Re-Evaluation of the Anticoagulant Properties of High-Density Lipoprotein—Brief Report

Jose A. Fernandez, Hiroshi Deguchi, Carole L. Banka, Joseph L. Witztum, John H. Griffin

Objective—This study was conducted to resolve the striking controversy between our previous report that high-density lipoprotein (HDL) enhances activated protein C (APC)/protein S anticoagulant actions and a subsequent, contradicting report that HDL lacks this activity.

Approach and Results—When fresh HDL preparations from 2 laboratories were subjected to Superose 6 column chromatography, fractions containing HDL-enhanced APC:protein S anticoagulant actions in clotting assays, thereby validating our previous report. Moreover, the ability of HDL to enhance the anticoagulant actions of APC:protein S was neutralized by anti-apoAI antibodies, further indicating that the activity is because of HDL particles and not because of contaminating phospholipid vesicles. Density gradient subfractionation studies of HDL showed that large HDL subfractions (densities between 1.063 and 1.125 g/mL) contained the APC:protein S—enhancing activity. Fresh HDL stored at 4°C gradually lost its anticoagulant enhancing activity for 14 days, indicating moderate instability in this activity of purified HDL.

Conclusions—These studies conclusively demonstrate that freshly prepared HDL fractions possess anticoagulant activity. Fractions from Superose 6 columns that contain HDL reproducibly enhance APC:protein S anticoagulant activity, consistent with the hypothesis that HDL has antithrombotic activity and with the observation that low HDL levels are found in male venous thrombosis patients. Understanding the basis for this activity could lead to novel therapeutic approaches to regulate venous thrombosis. (Arterioscler Thromb Vasc Biol. 2015;35:570-572. DOI: 10.1161/ATVBHA.114.304938.)

Key Words: high-density lipoproteins ■ protein C ■ protein S ■ thromboplastin ■ thrombosis

High-density lipoprotein (HDL) particles, defined by density of 1.063 to 1.21 g/mL, are heterogeneous in size and composition.1,2 Although there is some controversy, extensive epidemiological data show that endogenous HDL is positively associated with cardioprotection3–5 but much remain unknown about mechanism(s) for HDL’s benefits. HDL deficiency is associated with venous thrombosis (VTE).5–8 The multiple antithrombotic properties of HDL9 may help explain why the deficiency of HDL is associated with thrombotic diseases. In 1999, we discovered that purified HDL enhances activated protein C (APC):protein S anticoagulant action in plasma clotting and factor Va inactivation assays, giving rise to our hypothesis that HDL helps protect against venous thrombosis.10 However, in 2010, Oslakovic et al11 challenged our discovery in reporting that APC:protein S enhancement was an intrinsic property of HDL and that this APC:protein S enhancement activity was because of phospholipid contaminants in their HDL preparation based on fractionation of HDL on Superose 6. To resolve this striking conflict, here we also used Superose 6 chromatographic analyses and characterized 2 new sources for fresh HDL. Here, we provide new data that validate our initial report10 and extend the characterization of HDL’s ability to enhance APC:protein S activity.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Our original report demonstrating anticoagulant activity of HDL used fresh HDL prepared at the Scripps laboratory, as described.10 In these new studies, we again used freshly prepared, never-frozen, purified human HDL, but obtained HDL from 2 outside sources, the laboratory of Joseph L. Witztum at the University of California, San Diego, and a commercial product from Intracel Resources. When each HDL preparation was subjected to Superose 6 chromatography as described11 and column fractions were assayed, each HDL preparation gave similar results. For example, lipid, apoAI, and protein profiles of Superose 6 column fractions for 1 representative HDL preparation are seen in the Figure (A). ApoAI eluted in a typical pattern consistent with being on intact HDL particles, with the major retained peak of apoAI...
coeluting with the lipid fractions at elution volumes between 15 and 17 mL, which were well separated from the void volume (Vo) fractions (8–9 mL). Importantly, all the apoAI was associated with HDL particles.

To study the anticoagulant APC:protein S enhancing activity of Superose 6 column fractions, a modified prothrombin time clotting assay was used (see Methods in the online-only Data Supplement). As seen in the Figure (B), addition of Superose 6 fractions containing purified HDL that eluted between 15 and 17 mL caused a marked increase in the normalized APC:protein S ratio, reflecting enhancement of APC:protein S anticoagulant activity. In contrast, these HDL-containing fractions had no detectable prothrombinase activity. As reported by Oslakovic et al.,

11 void volume fractions slightly stimulated prothrombinase activity in reaction mixtures containing purified factors Xa and Va with prothrombin, and Vo fractions also had positive but minimal effects on APC:protein S anticoagulant activity. Thus, chromatography of fresh, never-frozen HDL on Superose 6 showed that fractions containing HDL contained >90% of the APC:protein S enhancing activity of the HDL preparation. This pattern was similarly observed for 3 different HDL preparations analyzed 6x on the Superose 6 column.

In the course of our multiple analyses of different HDL preparations using Superose 6 chromatography, we observed that fresh HDL showed the highest amount of anticoagulant enhancing activity in HDL fractions. On storage of never-frozen HDL at 3°C to 5°C, HDL gradually lost its activity for days. For example, when aliquots of the same HDL preparation were analyzed on days 8 and 12 after purification, ~40% of the activity was lost during the 4-day period (Figure [C]). After 3-week storage in the cold at 4°C, but not frozen, almost no APC:protein S enhancing activity (<10% of original activity) was measureable. When the HDL preparation was frozen for 24 hours at −20°C and later compared with the same non-frozen HDL preparation undergoing identical chromatography analyses, the pool of Superose 6 apoAI-containing fractions from the frozen HDL had less anticoagulant activity than the pool of apoAI fractions from fresh HDL (Figure IA in the online-only Data Supplement). However, the pool fractions from the Vo of the column showed an opposite effect. The Vo pool from the frozen–thawed HDL had more anticoagulant activity than the Vo pool from the fresh, never frozen HDL (Figure IA in the online-only Data Supplement). When the procoagulant activity of the fractions was tested with a prothrombinase assay, the Vo pool from the frozen HDL had an enhanced procoagulant activity compared with the Vo pool from the fresh HDL fractions (Figure IB in the online-only Data Supplement). No significant procoagulant activity was detected in fractions containing apoAI from either fresh or frozen–thawed HDL preparations (Figure IB in the online-only Data Supplement).

To confirm that the anticoagulant enhancing activity of HDL preparations was because of HDL particles, immobilized antibodies against apoAI were tested for their ability to absorb the anticoagulant activity of Superose 6 fractions. Immobilized anti-apoAI IgG adsorbed 86% of the anticoagulant activity, whereas no significant (<5%) adsorption of the activity was observed for control Sepharose-IgG beads (Figure II in the online-only Data Supplement). Thus, HDL fractions containing apoAI showed the anticoagulant activity observed, and anticoagulant activity was not because of contaminants in the HDL preparations.

To further characterize HDL’s anticoagulant property, we prepared HDL subfractions by sequential density gradient ultracentrifugation over the 1.063 to 1.21 g/mL density range.1 The density of each subfraction was measured by a densitometer at room temperature and ranged from 1.091 to 1.296 g/mL. Each fraction was dialyzed against TBS buffer containing 0.1% BSA and 0.2 mmol/L EDTA and then assayed for their anticoagulant enhancing activity, as shown in Figure (D). HDL subfractions (HDL2) ranging from 1.09 to 1.14 g/mL contained the predominant anticoagulant enhancing activity.

**Figure.** Characterization of high-density lipoprotein (HDL) using Superose 6 chromatography and density gradient subfractionation. **A**, Protein and lipid contents of the Superose 6 column fractions. **B**, Anticoagulant and procoagulant profiles for Superose 6 column fractions. **C**, Loss of anticoagulant activity over time for the HDL-containing fractions. **D**, Anticoagulant activity of HDL subfractions after density gradient subfractionation. Dotted vertical line indicates density of 1.125 g/mL and solid vertical line, a density of 1.21 g/mL. Enhancement of activated protein C (APC):protein S anticoagulant activity is reflected in the normalized ratio, which was calculated as the ratio of prothrombin times plus and minus APC:protein S normalized to 1.0 for samples lacking Superose 6 fractions.
Discussion
Our studies show that when fresh, never-frozen HDL preparations, sourced from 2 independent laboratories outside of Scripps Research Institute, were analyzed using Superose 6 chromatography, the column fractions containing HDL but not the Vo fractions contained >90% of the preparation’s ability to enhance APC:protein S anticoagulant activity in plasma clotting assays. Moreover, anti-apoAI antibodies removed most of the HDL preparation’s ability to enhance APC:protein S activity, and specifically, HDL2 subfractions were the major source of this anticoagulant activity. We further show that the ability of fresh HDL to mediate this activity is lost as the HDL ages and seems to be completely lost after storage in the cold for 3 weeks, even without freezing. Thus, the data in these new studies validate and extend our previous report10 about HDL’s ability to enhance APC:protein S activity. We speculate that the conflicting report from Oslakovic et al11 was because of differences in HDL preparation, notably, their inferior quality HDL preparations, that is, HDL which was stored frozen at −20°C before bioassays and which had been prepared from lipidic frozen blood bank plasma12 as we confirmed in our chromatography experiments using fresh, never frozen versus frozen–thawed HDL. Functional activity studies using lipoproteins usually use fresh, never-frozen preparations, as it is well known that freeze–thawing disrupts lipoprotein particle structure rendering such preparations unsuitable for biological studies unless they are specially cryopreserved.13 Thus, it is likely that freeze–thaw cycles promoted development of phospholipid vesicles eluting in the Vo of Superose 6 columns in their HDL preparation and that unknown factors related to the long-term, frozen storage of HDL led to the loss of HDL’s ability to enhance APC:protein S.

In summary, our studies confirm that fresh HDL possesses anticoagulant cofactor activity, as we previously reported.10 Understanding the components of HDL and the mechanisms by which this beneficial property occur could lead to novel therapeutic approaches to the prevention of venous thrombosis.

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Disclosures
None.

References

Significance
This study confirms and extends previous data showing that the activity profile of large high-density lipoprotein (HDL) subfractions includes an ability to enhance the anticoagulant actions of activated protein C:protein S. This activity may contribute to the multifaceted antithrombotic properties of HDL. Mechanisms for the anticoagulant-enhancing activity of HDL remain unidentified, and the moderate instability of this activity of fresh, never-frozen HDL for several weeks makes applications of lipidomics and proteomics methodologies to identify HDL’s active components challenging.
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SUPPLEMENTAL MATERIAL

Materials

Purified factors Va, Xa, and prothrombin were obtained from Haematologic Technologies Inc., Essex Junction, VT. Chromogenic substrate PefaChrome TH from Pentapharm Ltd., Basel, Switzerland, S2765 from DiaPharma, West Chester, OH, L-\(\alpha\)-Phosphatidylcholine (PC) (chicken egg) and L-\(\alpha\)-phosphatidylserine (PS) (Porcine brain) from Avanti Polar Lipids, Alabaster AL. L-3-PC-1,2-di [1-\(^{14}\)C]oleoyl from Amersham Biosciences. For some studies, L-\(\alpha\)-PS (bovine brain) and L-\(\alpha\)-PC (bovine brain) from Sigma-Aldrich Co. Phospholipid vesicles containing PC and PS in the molar ratio 9:1 for prothrombinase assays were prepared by sonication and centrifugation as described \(^1\), normal human plasma for clotting assays was purchased from George King Biomedical Inc., Overland Park, KA. HDL was either isolated in the Analytical Laboratory of Dr. Joseph L. Witztum at the University of California, San Diego from fresh fasting human plasma using classical sequential flotation techniques, at density values of 1.063 to 1.21 g/mL, as described \(^2,3\) or purchased from Intracel Resources, Frederick, MD. Anti-ApoA-I antibody came from Meridian Life Science Inc, Saco, ME and was covalently linked to Sepharose BrCN beads (GE Healthcare, Parsippany, NJ). HDL was subfractionated using density gradient centrifugation as described \(^2\) with the following modifications. 40 aliquots were collected from the centrifuged tube containing the density gradient, and the density of each aliquot was measured by density meter (Mettler-Toledo, Inc., Hightstown, NJ) at room temperature, with variations from 1.091 to 1.296 g/mL. Fractions with the density between 1.091 and 1.125 g/mL were designated as HDL2 and fractions with the density between 1.125 and 1.21 g/mL were HDL3.

Superose 6 Chromatography and Determination of the Concentration of ApoA-I and Lipids in Superose 6 HDL fractions
Purified HDL was subjected to chromatography using a Superose 6 HR column (0.5 x 28 cm) (GE Healthcare, Parsippany, NJ) at a flow rate of 0.5 ml/min at room temperature. Five hundred µl fractions were collected and used for protein, apoA-I antigen, and lipid assays and for clotting assays. Assay reagents were sourced as follows: Cholesterol (Wako Chemicals, Richmond, VA), choline phospholipid (Wako Chemicals), and apoA-I (DiaSorine, Stillwater, MN). Total protein was determined with the BCA kit from Pierce, Rockford, IL.

**Assay for ability of HDL to enhance anticoagulant activity of APC:Protein S**

To determine APC:Protein S enhancing anticoagulant activity of HDL, prothrombin time clotting assays were performed using an Amelung KC 4A micro apparatus (Sigma Diagnostics, St. Louis, MO). 7.5 µL normal plasma was mixed with 5 µL fibrinogen (267 nM), 25 µL APC (22 nM) or buffer (Tris buffered saline (TBS) containing 2% BSA), 25 µl of protein S (88 nM) or buffer, and 25 µl of Superose 6 column fractions or buffer, and this mixture was incubated 3 min at 37°C. Clotting times were then measured after addition of 25 µl containing 1:40 dilution of recombinant human tissue factor (Innovin; Baxter-Dade, Dade, FL), 30 mM CaCl₂, and TBS containing 2% BSA. Enhancement of APC:Protein S anticoagulant activity, defined as a “Normalized Ratio”, was calculated as the ratio of the prothrombin times plus and minus APC:Protein S, normalized to 1.0 for control values measured in the absence of Superose 6 column fractions.

**Prothrombinase activity assays**

Prothrombin activation in the presence of Superose 6 column fractions was determined using purified factor Xa (0.7 nM final) plus factor Va (15.5 nM final). Factor Xa and factor Va, were mixed with Superose 6 subfractions at room temperature for 5 min before addition of prothrombin (0.76 µM final). Then the rate of thrombin (IIa) formation was quantified by
measuring thrombin based on the rate of substrate (PefaChrome-TH, 0.4 mM final) hydrolysis monitored as Absorbance change at 405 nm.

References for Materials


**Suppl. Figure I.** Effects of freezing and thawing on the anticoagulant activity and procoagulant prothrombinase activity of HDL. **A.** Fresh and frozen-thawed HDL (-20° C) were chromatographed onto a Superose 6 column. Pooled fractions from the column void volume and from the fractions containing Apo AI from fresh and frozen-thawed HDL preparations were tested for APC/Protein S cofactor activity using clotting assays. The bars are the mean values and SD of three separated chromatography runs. **B.** Fractions from the Superpose 6 column
(from fresh and frozen-thawed HDL) were tested for procoagulant activity using a prothrombinase assay.

**Suppl. Figure II.** Effect of immunoabsorption of Apo AI on the anticoagulant activity of Superpose 6 fractions. Pools of apo AI-containing fractions from Superose 6 chromatography were immunoabsorbed with immobilized anti-ApoAI antibody or control IgG. Then, the ability of the immunoabsorbed fractions to prolong clot times in the presence of APC/Protein S was determined.