Protein unfolding allows use of commercial antibodies in an apolipoprotein M sandwich ELISA

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Abstract apoM is a member of the lipocalin superfamily and circulates in plasma attached to HDL particles. apoM plays a role in cholesterol metabolism and has recently been identified as transporter for the signaling lipid, sphingosine-1-phosphate (S1P), in plasma. S1P is implicated in several inflammatory diseases such as multiple sclerosis and rheumatoid arthritis. The ability to accurately measure apoM is crucial for investigating its biological functions and possible clinical implications. However, reliable commercial methods have been lacking so far. Therefore, we have developed an assay that specifically recognizes human apoM in plasma using commercially available reagents. Commercial apoM antibodies were screened for compatibility in a sandwich ELISA-based assay. One optimal pair of antibodies was chosen, and sample preparation, buffers, and incubation times were optimized to generate a simple and reproducible method. Validation and comparison to a previously described ELISA for apoM confirmed that the assay displays a high degree of sensitivity, specificity, and precision. Our results show that commercially available antibodies can be used to accurately measure human plasma apoM. This method can be implemented in every laboratory and will help promote high quality research.—Bosteen, M. H., B. Dahlbäck, L. B. Nielsen, and C. Christoffersen. Protein unfolding allows use of commercial antibodies in an apolipoprotein M sandwich ELISA. J. Lipid Res. 2015. 56: 754–759.

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apoM is an important carrier of sphingosine-1-phosphate (S1P) in plasma. In mouse models with elevated plasma apoM, S1P is increased ~267%, whereas apoM−/− mice have ~46% reduced plasma S1P levels compared with WT mice (1). S1P plays an important role in maintaining endothelial barrier function, vasodilation, and regulation of inflammatory mediators such as cytokines and adhesion molecules (2, 3). Considering the importance of S1P as mediator of vascular biology and inflammation, clinical implications for its chaperone, apoM, are numerous.

Plasma apoM is reduced in patients with sepsis and systemic inflammatory syndrome and behaves as a negative acute-phase reactant from the liver (4). Thus, apoM is a potential biomarker of disease severity. Leaking vessels are a feature of severe sepsis and systemic inflammatory syndrome causing hypovolemic shock. S1P can preserve endothelial function and prevent vascular leakage (5). Considering the ability of apoM to bind and affect levels of S1P, low plasma apoM may convey part of the clinical course of sepsis.

apoM has also shown promising potential as a biomarker for diabetes. Plasma apoM is significantly decreased in individuals with type 2 diabetes and can discriminate individuals with type 3 maturity onset diabetes of the young (MODY3) from those with type 1 diabetes and healthy controls (6). Earlier studies showed similar results (7, 8), while another study did not show such discrimination (9). The main differences between the studies were the specific antibodies used to measure apoM by ELISA. To date, none of the ELISA methods are commercially available. Hence, several studies have later been conducted using Western blot or dot blot to assess the plasma apoM concentration (10, 11). This has not improved the quality or accuracy of human plasma apoM measurements.

Several features of apoM can potentially affect its measurement in plasma. Human apoM contains 188 amino acids and exists in glycosylated and nonglycosylated forms. This gives rise to two distinct bands in Western blots, with apparent masses of approximately 20 and 25 KDa, respectively. In mouse apoM, the Asn135 glycosylation site does not exist and apoM is seen as a single band (12, 13). Human
apoM can be further modified by the addition of sialic acid, giving the protein more negative charge (13). apoM is a member of the lipocalin superfamily. Its specific fold is facilitated by six cysteine residues, all engaged in disulfide bridge interactions (14). The apoM gene encodes a 21 amino acid signal peptide, but lacks a cleavage site for a signal peptidase. Therefore, apoM is secreted from the liver to plasma with its signal peptide retained where it is responsible for anchoring apoM to lipoproteins (15, 16). Hence, apoM does not circulate in any free form. The plasma apoM concentration is ~0.9 μmol/l (17), and >95% is bound to HDL (the remaining apoM is bound to LDL and VLDL) (18). In humans, plasma apoM correlates positively with plasma HDL, LDL, and total cholesterol (17).

The clinical interest in plasma apoM has increased since the identification of ligands such as oxidized phospholipids (19), retinol (20), and SIP (1). The suggestion of plasma apoM as not only a marker of diabetes (6, 8), but also as a marker of severe sepsis (4), is of high clinical impact. Hence, the purpose of this study was to develop a stable, accurate, and valid method to measure human plasma apoM, using only commercially available reagents. This will allow all laboratories to perform high quality measurements of plasma apoM.

MATERIALS AND METHODS

Reagents and sample material

All antibodies tested were monoclonal and were raised in mice except for one clone (EPR2904), which was raised in rabbit. Clones A-10, 8F126G8S, 111.7 were from Santa Cruz Biotechnologies, Dallas, TX. Clone 1F10 was from Sigma-Aldrich, St. Louis, MO. Clone 10C3G5 was from Abcam, Cambridge, UK. Clones EPR2904 and 3H3 were from Genetex, Irvine, CA. Clones 1A2, 2A8, 2E5, 1G9, 4C7, 3C7, and 6H3 were from Abnova, Taipei City, Taiwan. Clone 16 was from BD Biosciences, Franklin Lakes, NJ. Anti-His tag antibody was from Abcam. Polyclonal anti-rabbit IgG HRP-conjugated antibody was from Dako, Glostrup, Denmark.

Bacterial recombinant 6xHis-tagged apoM was from MyBioSource, San Diego, CA. Human plasma was from healthy volunteer donors. apoM-TgN and apoM-TgH (containing approximately 2- and 11-fold more apoM than human plasma) as well as apoM-KO plasma, were from genetically modified mice generated as described previously (21). An EDTA plasma pool from 163 healthy donors was used to generate a standard curve in each assay run. The standard curve was generated by fourth order polynomial regression using the GraphPad Prism 4 software (GraphPad Software, San Diego, CA). The apoM concentration of the standard curve sample was determined to be 9.92 μmol/l by measuring it against a plasma sample of known apoM concentration determined previously (17). A separate internal standard plasma sample was used to calculate intra and inter assay coefficients of variation. Correlations between plasma apoM and cholesterol measurements were done by measuring apoM in a subgroup of 289 individuals from a previously described cohort of 389 subjects (22). Data were analyzed using the GraphPad Prism 4 software.

Immunoblotting

Dot blotting was performed by dotting a 2 μl sample to a nitrocellulose membrane. Recombinant apoM was prediluted in PBS (pH 7.4) containing 5% BSA to match the plasma concentration of apoM (0.9 μmol/l). The membrane was dried for 45 min before incubation with blocking buffer [5% skim milk powder in TBS (pH 7.4)] for 2 h. The membrane was incubated for 1 h with primary antibody diluted in blocking buffer with 0.1% Tween-20®, according to the recommendations of the antibody supplier. After washing the membrane three times with TBS (pH 7.4) with 0.1% Tween-20®, the membrane was incubated for 1 h with HRP-conjugated secondary antibody diluted in blocking buffer with 0.1% Tween-20®. After a final wash, the dots on the membrane were visualized using a LI-COR Odyssey Fc imaging system and software. Western blotting was performed by denaturing SDS-PAGE of 2 μl plasma, followed by electroblotting of proteins to a nitrocellulose membrane using the iBlot® (Invitrogen™) semi-dry blotting system from Life Technologies, Carlsbad, CA. The membrane was treated and analyzed as described for the dot blotting procedure above.

ELISA screening of antibodies

ELISA for bacterial recombinant apoM was performed as a sandwich ELISA exploiting the 6xHis tag on the recombinant protein as detection epitope. A 96-well plate was coated with the primary antibody (4–5 μg/ml in TBS) overnight at room temperature. After blocking with 2% BSA in TBS (pH 7.4), a 2-fold dilution series of recombinant apoM in dilution buffer (1% BSA in TBS with 1% Triton-X 100) was added and incubated overnight at room temperature. After washing three times with TBS (pH 7.4) with 0.1% Triton-X 100, anti-6xHis HRP-conjugated antibody (1:2,000) was added in dilution buffer [1% BSA in TBS (pH 7.4) with 0.1% Triton-X 100] and incubated for 2 h at room temperature. The wells were washed three times and apoM was detected by reading absorbance at 492 nm after incubation with o-phenylenediamine/hydrogen peroxide solution for 10 min followed immediately by addition of 1 M H₂SO₄. When testing plasma apoM, ELISA was performed as a competitive ELISA using bacterial recombinant 6xHis-tagged apoM as bait (fixed at 20 nmol/l) and plasma apoM as competitor (variable concentration). Buffers, reagents, and incubation times were the same as above.

Sandwich ELISA for human unfolded plasma apoM (disulfide bridge reduction and alkylation method)

An ELISA plate (Costar 3590 high binding) was coated with 50 μl of capture antibody (clone 1G9) by diluting to 5 μg/ml in TBS (pH 7.4) and incubated at room temperature overnight. The plate was blocked for 2 h with 200 μl of 2% BSA in TBS (pH 7.4). Ten microliters of each plasma sample were mixed with 90 μl of 50 mmol/l DTT, from Sigma-Aldrich, St. Louis, MO, in a 0.2 mol/l sodium phosphate buffer (pH 8.5). Disulfide bridges in apoM were broken by incubating samples at 30°C for 15 min. One hundred microliters of 0.6 mol/l iodoacetamide (Sigma-Aldrich) in a 0.02 mol/l sodium phosphate buffer (pH 8.0) were then added and samples were incubated for 1 h at room temperature in the dark to alkylate free cysteines in apoM and prevent reformation of disulfide bridges. After 1 h, the samples were diluted fifty times (variable for the standard curve) in TBS (pH 7.4) containing 1% BSA and transferred to the ELISA plate for overnight incubation at room temperature. The plate was washed three times with TBS (pH 7.4) and incubated overnight at room temperature with 75 μl of detection antibody (clone EPR2904) diluted to 125 ng/ml in TBS (pH 7.4) containing 1% BSA and 2% Triton-X 100. The plate was washed three times with TBS (pH 7.4) and 0.1% Triton-X 100 and incubated for 2 h with 75 μl of HRP-conjugated anti-rabbit IgG antibody diluted to 125 ng/ml in TBS (pH 7.4) containing 1% BSA and 0.1% Triton-X 100. Finally, the plate was washed three times with TBS (pH 7.4) and 0.1% Triton-X 100, and apoM was
detected by reading absorbance at 492 nm after incubation with 75 μl o-phenylenediamine/hydrogen peroxide solution for 10 min followed immediately by addition of 75 μl 1 mol/l H2SO4. The apoM concentration of measured samples was determined from the standard curve included in each assay run.

Validation testing of the human unfolded apoM sandwich ELISA

Potential interference with measurements of apoM by hemolysis was tested by spiking plasma samples with 1% (v/v) erythrocyte lysate. The lysate was generated from pelleted human erythrocytes that had undergone several freeze-thaw cycles to induce cell lysis. Potential interference with measurements of apoM by hyperlipidemia was tested by spiking plasma samples with 10% (v/v) Intralipid® (20% emulsion) from Fresenius Kabi AG, Germany. Comparative measurements of plasma apoM were performed using a previously described apoM sandwich ELISA and protocol (17). Measurements were carried out on a subset of samples (n = 64) from a previously described cohort of 389 subjects (22). The data were analyzed by ordinary Deming regression using the GraphPad Prism 4 software. Duplicate measurements in each assay run were used to determine the ratio of the variances. Intra assay variances were determined to be 2.1% and 2.4% for the unfolded human apoM ELISA and the previously described ELISA, respectively.

RESULTS

Testing of commercially available anti-human apoM antibodies

Initial attempts to create an ELISA-based detection system for human plasma apoM included screening of 15 commercially available monoclonal antibodies from various suppliers. The antibodies were screened for specificity and applicability using three different immunoassays, Western blotting (reducing/nonreducing), dot blotting, and ELISA. As seen in Table 1, the majority of antibodies tested were able to bind recombinant human apoM. However, even the most promising candidate antibodies selected by these assays failed to recognize plasma apoM in ELISA-based applications. Supplementary Fig. 1 shows representative data from each type of immunoassay employed. Human plasma displayed unspecified competition when assayed with antibody clone 3H3 in a competitive ELISA (supplementary Fig. 1D). This was consistent with a high molecular weight unspecific band seen for clone 3H3 in Western blotting experiments (data not shown).

Disulfide bridge reduction and alkylation to expose buried apoM epitopes

To test whether human plasma apoM had buried or shielded epitopes, we employed a disulfide bridge reduction and alkylation strategy to unfold plasma apoM and expose buried epitopes to the antibodies. Supplementary Fig. 2A describes the principle behind the disulfide bridge reduction and alkylation reaction. Using a sandwich ELISA approach, we screened for compatible antibody pairs that could detect human unfolded apoM. Only one antibody (EPR2904) was a rabbit mono clone, with the remainder being mouse mono clones. By using EPR2904 as secondary antibody and the mouse mono clones as capture antibodies, apoM sandwiching could be detected by an anti-rabbit IgG antibody conjugated to HRP (supplementary Fig. 2B). Screening with clone EPR2904 as secondary antibody identified mouse clone 1G9 as being the most compatible antibody partner for the detection of human apoM (Fig. 1). Recombinant human apoM and human plasma not subjected to disulfide bridge reduction and alkylation served as positive and negative control, respectively. As can be seen from the magnitudes of the absorbencies obtained (Fig. 1), unfolded human plasma apoM was only just detectable. Therefore, we speculated whether the disulfide bridge reduction reaction could be optimized to give a stronger signal. The reaction displayed a clear temperature and time dependence with apparent optimum at 30°C and an incubation time limited to 20 min (Fig. 2). These conditions were therefore used for the remainder of the experiments.

Validation of the human unfolded apoM sandwich ELISA

Human apoM was readily detectable in human apoM transgenic mouse plasma; whereas, no ELISA signal was detectable when testing mouse apoM KO plasma or WT mouse plasma containing only murine apoM (Fig. 3A). Thus, the assay displayed specificity for human apoM. The concentration of apoM in mouse transgenic plasma (1.89-fold higher than human plasma), as calculated from a standard curve of diluted human plasma (1.89-fold higher than human plasma), was determined from the magnitudes of the absorbencies obtained (Fig. 3A, B). (21). Comparison of alkylated recombinant apoM and alkylated human plasma

| Antibody | A10 | 16 | IF10 | 10C3G5 | SF21G6B8 | 11L7 | EPR2904 | 3H5 | 1G9 | 3C7 | 4C7 | 6H5 | 2E5 | 2A8 | 1A2 |
|----------|-----|----|------|--------|----------|------|---------|----|-----|-----|-----|-----|-----|-----|-----|
| WB       |     |    |      |        |          |      |         |    |     |     |     |     |     |     |     |
| Reducing | ++  | +  | ++   | +      | +++     | –    | +++     | ++ | +++ | +++ | ++  | +++ | +   | NA  |
| Nonreducing | +  | +  | –    | –      | –       | +    | NA      | NA | NA  | NA  | NA  | NA  | NA  | NA  |
| DB       |    |    |      |        |          |      |         |    |     |     |     |     |     |     |     |
| rhapoM   | ++  | NA | NA   | NA     | +++     | NA   | ++      | ++ | +   | +   | +++ | +++ | +   | NA  |
| Plasma apoM | –  | NA | NA   | NA     | –       | NA   | –       | +  | –   | –   | –   | –   | –   | –   |
| ELISA    |    |    |      |        |          |      |         |    |     |     |     |     |     |     |     |
| rhapoM   | +  | NA | NA   | NA     | –       | NA   | NA      | NA | NA  | NA  | +   | +   | ++  | NA  |
| Plasma apoM | NA | NA | NA   | NA     | NA     | NA   | NA      | NA | NA  | NA  | +   | –   | –   | NA  |

WB, Western blot (plasma apoM); DB, dot blot; rhapoM, bacterially expressed recombinant human apoM; ++++, strong signal; ++, medium signal; +, low signal, –, no signal; NA, not assessed.

*Nonspecific binding as evaluated by Western blot.

TABLE 1. Screening of commercially available anti-human apoM antibodies
apoM showed similar reactivity at high concentrations, but differed at lower concentrations (supplementary Fig. 3). Therefore, it was not possible to use recombinant apoM as calibration standard in the ELISA. To overcome this problem, a human plasma pool with a known apoM concentration was used instead.

Calculated from a mid-range control sample ([apoM] = 0.98 μmol/l) intra and inter assay coefficients of variation were 3.2% (n = 12) and 7.9% (n = 25), respectively. Recovery of human apoM was determined by spiking eight individual samples ([apoM] range = 0.44–0.99 μmol/l) with a human plasma pool ([apoM] = 0.94 μmol/l). Mean recovery for the eight samples was 100.9%. The highest detectable dilution of plasma tested was 1:8,000, corresponding to an apoM concentration of 115 pmol/l.

The quality (hemolyzed vs. nonhemolyzed) and type of sample (fasting vs. nonfasting), as well as type of anticoagulant used, could potentially influence measurements of apoM. Therefore, six different types of samples from each of four individuals were created by the use of various anticoagulants or by spiking the samples with erythrocyte lysate or Intralipid®. The latter two types were created to simulate hemolyzed and nonfasting samples, respectively. As shown in Table 2, neither the type of anticoagulant used, nor the quality/type of the sample, exhibited noteworthy effect on the measured apoM concentration. However, plasma from citrate-anticoagulated blood, as well as hemolyzed and nonfasting plasma, did display an ~5% lower apoM concentration.

Plasma apoM has previously been shown to correlate with total plasma cholesterol, HDL cholesterol, and LDL cholesterol (17). To further validate our assay, we investigated whether such correlations could be found in a cohort of 289 individuals. In agreement with previous reports, plasma apoM correlated significantly with total plasma cholesterol (Pearson r = 0.37, P < 0.0001), HDL cholesterol (Pearson r = 0.27, P < 0.001), and LDL cholesterol (Pearson r = 0.22, P < 0.01).

Finally, we investigated how the new human unfolded apoM sandwich ELISA performed compared with another previously described (17) sandwich ELISA for human plasma apoM. A set of 64 human plasma samples were measured in
uses specific antibodies and other simple reagents that are available from commercial suppliers.

The assay was validated in a number of ways. Precision was found to be satisfactory with intra and inter assay coefficients of variation below 10%. The specific type and quality of plasma used, whether anticoagulated with EDTA or any of several other standard chemicals, were found to have no or little influence on the results obtained. We further validated our assay by measuring a set of 64 human plasma samples and comparing the results with measurements from a previously published sandwich ELISA assay for human apoM. The two datasets were highly correlated and displayed no systematic bias in a Deming regression analysis. Furthermore, we correlated apoM to levels of total plasma cholesterol, HDL cholesterol, and LDL cholesterol and found significant correlations, as previously described by Axler, Ahnstrom, and Dahlbäck (17). These correlations provide strong evidence that our measurements are valid.

apoM is highly conserved through evolution, and the mouse homolog displays 79% sequence identity to the human protein (23). The assay was found to be specific for human apoM, as no signal could be detected in WT mouse plasma. These results did not agree with those obtained from Western blotting experiments in which both the capture and detection antibody of the ELISA assay reacted well with WT mouse apoM (data not shown). One explanation for this could relate to structural differences between the mouse and human protein. Human apoM contains a critical disulfide bridge at the N terminal from tandem by the same investigator using both ELISA assays. The data were analyzed by ordinary Deming regression (Fig. 4). The slope of the regression curve was 0.92 with a 95% confidence interval of 0.73–1.11. The 95% confidence interval of the y intercept was −0.22 to 0.19. Thus, no systematic bias could be found, because the 95% confidence interval of the slope and intercept spanned 1 and 0, respectively.

DISCUSSION

So far, investigations of apoM biology have been limited because high quality quantitative assays that specifically detect this protein in biological fluids have not been available. We have established a sandwich ELISA-based assay that accurately measures human apoM in plasma. This assay

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**TABLE 2. Influence of sample quality and sample type on measurements of apoM**

| Sample Type                        | Measured [apoM] Relative to EDTA-anticoagulated Sample (%) ±SD (%) (n = 4) |
|-----------------------------------|-----------------------------------------------------------------------------|
| EDTA                              | 100 —                                                                       |
| Serum                             | 103.2 3.43                                                                  |
| Heparin                           | 102 4.15                                                                    |
| Citrate                           | 94.8 3.25                                                                    |
| EDTA (with hemolysis)             | 95.4 2.10                                                                    |
| EDTA (intralipid enriched)        | 94.8 3.67                                                                    |

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**Fig. 2.** Effect of temperature and time of incubation in the disulfide bridge reduction and alkylation reaction on signal intensity obtained in the human unfolded apoM ELISA assay.

**Fig. 3.** A: Human unfolded sandwich ELISA displays specificity for human apoM. hp, human plasma; mp, mouse plasma; mp (Tg), human apoM transgenic mouse plasma. B: Example of a standard curve from the human unfolded sandwich ELISA.

**Fig. 4.** Ordinary Deming regression analysis of the human unfolded apoM ELISA versus the previously described apoM ELISA by Axler, Ahnstrom, and Dahlbäck (17).
which the protein structure can be opened when treated with reducing agents. In mouse apoM, this disulfide bridge is replaced by a salt bridge (23). Thus, it is possible that mouse apoM cannot be adequately unfolded by solely using reducing agents.

Sandwich ELISA screening experiments (Fig. 1) clearly identified one antibody pair (clone 1G9 and EPR2904) as the optimal combination among several tested. It is possible that apoM is not completely unfolded during the reduction and alkylation reaction. Hence, some epitopes may still be unavailable to some antibodies. It could also be that some capture antibodies recognize the same epitope as the detection antibody (EPR2904). These factors would explain the failure of several antibody combinations.

apoM has been implicated in several diseases. It has a strong association with S1P, which plays a role in diseases like multiple sclerosis (24), rheumatoid arthritis (25), and cancer (26), along with novel implications in protection against ischemia-reperfusion injuries and exacerbated immune reactions to infections (27, 28). Interest in investigating apoM and its importance in these biological contexts is continuously growing, and so is the need for reliable methods to measure it. In the present work, we provide a sandwich ELISA-based assay with high sensitivity and specificity, capable of measuring human apoM in various types of plasma. This method can greatly aid the research of functions for apoM in normal physiology and disease.

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