Anticoagulant Activity of Tissue Factor Pathway Inhibitor in Human Plasma Is Preferentially Associated With Dense Subspecies of LDL and HDL and With Lp(a)

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Human plasma contains a multivalent, Kunitz-type proteinase inhibitor termed tissue factor pathway inhibitor (TFPI), which specifically inhibits the action of the factor VII(a)-tissue factor complex in coagulation. In the present study, we examined the distribution and anticoagulant activity of TFPI among plasma lipoprotein subspecies separated by isopycnic density gradient ultracentrifugation; this procedure permitted the simultaneous fractionation of the major lipoprotein classes (very-low-density lipoprotein [VLDL], intermediate-density lipoprotein [IDL], low-density lipoprotein [LDL], high-density lipoprotein [HDL] 2 and 3, and very-high-density lipoprotein [VHDL]). Studies of eight normolipidemic subjects revealed two major lipoprotein carriers of TFPI activity: dense LDL subspecies \( d = 1.039 \) to 1.063 g/mL and both dense HDL particles and VHDL \( d = 1.133 \) to 1.190 g/mL, representing 33.8% and 35.9%, respectively, of the total lipoprotein-associated TFPI activity in plasma. TFPI activity was also associated with lipoprotein(a) (Lp(a)), whose density distribution \( d \leq 1.044 \) to 1.100 g/mL overlapped that of LDL and HDL; such association was related to Lp(a)'s particle size and phenotype. VLDL, IDL, and LDL, \( d \leq 1.019 \) to 1.039 g/mL, HDL \(_2\) \( d = 1.063 \) to 1.100 g/mL, and light subfractions of HDL \(_2\) \( d = 1.100 \) to 1.167 g/mL conveyed only 1.8%, 10%, and 18.5%, respectively, of lipoprotein-associated TFPI activity. Such anticoagulant activity was dependent on the presence of TFPI protein. The dense subspecies of HDL \(_2\) \( d = 1.133 \) to 1.167 g/mL with which TFPI was preferentially associated were small, displayed a cholesteryl ester to protein ratio of \( -0.2 \), and were deficient in phospholipid (13.6% to 18.3%). HDL subspecies of \( d = 1.110 \) to 1.167 g/mL mainly contained the higher relative molecular mass form of TFPI of 41 kD (a form that is known to be covalently associated with apolipoprotein [apo] A-II) and minor bands of the 35- and 52-kD forms. The second major localization of TFPI was within the hydrated density range of small, dense LDL particles \( d = 1.033 \) to 1.063 g/mL, which in comparison with light LDL \( d = 1.019 \) to 1.033 g/mL, exhibited a markedly lower proportion of triglyceride and enrichment in cholesteryl ester. Analysis of the ratios of the percent mass of cholesteryl ester to free cholesterol in LDL subfractions showed that the increase in cholesteryl ester content was positively correlated with an increase in TFPI activity \( r = .86, P < .0013 \); equally, a positive correlation between an increase in the free cholesterol to protein ratio in LDL and an increase in TFPI activity \( r = .89, P < .0006 \) was detected. In contrast to dense apo A-I-containing lipoprotein particles, dense LDL particles of \( d = 1.039 \) to 1.058 g/mL mainly transported the 35-kD form of TFPI. We conclude that among the spectrum of apo B-containing lipoprotein subspecies, small, dense LDL particles provide the most favorable surface structure for efficient TFPI binding and equally, for the expression of its anticoagulant activity.

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itiation of blood coagulation via the extrinsic pathway occurs when tissue factor (TF) is exposed to plasma. The resulting factor VIIa–TF complex then proteolytically activates factors IX and X.\(^1\) Physiological regulation of this pathway is mediated by a specific factor \( Xa \)-dependent inhibitor of the factor VIIa–TF complex, which has been termed lipoprotein-associated coagulation inhibitor (LACI),\(^2\) extrinsic pathway inhibitor (EPI),\(^3\) or tissue factor inhibitor (TFI); this factor is now denoted "tissue factor pathway inhibitor" (TFPI).\(^4\) TFPI is present in vivo in three separate pools: one is stored in blood platelets,\(^6\) and a second is released into plasma by intravenous heparin injection\(^7\) and is probably of endothelial cell origin,\(^8\) while a third is associated with lipoproteins.\(^9\) The TF inhibitor activity of normal

*The Scientific and Standardization Committee of the International Society on Thrombosis and Hemostasis recommended use of the term “tissue factor pathway inhibitor” (TFPI).
plasma, present at concentrations of 50 to 100 ng/mL, was separated by gel filtration into three distinct fractions. More than 50% of plasma TFPI was isolated in the high-molecular-weight fraction and was associated with apolipoprotein (apo) B–containing lipoprotein particles, ie, low-density lipoproteins (LDLs) and very-low-density lipoproteins (VLDLs). A second form, of intermediate molecular weight and representing 44% of plasma TFPI, was present in covalent association with apo A-II in apo A-I–containing lipoproteins, ie, high-density lipoproteins (HDLs), while the low-molecular-weight form (6% of TFPI) was probably uncomplexed.

The functional and structural properties of the lipoprotein-associated forms of TFPI are, however, largely indeterminate.

It is now established that HDL appears to afford protection against coronary artery disease (CAD), probably as a result of the central role of these particles in the reverse cholesterol transport system. In contrast, elevated plasma levels of LDL particles or of lipoprotein(a) (Lp[a], a structural variant of LDL) are a primary risk factor. A number of therapeutic studies have clearly demonstrated the beneficial effects of a reduction in LDL levels, and conversely of an elevation in those of HDL, on both the prevention of CAD and the regression of atherosclerotic plaques. Nevertheless, quantitative elevation of plasma LDL or subnormal concentrations of HDL do not of themselves provide an explanation for the complex mechanisms underlying CAD.

Both LDL and HDL are not structurally and metabolically homogeneous species of lipoproteins, but rather they consist of a spectrum of lipoprotein particle subpopulations of varying molecular weights, buoyant density, and composition. The physicochemical heterogeneity of LDL particles is related to differing degrees of atherosclerosis risk. Indeed, dense LDL, which possesses diminished resistance to oxidative stress, may be potentially more atherogenic. Equally, plasma HDLs are highly heterogeneous, reflecting the diverse pathways of formation and interconversion of these particles. Two major subclasses of HDL have been defined on the basis of density: HDL$_1$ and HDL$_2$. The HDL$_1$ subclass appears to consist of particles that may correspond to primary acceptors of cellular free cholesterol.

In the present study, our aims were to evaluate the possible contribution of TFPI to the physicochemical heterogeneity and metabolic behavior of apo B– and apo A-I–containing lipoprotein subspecies and to compare the antithrombotic capacity of individual subspecies with their potential atherogenicity. We therefore determined the distribution of TFPI among lipoprotein subspecies, the nature of the interaction of TFPI with lipoprotein particles, and the antithrombotic activity associated with such particles. Our data demonstrate that expression of antithrombotic activity and the presence of TFPI protein are closely correlated and that such activity is primarily due to the preferential interaction of TFPI with small, dense LDL subspecies, with a particle population of the densest HDL subclass (HDL$_1$), and with very-high-density lipoprotein (VHDL); equally, TFPI activity was associated with Lp(a).

**Methods**

**Materials**

Purified coagulation factors were of human origin except for TF, which was prepared from rabbit brain (Sigma Diagnostics). Factors VIIa, X, and Xa were obtained from Diagnostica Stago (Asnières, France). Three reagents were dissolved according to the instructions of the manufacturer and further diluted in a buffer containing 0.05 mol/L tris(hydroxymethyl)aminomethane (Tris) HCl, 0.1 mol/L NaCl, 0.01 mol/L sodium citrate, and 0.02% NaN$_3$; this solution is hereafter referred to as TSC buffer. Bovine serum albumin (BSA, Sigma) was added to this solution (final concentration, 2 mg/mL) to constitute the TSC/BSA buffer. Rabbit TF was adjusted to optimal concentration with distilled water, and under such conditions the clotting time of normal plasma was 16 seconds (equal volumes of plasma, 35 mmol/L CaCl$_2$, and TF). The chromogenic substrate S2222 (benzoyl-Ile-Glu-Arg-p-nitroanilide; Kabi) was dissolved in distilled water at a final concentration of 2.7 mmol/L. Low-molecular-weight standards for sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. A rabbit polyclonal antibody to human TFPI was the kind gift of Dr G.J. Broze. An $^{125}$I-labeled antibody to rabbit immunoglobulin G (IgG) was supplied by Amersham International.

**Blood Samples**

Subjects were healthy normolipidemic volunteers (5 males and 3 females) who had fasted overnight. Venous blood was collected into glass bottles containing Na$_2$EDTA (1 mg/mL), from which plasma was rapidly separated by low-speed centrifugation (1000g, 20 minutes) at 4°C. Immediately after collection of plasma samples, gentamicin (50 µg/mL), EDTA (0.01%), and NaN$_3$ (0.01%) were added. The concentrations of lipids (total cholesterol, 199±43 mg/dL plasma; total triglyceride, 61±15 mg/dL plasma) in each plasma sample corresponded to values typical of a normolipidemic population.$^{14}$ The mean concentration of Lp(a) was 11±5 mg/dL plasma.

**Fractionation of Lipoproteins**

**Density gradient ultracentrifugation.** Lipoproteins were fractionated by isopycnic density gradient ultracentrifugation using a Beckman SW41 Ti rotor at 40 000 rpm for 44 hours in a Beckman XL70 centrifuge at 15°C by a slight modification of the method described by Chapman et al.$^{23}$ Briefly, plasma density was increased to 1.21 g/mL by addition of dry, solid potassium bromide. Construction of a discontinuous density gradient at ambient temperature was initiated by pumping 2 mL of a sodium chloride–potassium bromide solution of $d=1.24$ g/mL into the bottom of the tube. The following solutions were then layered above: 3 mL of plasma at 1.21 g/mL; 2 mL of a sodium chloride–potassium bromide solution of $d=1.063$ g/mL; 2.5 mL of $d=1.019$ g/mL; and 2.5 mL NaCl solution of $d=1.006$ g/mL. All density solutions contained 0.01% NaN$_3$, 0.01% EDTA, and 0.005% gentamicin at pH 7.4. After ultracentrifugation, gradients were collected by successive aspiration of 24 fractions, 0.4 mL each, from the meniscus downward. All fractions were dialyzed in Spectrapor mem-
Dissociation of Lipoprotein-Associated TFPI with the same buffer as that used for LDL incubation. The VLDL-deficient plasma (d<1.017) was then applied to a column of Ultrogel AcA 22 (height, 50 cm; diameter, 2.5 cm) at a flow rate of 0.6 mL/min. Size exclusion chromatography was carried out at 4°C; the elution buffer contained phosphate-buffered saline (PBS) and EDTA (0.1%). Fractions of 0.6 mL each were collected and the contents of each subtraction as the sum of the mass of the individual components (free cholesterol, cholesteryl ester, triglyceride, phospholipid, and protein).

Gradient Ultracentrifugation

Density Distribution and Chemical Composition of Lipoprotein Subfractions Isolated From Normolipidemic Plasma by Density Gradient Ultracentrifugation

<table>
<thead>
<tr>
<th>Gradient fraction* and corresponding range of density limits†</th>
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<tr>
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<tr>
<td>&lt;1.017</td>
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**Components‡**

- Free cholesterol
- Cholesteryl ester
- Triglyceride
- Phospholipid
- Protein

**Lipoprotein species**

- VLDL
- IDL
- LDL₁
- LDL₂
- LDL₃
- LDL₄

**Percent of lipoprotein mass§**

- 2.7
- 1.9
- 2.5
- 5.4
- 19.9
- 15.4

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*All fractions were 0.4 mL each.
†Density limits were taken from a standard curve of density versus volume derived from control gradients containing only salt solutions (see Reference 25) and are in grams per milliliter.
‡Values are the means of duplicate analyses of each fraction isolated from the plasmas of eight normolipidemic subjects and are in weight percent.
§Lipoprotein mass corresponds to the sum of all lipid and protein components in each fraction.

brane tubing (relative molecular mass cutoff, 3500) at 4°C against 20 mmol/L NH₄HCO₃ buffer containing 0.1% EDTA at pH 7.4 before analysis.

**Gel filtration chromatography.** Plasma samples (up to 250 mL) were ultracentrifuged in a 50 Ti rotor for 18 hours at 15°C and 45 000 rpm. The top 1-mL fraction of d<1.006 g/mL containing triglyceride-rich lipoproteins was eliminated. The VLDL-deficient plasma (d>1.006 g/mL; 60 mL) was then applied to a column of Ultrigel AcA 22 (height, 50 cm; diameter, 2.5 cm) at a flow rate of 0.6 mL/min. Size exclusion chromatography was carried out at 4°C; the elution buffer contained phosphate-buffered saline (PBS) and EDTA (0.1%). Fractions of 2 mL were collected and the contents of each subtraction as the sum of the mass of the individual components (free cholesterol, cholesteryl ester, triglyceride, phospholipid, and protein).

**Lipid and Lipoprotein Analysis**

Lipids in plasma or isolated lipoprotein fractions were analyzed by enzymatic determinations using Bio-Merieux kits (Marcy l'Etoile, France) for total cholesterol, free cholesterol, phospholipids, and triglycerides. Cholesteryl ester mass was calculated as 1.67x(free cholesterol mass). The protein content of lipoprotein fractions was determined by the procedure of Lowry et al. Lipoprotein mass was calculated for each subtraction as the sum of the mass of the individual components (free cholesterol, cholesteryl ester, triglyceride, phospholipid, and protein).

**Assay of TFPI Activity**

TFPI activity was measured by a slight modification of the method described by Sandset et al. The assay was performed in microtiter plates in a 37°C water bath. Duplicate aliquots (25 µL) of the test sample or standard were first distributed into wells. The amido-lytic assay was performed in two stages by incubation of 100 µL of combined reagent I for 20 minutes, followed by incubation of 50 µL of combined reagent II for 25 minutes. Reagent I contained TF, factor Xa, factor VIIa, and CaCl₂ at final concentrations of 1% (vol/vol), 5 mU/mL (0.8 mmol/L), 2.5 mU/mL (25 pmol/L), and 15 mmol/L, respectively. Reagent II was a mixture (vol/vol) of factor X (0.4 U/mL; 60 mmol/L) and S2222 (2.7 mmol/L). The reaction was stopped by the addition of 50 µL of 50% acetic acid. Absorbance (OD) was read at 405 nm by using a microtiter plate reader (Dynatech). Standard curves were prepared by assaying dilutions (0 to 1%) of pooled, heated, citrated plasma diluted in TSC/BSA buffer containing 2 µg/mL heparin. Heparin was added to a final concentration of 100% to prevent any influence of heparin, as was described earlier. TFPI activity in 1 mL of plasma was arbitrarily defined as 100% activity (or 1 U/mL).
plot of TFPI activity (as OD₄₅₀ units) as a function of progressive dilutions of a pooled plasma sample was used for calibration purposes. TFPI activity in lipoprotein subfractions was then expressed relative to the lipoprotein mass content of each subfraction. Our dilutions ranged from 0 to 25 pmol/L in our standard when using a pooled plasma at a final concentration of 2.5 nmol/L.² When samples were incubated with a specific polyclonal antibody to human TFPI, proteolytic cleavage of S2222 was not inhibited, demonstrating the TFPI dependence of the inhibition assay.

Enzyme-Linked Immunoassay for Lp(a)

Lp(a) was quantified by a noncompetitive enzyme-linked immunosorbent assay (ELISA) with two polyclonal antibodies. An anti-human apo(a) antibody prepared in sheep was used as the “capture” antibody and a peroxidase-conjugated anti-human apo B-100 antibody as the detecting antibody.¹⁷ Both antibodies were purified by caprylic acid precipitation and then concentrated by ammonium sulfate precipitation (40%). Polystyrene microtiter plates (96-well immunoplates, Costar, Cambridge, UK) were coated and left overnight at 4°C with a 100-µg/mL dilution of anti-apo(a) antibody in PBS at pH 7.4. After washing to remove unbound antibodies (washing buffer was PBS containing 0.05% Tween), the wells were blocked by incubation with 3% BSA in PBS at room temperature for 1 hour. After another wash, 100 µL of appropriately diluted Lp(a) samples in PBS containing 0.05% Tween and 1% BSA at pH 7.4 was added to the wells. The plates were incubated for 2 hours at 37°C; they were subsequently washed and a peroxidase-labeled antibody against apo B-100 (diluted 1:800 in PBS/Tween/BSA) was added. After an additional incubation for 90 minutes at 37°C, the plates were washed and the substrate (1 mg/mL of o-phenylenediamine dihydrochloride, Sigma, in 0.05 mol/L citrate buffer at pH 5.5 containing 0.01% H₂O₂) was added. The reaction was allowed to proceed in the dark for 20 minutes and was stopped with 3N HCl. Absorbance was then read at 492 nm on a Titertek multiscan microplate reader. Each plate contained eight dilutions (4 to 500 ng total Lp[a]/well) of a reference standard (Immunofrance). Periodically, purified Lp(a) was compared with the reference standard for calibration purposes. For the present ELISA, the working concentration range was 40 ng to 5 µg/mL Lp(a) lipoprotein mass.

Electrophoretic Analyses

Native lipoproteins. For evaluation of lipoprotein particle size and heterogeneity, nondenaturing gradient gel electrophoresis of native lipoproteins was performed in a Pharmacia GE-2/4 LS electrophoresis apparatus loaded with gels containing a 2% to 16% gradient (PAA 2/1, Pharmacia). Approximately 20 µg of protein was applied to each well, and electrophoresis was carried out at 90 V for 12 hours at 4°C in a Tris/borate buffer (0.09 mol/L Tris, 0.08 mol/L boric acid, and 0.003 mol/L EDTA, pH 8.35).³⁰ A set of standard markers with known hydrated diameters (thyroglobulin, 170 Å; ferritin, 122 Å; catalase, 104 Å; and lactate dehydrogenase, 81 Å) was run on each slab as a reference. Gels were subsequently equilibrated in transfer buffer (see above).

Apolipoproteins. Apolipoprotein electrophoresis was performed in 5% to 19% gradient SDS polyacrylamide gels according to the method of Laemml.¹¹ Lipoprotein samples were solubilized and heated at 100°C for 5 minutes in 30 µL sample buffer containing SDS (1% SDS, 10 mmol/L Tris with or without 10 mmol/L dithiothreitol at pH 6.8). Twenty microliters of 50% sucrose/bromophenol blue was then added to each sample. Samples were loaded and gels electrophoresed at a current of 30 mA/slab at 15°C for 2 hours. Molecular-weight protein standards ranging from 29 to 205 kD (Sigma) were electrophoresed concomitantly.

Western Blots

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was used as the method for detection of TFPI in the protein moieties of the various subfractions. Immunoblotting was performed after electrophoresis of either native lipoproteins or apolipoproteins. Apolipoprotein or lipoprotein bands were electrophoretically blotted onto nitrocellulose sheets (Bio-Rad) for 1 hour and 12 hours, respectively. A transfer buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol at pH 8.3 was used in a Transblot cell (Bio-Rad). Electrophoretic transfer was performed at 90 V.³² The nitrocellulose paper was then blocked with 2.5% nonfat milk in TS buffer (0.05 mol/L Tris HCl, 0.15 mol/L NaCl, and 0.05% NaN₃) at
pH 7.5 for 1 hour at room temperature and incubated overnight at room temperature with rabbit anti-TFPI IgG's diluted 1:2500 in TBSA buffer (0.05 mol/L Tris HCl, 0.15 mol/L NaCl, 0.05% NaN3, and 0.17% BSA). The paper was then washed three times in TBS containing 2.5% nonfat dry milk; the blots were subsequently incubated for 1 hour with a goat anti-rabbit IgG radio-labeled with 125I (diluted 1:1000 in TBSA). After washing three times, blots were exposed to Kodak XAR film. Western blotting and immunodetection of Lp(a) were performed according to the protocol of Bronstein et al.32 Antibody and antibody conjugate used for immunodetection were, respectively, an anti-human apo(a) (1:2000) prepared in sheep32 and an anti-sheep IgG-alkaline phosphatase (1:20 000) (Sigma).

Results
Characterization of Lipoprotein Subspecies
To evaluate the distribution of TFPI among the apo B–containing and apo A-I–containing lipoprotein subspecies, we fractionated the plasma lipoprotein spectrum by isopycnic density gradient ultracentrifugation.23 We initially determined the density distribution, concentration, chemical composition, and apolipoprotein content of lipoprotein subspecies. The quantitative distribution of the various lipoprotein subspecies and of their triglyceride, phospholipid, cholesteryl ester, free cholesterol, and protein components are shown over the density range from 1.016 to 1.190 g/mL in the Table. This pattern was obtained from analyses of the 24 successive gradient fractions of 0.4 mL from plasma samples of eight subjects. The correspondence of density subfractions to lipoprotein subspecies was assessed on the basis of chemical, physical, and immunological analyses.28 Note that VLDL and IDL (d<1.019 g/mL) represent 4.6% of total lipoprotein mass, LDL (d=1.019 to 1.063 g/mL) some 47.9%, and HDL (d=1.063 to 1.167 g/mL) some 39.7%. The profile of lipoprotein mass concentration as a function of density is shown in Fig 1A. The first fraction contained a mixture of VLDL of d<1.006 g/mL and of IDL of d=1.006 to 1.018 g/mL (Table). Fraction 2 corresponded to a subfraction of LDL (d=1.018 to 1.019 g/mL). The following subfractions (No. 3 to 12) corresponded to LDL ranging in density from 1.019 to 1.063 g/mL. Fraction 13 (d=1.063 to 1.072 g/mL) displayed a composition between those considered typical of LDL and HDL. On the basis of our earlier studies,22 the hydrated densities of HDL2 and HDL3 ranged, respectively, from 1.063 to 1.100 g/mL (subfractions 13 to 16) and from 1.100 to 1.167 g/mL (subfractions 17 to 22). Gradient fractions 23 and 24, of d=1.167 to 1.190 g/mL and containing ~80% of protein by weight, represent forms of VHDL, notably VHDL1 (hydrated density ~1.16 g/mL).

Distribution of TFPI Activity and TFPI Protein Among Lipoprotein Subspecies
All 24 lipoprotein subfractions were subsequently analyzed for TFPI activity (Fig 1B) by the use of a chromogenic inhibition assay. While all lipoprotein subspecies displayed a basal level of TFPI activity (~20 arbitrary units of TFPI activity expressed as percent plasma per milligram of total lipoprotein mass), TFPI activity was specifically colocalized with apo B–containing lipoprotein subspecies ranging in density from 1.033 to 1.081 g/mL (subfractions 8 to 14; >40 arbitrary units of TFPI activity expressed as percent plasma per milligram of total lipoprotein mass) and with apo A-I–containing subspecies ranging from d=1.133 to 1.190 g/mL (subfractions 20 to 24).

In the series of eight normolipidemic plasmas studied, the density distribution of the first peak of TFPI activity typically showed partial overlap with that of Lp(a) (Figs 1B and 1C). To verify whether TFPI activity and Lp(a) were codistributed, we quantified the Lp(a) content of all subfractions by ELISA (Fig 1C). Such immunological detection revealed a major unique peak of Lp(a) near fraction 13 (d=1.063 to 1.072 g/mL), thereby suggesting that TFPI was not codistributed with Lp(a). By contrast, TFPI appeared to be present mainly in denser LDL (subfractions 10 to 13) and in certain subfractions (12 to 15) containing both Lp(a) and HDL particles. The plasma profiles in two individual donors (Fig 2) revealed distinct density ranges for LDL and HDL and Lp(a) in these subjects. Thus, TFPI comigrated with dense LDL subfractions 9 to 11 (d=1.039 to 1.058 g/mL) in one individual (Fig 2A); in the second subject, however, the peaks of TFPI activity and Lp(a) occurred simultaneously in subfractions 10 to 13 (d=1.044 to 1.072 g/mL). Differences in circulating Lp(a) isofoms in these subjects (S3 in Fig 2A and F in Fig 2B, respectively) may partially explain such distinct distributions. Furthermore, TFPI was present in the densest HDL fraction (20 to 24; d=1.133 to 1.190 g/mL) in both subjects and may represent that bound to apo A-II by covalent linkage.

To provide direct evidence for the association of TFPI protein with lipoprotein particles, qualitative immunodetection was performed on native lipoprotein subspecies in nondenaturing 2% to 16% gradient gels (Fig 3) as indicated above. The electrophoretic conditions did not appear to affect the potential relation between lipoprotein particles and TFPI, as TFPI was observed to comigrate with all lipoprotein species. Furthermore, the TFPI band associated with LDL particles whose hydrated diameters were in the range from 255 to 260 Å had the strongest intensity by comparison with that associated with LDL subfractions of larger particle size (3 to 7; diameter of 260 to 280 Å). The overlap in the density distribution of Lp(a) and HDL particles in subfractions 12 to 14 was resolved by nondenaturing and nonreducing gel electrophoresis. This methodology allowed discrimination between the preferential association of TFPI with Lp(a) or with light HDL1. Indeed, the band corresponding to the particle size of Lp(a) (~280 Å) (Fig 3, fractions 11 to 15) had a higher reactivity after immunological detection than did the bands corresponding to the diameters of HDL2 particles (subfractions 14 and 15). TFPI protein was also identified in HDL subfractions and more specifically in those of small particle size (subfractions 17 to 21; range in diameter of 82 to 112 Å). The intensity of staining of HDL2 (subfractions 17 to 21; Fig 3) and VHDL (subfraction 23; Fig 3) was markedly greater than that of HDL1 (subfractions 14 and 15; Fig 3). The association of TFPI with Lp(a) particles was further analyzed by immunodetection of Lp(a) and TFPI under the same conditions as above (Fig 4). In these studies, TFPI protein (Fig 4, lane 3) was located at the same position as that to which Lp(a) migrated (Fig 4, lane 2),
clearly indicating a collocalization of the two proteins. We next verified whether TFPI activity was dependent on the presence of TFPI protein. Lipoprotein fractions (subject A) were analyzed under reducing conditions (10 mmol/L dithiothreitol) by SDS-PAGE on a 5% to 19% gradient gel. After Western blotting, a rabbit antibody against TFPI, as revealed by addition of an 123I-labeled anti-rabbit IgG, recognized a unique band that migrated with a relative molecular mass of 35 kD (Fig 2A). The distribution of immunologically reactive TFPI among lipoprotein subspecies thus correlated well with TFPI activity (Fig 2A).

**Molecular-Weight Species of Lipoprotein-Associated TFPI**

Several molecular-weight forms of TFPI were detected among lipoprotein subfractions, and these were visualized after SDS-PAGE on a 5% to 19% gel, followed by Western blotting, immunodetection, and autoradiography after treatment of the lipoprotein samples under denaturing and nonreducing conditions (Fig 5). The results demonstrate that dense LDL subspecies of $d=1.039$ to 1.058 g/mL (8 to 12) transport mainly the 35-kD form of TFPI, whereas dense subspecies of HDL (subfractions 18 to 23 of $d=1.110$ to 1.167 g/mL) contain mainly the higher relative molecular mass form of 41 kD (a form that is known to be covalently associated with apo A-IP) and minor bands of the 35-kD and 52-kD forms.

**Dissociation of Lipoprotein-Associated TFPI**

We next asked whether the specific distribution of TFPI detected among LDL subspecies was not due to uncoupling of TFPI from LDL after contact with a salt solution (potassium bromide) of high ionic strength. LDLs were therefore isolated by gel filtration (see "Methods"). Subsequently, chromatographically isolated LDLs were treated separately with increasing concentrations of sodium chloride (0.15 to 2 mol/L). To separate any free TFPI dissociated from LDL, such LDLs were applied to a gel filtration column (see "Methods"). Autoradiography of TFPI (data not shown) on a Western blot of eluted LDL pretreated under reducing conditions did not reveal any difference in TFPI content, thus indicating no liberation of TFPI.
by 2 mol/L NaCl. Identical experiments were performed with heparin (2 U/mL), a polyanion known to liberate TFPI from vascular endothelial cells, e-amino-n-caproic acid (5 mmol/L), and dithiothreitol (50 mmol/L) (data not shown). No additional TFPI was liberated from LDL by treatment with such agents.

Discussion

The present studies demonstrate for the first time the heterogeneity of lipoprotein-mediated TFPI transport and more specifically the preferential association of TFPI activity and TFPI protein with subspecies of apo B- and apo A-I-containing particles. Furthermore, the
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major plasma carriers of TFPI correspond to small, dense forms of both LDL and HDL. Equally, our data indicate that TFPI is associated with Lp(a) and that this association is related to Lp(a) particle size and phenotype.

Use of our density gradient ultracentrifugation procedure has permitted the simultaneous fractionation of the major lipoprotein classes (VLDL, IDL, LDL, HDL1, and HDL2) in a single step. Moreover, this procedure allowed us to analyze the distribution of TFPI among subfractions of each lipoprotein class, whose physicochemical and metabolic heterogeneity is well established.17-21,22,26 Our studies of eight normolipidemic subjects have revealed two major lipoprotein carriers of TFPI: dense subspecies of LDL (d=1.039 to 1.063 g/mL) and of HDL (d=1.133 to 1.190 g/mL); together, these subspecies accounted for ~70% (33.8% and 35.9%, respectively) of the total lipoprotein-associated TFPI activity in plasma. By comparison, VLDL and IDL, LDL1 through LDL2 (d=1.019 to 1.039 g/mL), and HDL2 (subfractions 13 to 16; d=1.063 to 1.100 g/mL) together with light subfractions of HDL (17 to 20; d=1.100 to 1.167 g/mL) convey only 1.8%, 10%, and 18.5%, respectively, of lipoprotein-associated TFPI activity. When compared with the data of Novotny et al,9 our results indicate a slightly greater association of TFPI activity with HDL (54.4%) than with VLDL and LDL (45.6%). Indeed, the latter investigators found 50% of TFPI to be associated with VLDL and LDL, 44% with HDL, and 6% with free TFPI. In the latter study, lipoproteins were separated by gel filtration and TFPI was quantified by immunoassay. Despite differences in the methodologies employed, these results are largely consistent, the minor discrepancy probably arising from use of different TFPI assays, which were functional in the present study and antigenic in that of Novotny et al.9

The dense subspecies of HDL (d=1.133 to 1.167 g/mL) with which TFPI was preferentially associated were of small size, displayed a cholesteryl ester to protein ratio of ~0.2, and were deficient in phospholipid (range, 13.6% to 18.3%). The presence of TFPI among HDL particles has been well documented. Indeed, several authors have suggested that TFPI may be associated with HDL as a result of its binding to apo A-II.11-35 This hypothesis was confirmed by Novotny et al,9 who demonstrated that the 46-kD form of TFPI is in covalent linkage with apo A-II. Our findings also document the association of two distinct molecular-weight forms of TFPI (35 kD and 41 kD) with HDL particles. A note added in proof in the article of Novotny et al9 indicated an error in the apparent molecular weights of standards used for SDS-PAGE by the manufacturer. Comparison of our data with the recalculated data of Novotny et al9 gave similar values for the two major size species of TFPI. In addition, significant amounts of TFPI were associated with VHDL subfractions of d=1.167 to 1.190 g/mL.

The second major localization of TFPI was within the hydrated density range of dense LDL particles. Earlier studies of the distribution of TFPI among plasma lipoproteins did not discriminate between the presence of TFPI in triglyceride-rich compared with cholesterol-rich apo B-containing subspecies (VLDL and LDL, respectively). Our results demonstrate a major localization of TFPI with dense LDL (d=1.033 to 1.063 g/mL) of small particle size, which in comparison with light LDL (d=1.019 to 1.033 g/mL) exhibit a markedly lower proportion of triglyceride (5% versus 19%) and enrichment in cholesteryl ester (38% versus 30%). Analysis of the mean ratios of the percent mass of the various

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**FIG 4.** Association of tissue factor pathway inhibitor (TFPI) protein with lipoprotein(a) (Lp(a)). Aliquots (20 μg protein) of gradient fraction 12 were electrophoresed under nondenaturing and nonreducing conditions in a 2% to 16% polyacrylamide gradient gel (lane 1). Samples were electrophoresed for 12 hours, and gels were subsequently equilibrated in transfer buffer (see "Methods"). After electrophoretic transfer, TFPI protein (lane 3) was immunodetected as described in the legend to Fig 3. Immunological detection of Lp(a) (lane 2) on the nitrocellulose sheet was performed after incubation with purified polyclonal anti-human apolipoprotein (a) obtained from sheep and revealed with an anti-sheep immunoglobulin G conjugated to alkaline phosphatase under conditions described in "Methods."

**FIG 5.** Western blots of individual lipoprotein gradient subfractions (1 to 23) electrophoresed under denaturing conditions on sodium dodecyl sulfate-polyacrylamide gradient gels of 5% to 19%. Aliquots (60 μg protein) were applied to each well and electrophoresed for 2 hours (see "Methods"). The protein moieties of individual subfractions were electrotransferred, and tissue factor pathway inhibitor protein was immunodetected as described in the legend to Fig 3. PM, 29-, 45-, 66-, 97.4-, 116-, and 205-kD.
components (cholesteryl ester/free cholesterol, cholesteryl ester/triglyceride, cholesteryl ester/phospholipid, free cholesteryl/phospholipid, free cholesteryl/protein, triglyceride/protein, phospholipid/protein, and cholesteryl ester/protein; ratios calculated from the data in the Table) indicated a positive relation between an increase in the cholesteryl ester to free cholesterol ratio in LDL subfractions and the increase in TFPI content ($r = 0.66; P < 0.013$) and equally a positive correlation between an increase in the free cholesteryl to protein ratio in LDL and an increase in TFPI activity ($r = 0.89; P < 0.0006$). The preferential binding of TFPI to dense LDL does not appear to reflect apolipoprotein content per se, as one apo B-100 is present per particle in all LDL subfractions. Substantially lower amounts of apo E (< 0.5%) were, however, detected in the lightest and in densest LDL subfractions. The presence of apo E at each extreme of the LDL spectrum does not correspond to the distribution of TFPI among LDL subfractions and therefore tends to exclude a specific association of TFPI with apo E. Furthermore, the apo E-rich fractions, i.e., VLDL and IDL, did not appear to be efficient vectors of TFPI.

The third and new localization of TFPI was among Lp(a) particles. The protein moiety of Lp(a) is composed of one copy of apo B-100 disulfide-linked to apo(a), a highly glycated protein. Apo(a) is heterogeneous in size, exhibiting molecular mass isoforms ranging from ~350 to 700 kD. The variation in the number of repeat copies of the kringle-4-like domain accounts for the wide size range of apo(a). The various molecular-weight forms of apo(a) may provide an explanation for the distinct distribution of TFPI among Lp(a) isoforms (Fig. 2). High relative molecular mass isoforms display lower TFPI content than smaller forms. It is, therefore, tempting to speculate that the interaction of TFPI with Lp(a) is at least partially dependent on the size of apo(a), which for reasons of steric hindrance may prevent an efficient interaction between TFPI and the surface components of Lp(a) particles. Such a hypothesis may indirectly argue in favor of an interaction of TFPI with the "LDL" moiety of Lp(a). Furthermore, recent studies have demonstrated a proteolytic activity of human Lp(a) on synthetic peptide substrates, and especially on peptide substrate S2222. Therefore, we asked whether Lp(a)-rich subfractions might interfere in the amidolytic assay of TFPI activity (data not shown). This assay was therefore performed in TSC buffer, but in the absence of coagulation factors, as described in the "Methods" section. Under these conditions no cleavage of S2222 was detected, thereby excluding the possible interference of apo(a) proteolytic activity in our evaluation of TFPI activity.

Although the association of TFPI with HDL appears to be covalent, the type of interaction involved in the association of TFPI with LDL and Lp(a) is indeterminate. We have ruled out a key role for ionic/polar interactions, since TFPI could not be dissociated by 2 mol/L NaCl or heparin (2 U/mL), which we expected would compete for possible binding with TFPI at the carbohydrate side chains of apo B or apo(a), respectively. Indeed, the common content of apo B-100 among LDL particles despite a discontinuous distribution of TFPI provokes the hypothesis that TFPI may interact with surface-exposed lipid components of LDL rather than with apo B. Nevertheless, alterations in apo B-100 conformation that parallel the modifications in the radius of curvature of LDL particles across the density distribution would equally explain the differential distribution of TFPI between dense, intermediate, and light LDL. The predicted amino acid sequence of TFPI contains no obvious hydrophobic region that might facilitate lipid binding. Certainly, the binding of TFPI to LDL does not appear to be exclusively electrostatic. We therefore conclude that among the spectrum of apo B-containing lipoprotein subfractions, small, dense LDL particles provide the most favorable surface structure for efficient TFPI binding.

Previous reports have established the existence of a positive correlation between plasma levels of dense LDL and atherosclerosis. On the other hand, expression of TF on the surface of lipid-laden macrophages might play a role in fibrin deposition in atherosclerotic plaques; penetration of TFPI-bearing, dense LDL into the subendothelial space would potentially inhibit this process, in which case dense LDL would play an antiatherosclerotic role. Equally, however, the elevated susceptibility of dense LDL to oxidation, coupled with the subsequent inactivation of LDL-bound TFPI (P. Lesnik et al, unpublished results), would result in the neutralization of such an antiatherogenic and anti-thrombotic role. Our hypothesis would then explain the atherogenicity of dense LDL and provide a new link between thrombus formation and atheroma.

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Lesnik et al. TFPI, Lp(a), and Dense LDL and HDL


Anticoagulant activity of tissue factor pathway inhibitor in human plasma is preferentially associated with dense subspecies of LDL and HDL and with Lp(a).
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