is designated hereafter as hedgehog plasminogen.  

Human and hedgehog fibrinogens were purified from fresh plasma according to Kazal et al and were depleted in plasminogen and fibrinectin as described.  

**Polyclonal Antibody Against Plasminogen**

A polyclonal antibody directed against the protein content of the third peak obtained after gel-filtration chromatography was prepared as follows. Antigen (0.5 mg) in Freund’s complete adjuvant was injected subcutaneously into sheep. The same amount, in Freund’s incomplete adjuvant, was similarly injected at 3 and at 6 weeks after the initial immunization. After immunologic testing of the antiserum, the sheep was bled, and serum was separated. The immunoglobulin G fraction of the antiserum was then separated by ammonium sulfate precipitation, ion-exchange chromatography on DEAE-Trisacryl, and affinity chromatography on protein A–Sepharose.  

**Preparation of Solid-Phase Fibrin Surfaces**

The solid-phase fibrin surfaces were prepared as previously described. In brief, polyvinyl chloride-bound stable polyglutaraldehyde derivatives were first produced by treating U-shaped microtitration polyvinyl chloride plates with 2.5% (vol/vol) glutaraldehyde in 0.1 M NaHCO₃ buffer, pH 9.5, for 2 hours at 22°C. Then fibrinogen (0.3 μM in 0.1 M NaH₂PO₄ buffer, pH 7.4, containing 1 mM CaCl₂) was covalently fixed for 18 hours at 4°C. After washing, a fibrin network was generated by thrombin treatment (1 National Institutes of Health unit/ml in assay buffer containing 1 mM CaCl₂). The excess thrombin was eluted by three washes with a high-ionic-strength solution (0.5 M NaCl/8 mM CaCl₂/0.05% [vol/vol] Tween 20). A final wash with 5 mM NaH₂PO₄ buffer (pH 6.8 containing 0.05% [vol/vol] Tween 20) was performed, and 100 μl per well of buffer B, containing 0.02 M lysine, 0.2% (wt/vol) bovine serum albumin, and 0.01% NaN₃ was added. The plate was sealed and stored in this state at 4°C until further use. Fibrin surfaces were prepared with both human and hedgehog fibrinogens. The ability of thrombin to cleave human fibrinogen was monitored by use of a horseradish peroxidase–labeled mouse monoclonal antibody (Y18-HRP) kindly provided by Organon Teknika (Fresnes, France). The immunoreactivity of this antibody with the Aα stretch 1–51 of human fibrinogen disappeared on treatment with thrombin.  

**Assay Methods**

Lp(a) in hedgehog plasma was determined by an immunonephelometric method (Behring nephelometer analyzer) with a monospecific sheep antisera to human Lp(a) (Immuno AG, Vienna, Austria) and a reference human standard in which the Lp(a) mass content had been previously determined by electroimmunodiffusion (Immuno AG). The validity of this technique for measuring plasma Lp(a) concentrations in the hedgehog was verified as follows. The lipoprotein concentration of Lp(a) purified from a pool of hedgehog plasma as indicated above was ascertained by chemical analysis. Immunonephelometric measurements of Lp(a) in both serial dilutions of the purified Lp(a) and in plasmas supplemented
with increasing concentration of the same Lp(a) preparation were then performed. In both series of experiments, results showed that the method in use was satisfactory for hedgehog Lp(a) measurements. More specifically, the range of results obtained for a given hedgehog Lp(a) concentration was within 89–106% of those obtained for the same concentration of human Lp(a); in addition, a linear response to supplementation in Lp(a) was obtained throughout the range of concentrations of this lipoprotein encountered in our animals according to season, that is, 0–100 mg/dl.

Plasminogen antigen concentration in hedgehog plasma was determined by radial immunodiffusion according to Mancini et al.18 by using the sheep antibody described above and an aliquot of the content of the third peak obtained during gel-filtration subtraction fractionation of the lysine-absorbable proteins of hedgehog plasma as a standard.

The activatable plasminogen was determined in plasma by spectrophotometric assay consisting of fibrin-bound t-PA (see below), a volume dilution (final, 1:80) of plasma in binding buffer, and 1.5 mM CBS 1065. Human plasmin was used as a reference, and the initial rate of activation under first-order conditions was determined as indicated below.

The antiplasmin activity of hedgehog plasma was determined by titration of added human plasmin in a spectrophotometric assay as described previously,19 except that residual plasmin activity was detected with 1.5 mM of the chromogenic substrate CBS 1065 at a double-wavelength absorbance ratio (405 nm/490 nm).

Activation of Plasma Plasminogen by Fibrin-Bound Tissue-Type Plasminogen Activator

The activation of hedgehog plasminogen by human t-PA on a fibrin surface was performed as indicated elsewhere.20 In brief, a solid-phase fibrin plate was washed three times with binding buffer, and 50 µl per well of the same buffer containing 20 IU/ml human t-PA was incubated for 1 hour at 37°C. The plate was then extensively washed with binding buffer to eliminate unbound proteins, and the reaction was started by adding 50 µl per well of the activation mixture. This mixture was either native plasma or Lp(a)-free plasma supplemented with the equivalent of 0.25 mg/ml of either Lp(a) or albumin. The activation was performed in a series of three wells and was monitored for 4 hours. Every 5–15 minutes, the reaction was stopped by removing the fluid phase and washing the wells with assay buffer. When indicated, a trace amount (final concentration, 0.3–0.7 µg/ml) of hedgehog 125I-plasminogen was added. Products present in the solid phase were analyzed by spectrophotometry, radioisotopic counting, SDS-PAGE autoradiography, and specific immunoblotting as follows. Fibrin-bound plasmin activity was detected by adding 50 µl 1.5 mM CBS 1065 in assay buffer per well and measuring the change in absorbance (ΔA405/min) at a double-wavelength absorbance ratio (A405/A490) with a microtitration plate counter (MR 610, Dyna-tech) equipped with a thermostatic device to maintain temperature at a constant 37°C. Initial velocities of each reaction were calculated by using an appropriate statistical analysis of data in its original coordinate system, that is, ΔA405 versus time, as described previously.15 Because CBS 1065 is a plasmin-selective chromogenic substrate and fibrin-bound plasmin was measured at the end of the activation in the absence of plasminogen, it is very unlikely that fibrin-bound t-PA was interfering with the assay. Moreover, experiments were performed to detect any possible hydrolysis of the chromogenic substrate by Lp(a).

To quantify the amount of fibrin-bound plasminogen and to identify the molecular derivatives of plasminogen bound to fibrin, the experiments were performed in the presence of 125I-hedgehog plasminogen, and the reaction was stopped with assay buffer supplemented with 1 mM benzamidine and 10 µM GGACK. At the end of activation, the radioactivity in the wells was counted in a gamma radiation counter. In parallel experiments, the molecular species bound to the fibrin surface were eluted by incubating 50 µl 500,000 cpm/ml. The nitrocellulose was washed and autoradiographed on Kodak XS-1 films for 8–12 hours at 22°C. The eluates were used to identify the plasminogen-derived products by SDS-PAGE and immunoblotting.

Electrophoretic Methods

Gradient gel electrophoresis. Continuous-gradient slab-gel electrophoresis was performed in a Pharmacia electrophoresis apparatus GE 2/4 loaded with gradient gels PAA 2/16 (Pharmacia), according to the conditions of Nichols et al.21 For particle-size calibration, we used a series of markers ranging in hydrated diameters from 71 to 170 Å (High Molecular Weight electrophoresis calibration kit, Pharmacia) and a solution of latex particles (Dow Chemical, 380 Å diameter). The Stokes’ diameters of the particles were calculated by the Stokes-Einstein equation described by Anderson et al.22

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting. SDS-PAGE and immunoblotting were performed essentially as described in References 23 and 24, with minor modifications. After blotting the proteins from the polyacrylamide gel, the nitrocellulose paper was incubated with 5% (wt/vol) nonfat dry milk in 10 mM Tris buffer, pH 7.4, to block free binding sites. Blotted proteins were probed with the antiserum against hedgehog plasminogen diluted 1:10 with assay buffer. After washing, the membrane was soaked in a solution of radiolabeled protein A (~500,000 cpm/ml). The nitrocellulose was washed and autoradiographed on Kodak X-Omatic intensifying screen.

Radioiodination of Proteins

Hedgehog plasminogen and protein A were radioiodinated with 125I using the lodogen method of Fraker and Speck23 with the following modifications: an iodination time of 4 minutes at 4°C and removal of free 125I by molecular sieving on a PD-10 Sephadex
Activation of Plasminogen by Fibrin-Bound
Hedgehog Lysine-Adsorbable Proteins

Proteins eluted from the lysine-Sepharose 4B column were concentrated and then rechromatographed on a column of Ultrogel AcA 44. Typically, three peaks were obtained. In each series of experiments, proteins in each fraction were analyzed by SDS-PAGE. In one experiment, proteins in each peak were examined equally by gradient-gel electrophoresis, thus demonstrating that the first peak contained a protein of apparent M_r of ~620,000. Such a value was comparable to that already reported by us for certain forms of hedgehog apo(a).\(^{10}\)

This molecule exhibited cross-immunoreactivity with the sheep antibody against hedgehog plasminogen, as determined by immunoblotting (see below). Proteins in the third peak were resolved into three protein bands by SDS-PAGE. Plasminogen was identified by fibrin autography in the protein band migrating with an M_r of ~93,000 (Figure 1), a molecular weight similar to that of human plasminogen. Two other bands migrating in a more anodic position and exhibiting relative molecular weights of 79,000 and 72,000 did not produce fibrinolytic activity, either spontaneously or in the presence of activators. Although a trace of plasminogen was also detected in the last fractions of the third peak, only the third peak was considered for certain forms of hedgehog apo(a).\(^{10}\)

A portion of the gel was soaked for 1 hour in 2.5% (vol/vol) Triton X-100 to remove SDS and then in distilled water for 5 minutes. The gel was then overlaid on a fibrin-agar indicator gel prepared in a moist chamber as described previously.\(^{26}\)

Autographs were allowed to develop at 37°C for 6–48 hours and then photographed with dark-ground illumination.

Results

Hedgehog Lysine-Adsorbable Proteins

Plasminogen was also detected in the last fractions of the second peak, only the third peak was considered to contain plasminogen for the purpose of the experiments described here. Although proteins in the second chromatographic peak were eluted with an apparent molecular weight corresponding to that of 7S gamma globulins, they were resolved by SDS-PAGE into two protein bands identical to those with M_r 79,000 and 72,000 found as contaminants of plasminogen in the third peak. Fibrinogen purified from hedgehog plasma migrated as human fibrinogen purified and electrophoresed under similar conditions (not shown).

Activation of Plasminogen by Fibrin-Bound Tissue-Type Plasminogen Activator

Activatable plasminogen in hedgehog plasmas (from eight animals) was equivalent to 226 ± 97 nM human plasminogen. The corresponding value in humans is 721 ± 98 nM (10 donors). The difference is most probably related to the higher level of antiplasmin activity observed in hedgehog plasma (145–200% compared with 80–125% for normal human plasmas) and to the presence of a substantial amount of nonactivatable lysine-binding proteins. The higher plasminogen antigen value (350–500 mg/l) found in these animal plasmas relative to the value in humans (150–200 mg/l) is in agreement with the latter observation and with the existence of a cross-reaction between the lysine-adsorbable proteins and the antibodies raised against plasminogen present in the third chromatographic peak.

Plasminogen activation experiments were performed using surfaces of fibrin prepared from monolayers of human or hedgehog fibrinogen covalently bound through polyglutaraldehyde to a solid support. The validity for use of such a surface of fibrin for plasminogen activation experiments at the plasma-fibrin interface has been previously demonstrated.\(^{20}\)

In these experiments, human t-PA was first bound to the fibrin surface, unbound proteins were eliminated by washing, and hedgehog plasma was added to initiate the activation. The extent of plasmin generation at the surface of fibrin was measured with a plasmin-selective chromogenic substrate. The possible hydrolysis of the chromogenic substrate by Lp(a) was investigated before these experiments were performed; even at high Lp(a) concentrations and after 3–4 hours of incubation at 37°C, we were unable to obtain an increase in the absorbance at 405 nm relative to that of the substrate alone. The results
The amounts of plasmin generated as a function of time are represented by the initial velocities (v) expressed as

\[ v = \frac{\Delta A_{405}}{t} \]

where \( \Delta A_{405} \) is the change in absorbance per minute at 405 nm. At the end of activation, the wells were washed, dried, and cut out, and their respective radioactivities were counted in a gamma radiation counter.

**Role of Lipoprotein(a) in the Activation of Plasminogen**

To determine whether Lp(a) bound to the fibrin surface was interfering with the binding of plasminogen, comparative activation experiments were performed with native hedgehog plasma, Lp(a)-free plasma, and Lp(a)-free plasma supplemented with the equivalent of 0.25 g/l protein mass of either Lp(a) or albumin. Typical activation profiles obtained under these conditions are shown in Figure 4. The amount of plasminogen derivatives bound to fibrin as a function of time was markedly decreased when the activation was performed with native plasma (80% decrease at 4 hours; Figure 4A) and with Lp(a)-free plasma supplemented with 0.25 g/l Lp(a) (60% decrease at 4 hours; Figure 4B) compared with the activation obtained in the absence of Lp(a). It is of note that the respective antiplasmin activities of Lp(a)-free and native plasmas were similar, thus indicating that the increased generation of plasmin was actually related to the content in Lp(a).

**Discussion**

In the human species, the generation of plasmin at the plasma–fibrin interface is finely regulated at the level of both t-PA and plasminogen. Plasminogen interactions with two plasma proteins, histidine-rich...
glycoprotein and α2-antiplasmin, and with fibrin are mediated through lysine-binding sites present in the kringle domains of plasminogen.19,28,29 As a consequence, the amount of plasminogen available for fibrin and its transformation into plasmin are lowered by 50% in plasma (activatable plasminogen was 721 ± 98 nM in 10 normal volunteers). Exposure of new plasminogen-binding sites on plasmin-degraded fibrin is considered to be a potent effector function that increases the rate of plasmin formation by t-PA.30,31 A mechanism that would modulate the extent of fibrinolysis by decreasing the number of such new binding sites has not yet been described. In particular, under physiological conditions, no molecule or circulating protein has been shown to behave as plasminogen vis-a-vis its fibrin-binding properties. The demonstration that apo(a) is composed mainly of structural domains homologous to kringle 4 and 5 of human plasminogen suggests that this protein may be a likely ligand for plasminogen-binding sites in fibrin. Indeed, we have recently shown that in human subjects with high levels of Lp(a), a decreased generation of plasmin at the plasma–fibrin interface was associated with the binding of Lp(a) to lysine residues of fibrin.8,9 In the present work, we have further investigated the possible role of apo(a) as a modulator of plasminogen activation. For this purpose, we have studied an animal model, the hedgehog, which has been shown to possess Lp(a) as its prominent apolipoprotein B–containing plasma lipoprotein.10 Except for the human source of t-PA, these studies were performed with hedgehog plasma or its derived products, including Lp(a), apo(a), fibrinogen, and plasminogen. From the results reported in the present study, two main aspects should be stressed. First, a group of lysine-adsorbable proteins was separated from plasma by lysine affinity chromatography and subsequently fractionated by gel filtration. One of these proteins had an unusually high Mr (~620,000), resembling that of purified hedgehog apo(a) and reacting with antibodies against hedgehog plasminogen and against human Lp(a) on immunoblotting. Another protein had the same molecular mass as human plasminogen (Mr 93,000) and was efficiently transformed into a fibrinolytic enzyme by plasminogen activators; it was therefore considered to be plasminogen. Two other protein bands migrating with a molecular mass (Mr 79,000 and 72,000) lower than that of plasminogen had no proteolytic activity and were not activated by plasminogen activators. These proteins were the main components of the second chromographic peak of the lysine-adsorbable proteins, and their presence in the peak of plasminogen was due to incomplete chromatographic resolution. It is of note that their relative molecular weights determined by SDS-PAGE were lower than expected from their chromatographic behavior, suggesting the existence in plasma of a molecular form of relatively higher molecular weight. Because the isolation of these proteins was performed under conditions that avoided proteolytic degradation immediately after sampling and during the purification procedure, we suggest that such forms are probably present in circulating plasma. Furthermore, electrophoresis of the purified products under nonnaturating conditions also showed the presence of such molecular forms. Further biochemical and functional characterization of these lysine-adsorbable proteins is in progress in our laboratory.

Second, the preparation of a hedgehog fibrin surface was feasible and proved to be useful for the binding of human t-PA and the activation of hedgehog plasminogen either in a reconstituted system or in native plasma. The generation of plasmin by fibrin-bound t-PA was markedly decreased when the activation was performed with Lp(a)-free plasma supplemented with purified Lp(a). In addition, we have positively documented the binding of the Mr ~620,000 apo(a)-like protein and, to a lesser extent, of other lysine-absorbable proteins to fibrin during ongoing plasminogen activation. Considered together, these data indicate that in the hedgehog the activation of plasminogen at the plasma–fibrin interface is modulated by proteins containing kringle structures, mainly apo(a), that bind to fibrin. Because this phenomenon was documented in intact animals bred under natural conditions, our data strongly sug-
Concentration of plasminogen was kept constant by adjusting the final volumes (•). Plasma (0.25 g/l) or with a solution of albumin

Activation with plasma, that is, Lp(a)-free plasma. Final plasminogen concentration was similar in the two samples.

The modulation of plasminogen activation by Lp(a) in humans are a risk factor for atherosclerotic cardiovascular disease and to support the view of Lp(a) as a link to the observation that high levels of Lp(a) in obviously a seasonal physiological phenomenon. There-

gest that, at least in the hedgehog, Lp(a) may constitute an important regulatory factor of fibrinolysis.

The European hedgehog exhibits naturally occurring seasonal variations in the plasma concentration of Lp(a). These variations are reproducible from one year to another and are, at least in part, under endocrine control. In the human species in contrast, the plasma level of Lp(a) appears to be genetically deter-

minded and is relatively constant, being insensitive to most dietary changes and hypocholesterolemic drugs. The modulation of plasminogen activation by Lp(a) in the hedgehog, as demonstrated in the present report, is obviously a seasonal physiological phenomenon. Therefore, such seasonal variations may help to provide a clue to the observation that high levels of Lp(a) in humans are a risk factor for atherosclerotic cardiovascular disease and to support the view of Lp(a) as a link between atherosclerosis and thrombosis.

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