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Depletion of High-Abundance Serum Proteins from Human Uterine Lavages Enhances Detection of Lower-Abundance Proteins

Natalie J. Hannan,*[,†,‡] Chelsea J. Stoikos,[†,‡] Andrew N. Stephens,[†] and Lois A. Salamonsen[†]

Prince Henry's Institute of Medical Research, and Department of Obstetrics and Gynecology, Monash University, Clayton, 3168 Australia

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Abstract: Two-dimentional electrophoresis, 2-DE, of human uterine lavages demonstrated the abundance of serum proteins. Two methods of serum protein removal were assessed. Multiple affinity removal system was most effective in depleting abundant serum proteins. 2D-DiGE comparing depleted versus nondepleted lavages resulted in increased protein detection following depletion, further enhanced by assessing the protein profile over a narrower p*I* 4−7 range. Demonstrating depletion of abundant serum proteins is important for sensitive proteomic analysis.

Keywords: Serum protein removal • prefractionation • uterine fluid • multiple affinity removal system

Introduction

Protein secretions from the lining of the uterus (endometrium) contain important mediators that may modulate the endometrium in preparation for pregnancy and also contribute factors essential to the conceptus prior to and during implantation. To date, there has been limited identification of the protein content of human uterine fluid; most studies have been biased toward particular molecules of interest[1,2] or have revealed little information on the specific proteins secreted into the lumen.[3] Other limitations with analysis of uterine lavages are that only one sample can be obtained from each woman and this has relatively low total protein content. Recent advances in proteomic analysis provide the potential to assess the content of uterine fluid. However, this is particularly difficult due to presence of high-abundance serum proteins that reduce the detection sensitivity of current protein identification methods. This was demonstrated in a recent publication, that used two-dimentional electrophoresis (2-DE) to identify proteins present in human uterine fluid; this study resolved around 600 spots across a p*I* 4−7 range and mass spectrometry revealed that the most abundant proteins were serum proteins (and their isoforms) including immunoglobulins (Ig), alpha-1 antitrypsin precursor, haptoglobin and transferrin.[4]

A number of strategies have been developed to selectively remove high-abundance proteins to enrich for lower-abundance molecules in plasma proteome mining. These include protein precipitation by trichloroacetic acid (TCA)[5,6] or a combination of TCA/acetone or TCA/ethanol. Several affinity matrices for abundant serum protein depletion have been developed including cibachron blue (a chlorotriazine dye) and hexadecanedionic acid immobilized to Sepharose resin, both of which have a high affinity for albumin and can effectively remove albumin from plasma,[7−9] and Protein A or G[10,11] which depletes immunoglobulin (Ig). However, such affinity materials, particularly those that are dye-based, often bind many other proteins nonspecifically. Furthermore, Protein A and G may not bind all of the Ig subgroups, leaving a portion of these very heterogeneous abundant proteins behind. Individual antibody methods have proven to be more specific in depleting targeted proteins and give more complete removal of abundant proteins, allowing detection of low-abundance proteins. The commercially developed multiple affinity removal system (MARS) encompasses highly specific polyclonal antibodies to deplete the six most abundant proteins in human plasma (albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin). This system has shown rapid and efficient depletion of the abundant serum proteins in a single purification step. Cho et al., using this depletion method prior to 2D-gel analysis, were able to detect tissue plasminogen activator (tPA) in human plasma, where its concentration is estimated at 10−60 ng/mL.[12] Another study revealed that depletion of these six abundant proteins removed about 85% of the total protein from human serum or plasma, enabling a 10- to 20-fold increase in amounts of depleted serum or plasma sample applied to 2-D gels.[13] Freeman et al. demonstrated the novel application of MARS to nonhuman primate serum, demonstrating depletion of abundant serum proteins from cynomolgus monkey serum, enhancing sensitivity in both 1-D and 2-D gels.[14] Abundant protein depletion strategies have been criticized in the past, largely due to the potential loss of important proteins that may be bound to the abundant targeted proteins. For example, albumin can bind to peptides and proteins of interest, including cytokines. A recent study demonstrated variable and nonspecific loss of cytokines in the eluted fraction from a commercially available Montage Albumin Depletion kit.[15] The authors recommended analysis of the eluted fraction and disruption of the bonds between albumin and low molecular weight compounds, prior to albumin depletion. These results were consistent with another study comparing methods of abundant protein depletion, which demonstrated large differences in the amount of nontargeted protein losses using different types of affinity

* To whom correspondence should be addressed. Natalie J. Hannan, PHIMR, P.O. Box 5152, Clayton, Victoria 3168, Australia. Fax, +61-3-9594 6125; e-mail, natalie.hannan@princehenrys.org.

† Prince Henry's Institute of Medical Research, Monash University.

‡ Department of Obstetrics and Gynecology, Monash University.

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media and with different wash buffers. The most extensive losses occurred with all dye-based affinity resins utilized, whereas the analysis of MARS column eluates revealed the lowest level of nontargeted protein losses.[13] The MARS antibody columns can effectively deplete the six most abundant proteins including their different molecular forms and many proteolytic products of these proteins, with low nonspecific losses of other proteins[13] and thus provide an efficient single-step approach for the depletion of multiple abundant serum proteins with minimal losses of other proteins. In addition, MARS spin columns allow processing of multiple samples without additional complex instrumentation.

Separation of serum and plasma proteins using 2-DE remains a powerful method for plasma characterization following removal of the most abundant plasma proteins; whether this holds true for other biological fluids remains to be established. We proposed that removal of high-abundance proteins by sample prefractionation would enhance the detection of low-abundance proteins in uterine lavages.

In this study, we compared the depletion of abundant serum proteins human uterine lavages using two methods previously used for serum proteomic analysis, the modified TCA/acetone approach described by Chen[16] and the commercially developed MARS. However, modifications to basic protocols were found to be necessary for assessment of proteins in uterine lavages by 2-DE.

Methods

Ethical approval was obtained from appropriate Institutional Ethics Committees for all lavage collections and written informed consent was obtained from all subjects prior to sampling. Human uterine lavages (uterine washings) ($n = 6$) were obtained during the midsecretory phase of the menstrual cycle. In brief, 5 mL of sterile saline was infused into the uterine cavity through a fine flexible catheter; the saline solution was then aspirated, transferred into a tube, and centrifuged (900*g*) to remove any cellular debris, and a protease inhibitor cocktail set (Calbiochem; Protease inhibitor cocktail set (#539134)) was added prior to snap-freezing and storage at −80 °C in 0.5 mL aliquots. The protein concentrations of the lavage sample were assessed using Bradford Assay kit (Bio-Rad, Hercules CA) according to manufacturer's instructions. Proteins were precipitated with acetone for 90 min, centrifuged at 20 000*g* for 20 min at room temperature (RT), and resuspended in solubilization buffer no. 1 [containing 40 mM Tris, 7 M urea, 2 M thiourea, 1% dimethylammoniopropanesulfonate (C7BzO) and protease inhibitor cocktail set (as used above)]. Subsequently, 150 μg (comprising a pool of 25 μg from each lavage; $n = 6$) of protein was applied to p*I* 3−10 11-cm IPG strips (Amersham). Proteins were then resolved by 2-DE and stained with colloidal Coomassie Brilliant Blue (CBB).

Results and Discussion

Initial 2-DE analysis of uterine lavages demonstrated high abundance of serum proteins (Figure 1A), with the major constituent being human serum albumin. To improve the detection of low-abundance proteins in the lavages, two methods of albumin removal were trialled. Albumin was removed from the samples using TCA and acetone as described by Chen et al.[16] TCA forms a complex with albumin that is soluble in organic solvents such as acetone, causing albumin to remain in solution and thus allowing selective precipitation

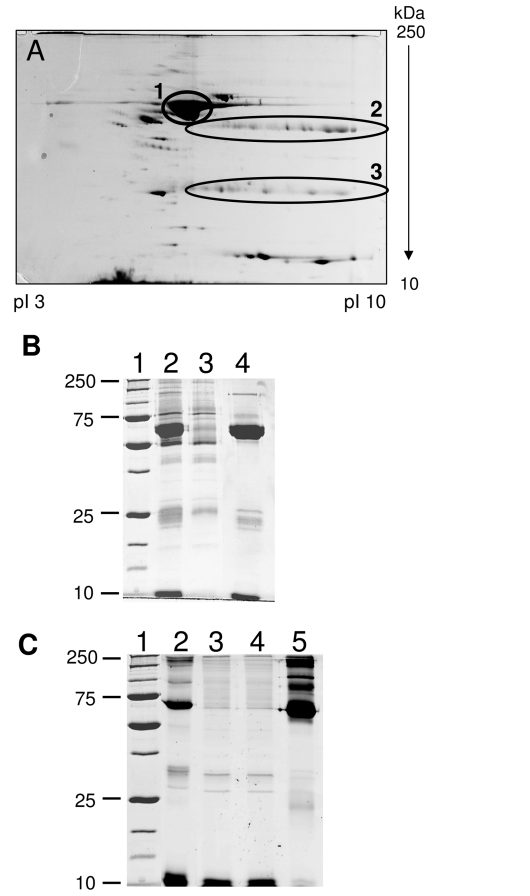

Figure 1. (A) 2-DE analysis of an endometrial lavage sample (p*I* 3−10; stained with colloidal CBB) demonstrates an abundance of serum proteins, predominantly human serum albumin (1) and immunoglobulins (Ig) heavy (2) and light (3) chains (determined by their p*I* and M_r). (B) Assessment of albumin removal (1) by 1-DE following TCA/acetone treatment. 1-DE of a uterine lavage: lane 1, molecular weight (MW) markers; lane 2, nondepleted lavage; lane 3, lavage following albumin depletion (TCA/acetone treatment), demonstrates the removal of a large 66 kDa band corresponding to human serum albumin; lane 4, TCA/albumin complex (removed fraction) demonstrating the presence of the removed albumin (66 kDa band) and multiple other bands indicating nonspecific protein removal. (C) Assessment of MARS depletion of abundant serum proteins. 1-DE of a uterine lavage: lane 1, MW markers; lane 2, nondepleted lavage; lane 3, depleted lavage flow through 1; lane 4, depleted lavage flow through 2; and lane 5, eluted bound serum protein fraction.

of the other proteins (except albumin). The resultant pellet was resuspended in solubilization buffer no. 1 containing protease inhibitors (as above). Albumin was precipitated from the first fraction with acetone and centrifuged as above, and the pellet containing albumin was resuspended in solubilization buffer no. 1. 1-DE followed by staining with Sypro Ruby Protein Gel stain (Molecular Probes; Invitrogen, Eugene, OR) demonstrated the removal of a protein band approximately 66 kDa in size, corresponding to human serum albumin (Figure 1B). While this method successfully depleted considerable amounts of albumin in the lavages (Figure 1B; lane 3), other bands were observed by 1-DE in the precipitated fraction (Figure 1B; lane 4) containing the albumin.

A more efficient enrichment of lower-abundance proteins was achieved using the Multiple Affinity Removal System (MARS) spin cartridges (Agilent Technologies, Santa Clara, CA)

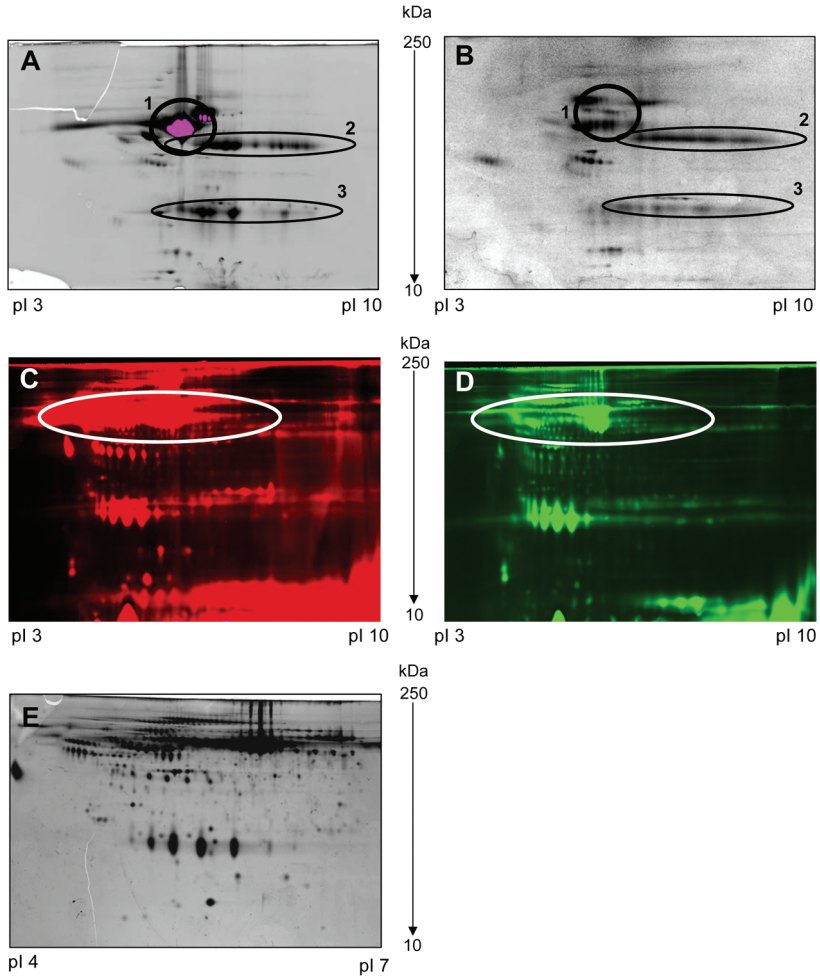

Figure 2. Comparison of depleted and nondepleted lavage samples by 2-DE. (A) Nondepleted lavage sample compared with (B) albumin depletion by TCA/acetone treatment over a p*I* 3−10 range. Successful depletion of albumin from the sample following treatment is evident (1), but large amounts of abundant proteins remained in the sample including Ig heavy (2) and light (3) chains. 2D-DIGE analysis of abundant protein removal using MARS; (C) prior to depletion (Cy5-*red*) (D) and following depletion (Cy3-*green*) (over a p*I* 3−10 range). Removal of the abundant proteins illustrates the effectiveness of depletion and the increased resolution. (E) 2-DE of the depleted lavage over a narrower pH range (p*I* 4−7) further increases the resolution and detection of protein spots.

designed to bind and remove the six most abundant proteins from human serum and plasma (albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin). Manufacturer's instructions devised for use with human serum were modified for high-abundance protein removal from the lavage samples. Lavage samples were thawed on ice and the protease inhibitor cocktail set (Calbiochem) was added. Samples were diluted with 1 vol of supplied buffer A (Buffer A; Agilent Technologies) and filtered (0.22 μm filter) and 400 μL aliquots were loaded onto the spin cartridge, which was then centrifuged. High-abundance proteins were retained in the cartridge and the depleted endometrial lavage flowed through the column and was collected as flow through (F) 1. The column was washed and the wash (F2) was combined with F1. Once the entire sample was depleted, the high-abundance proteins bound to the cartridge were eluted with the supplied buffer B (Buffer B; Agilent Technologies). Following removal of salts and buffer exchange, the depleted protein fraction (F1 + F2) was diluted with 1 vol of solubilization buffer no. 1, and centrifuged through individual Macrosep UF spin filter columns (3 kDa molecular weight cutoff, 15 mL capacity) (PALL Life Sciences, NY) at 4500*g* until the volume was reduced to 800 μL. The retentates were snap-frozen and stored at −80 °C. Total protein was measured in uterine lavage samples prior to and following MARS depletion of abundant proteins, which demonstrated that 90 ± 0.93% (mean ± SEM; $n = 16$) of the total protein was removed by the MARS column.

An aliquot of each of the fractions was subjected to 1-DE and stained as above. This confirmed the high abundance of the albumin (MW 66 kDa) in the original lavage (Figure 1C; lane 2); removal of targeted protein bands can be seen in the depleted fractions (Figure 1C; lanes 3 and 4), while bands corresponding to the targeted proteins can be seen in the eluted bound fraction (Figure 1C; lane 5).

Both methods were then assessed by 2-DE for a more accurate analysis and to determine whether removal is a useful tool for analysis of these samples. Proteins from the different fractions from each method were reduced with tributylphosphine (TBP; 5 mM (Sigma)) and cysteines were alkylated using acrylamide monomer (10 mM; Sigma) for 90 min at room temperature (RT). The reaction was quenched with DTT (10 mM; Sigma), at RT for 5 min. Citric acid (1 M; Sigma) was added and samples were incubated for 2 min and then centrifuged at 20 000*g* for 20 min at RT. Superna-

tants were carefully transferred to fresh tubes and the proteins were precipitated and pelleted using acetone for 90 min at RT, followed by centrifugation at 20 000*g* for 20 min at RT. Protein pellets were air-dried and resuspended in solubilization buffer no. 2 containing 7 M urea, 2 M thiourea and 1% C7BzO. Protein content was measured in each sample using a Bradford Assay kit (Bio-Rad) according to manufacturer's instructions. Following reduction and alkylation, endometrial proteins were separated by 2-DE. Briefly, 150 μg (pooled lavage; $n = 6$) of protein was adjusted to 450 μL with solubilization buffer no. 2. Ampholytes 0.5% (Amersham), DTT (10 mM) and Orange G dye (Proteome Systems; no. O10002-6) were added to the sample. The samples were then applied to 24-cm IPG strips, pH 3−10 or 4−7 gradient (Amersham). Proteins were isoelectrically focused using the IPGphor (Amersham) IEF system. IPG strips were then equilibrated and applied to 24 cm, 8−16% gradient second-dimension Tris-HCl gels. Gels were then stained using Sypro Ruby Protein Gel stain as above and images were acquired using Image Reader (FLA-500 Series, Version 1; Fuji Photo Film Co. Ltd. 2001−2003). Gel editing, spot matching and spot numbers were assessed using Same Spots & Progenesis software (PG240; version 2006, Non Linear Dynamics Ltd.).

2-DE again demonstrated effective removal of albumin using the modified TCA/acetone approach (Figures 2,B). However a high abundance of other serum proteins (e.g., Ig heavy and light chains (determined by p*I* and MW)) remained in the sample (Figure 2B) after depletion. The TCA/acetone approach also resulted in decreased resolution of protein spots by 2-DE (Figure 2B); thus, DIGE was not performed.

To assess the effect of MARS fractionation, lavage proteins were examined using 2D-Differential in Gel Electrophoresis (DiGE). In brief, proteins (from the depleted and nondepleted fractions) were resuspended in CyDye labeling buffer containing 30 mM Tris, 7 M urea, 2 M thiourea and 1% C7BzO and labeled with Cy3-*N*-hydroxysuccinimide ester (Cy3) and Cy5-*N*-hydroxysuccinimide ester (Cy5) (Amersham) according to manufacturer's instructions. The Cy3 and Cy5 labeled proteins were then combined. As described above, the labeled samples were resolved using 2-DE over p*I* range 3−10 (Figure 2C and D). DiGE imaging was performed at two excitation wavelengths, 532 and 633 nm, for Cy3 and Cy5, respectively. Figure 2C and D shows the same sample prior to (Figure 2C) and following depletion using MARS (Figure 2D), demonstrating the efficient removal of the targeted proteins and a marked increase in the number of spots detectable in the uterine lavage previously masked by the abundant serum proteins in the sample. Image analysis revealed approximately 900 protein spots in the MARS depleted uterine lavages assessed over a p*I* range 3−10 (Figure 2D). The majority of proteins present within the lavage samples appeared to lie within a narrower p*I* range of approximately 4−7; very few proteins were detected by 2-DE in the acidic and basic regions (Figures 1A and 2A−D) following resolution over a p*I* 3−10 range. Protein resolution and spot number were enhanced by separation over a narrower pH range (p*I* 4−7): approximately 1050 spots were detected in the same sample (Figure 2E), which was subsequently repeated with a separate sample and consistently spot numbers detection was enhanced.

In summary, this study describes and validates a method for the efficient depletion of abundant serum proteins from human uterine lavages using the MARS. Since these proteins comprised a consistent 90% of the total protein in the lavages, this technique provides a practical, relatively inexpensive method to enrich uterine lavages for less abundant proteins, including those secreted into the uterine cavity from the endometrial epithelium. These will include growth factors, cytokines and chemokines. Identification of the human uterine secretome has clinically relevant applications since these proteins play important roles in preimplantation development of the conceptus and during implantation, both critical steps in establishing pregnancy and in determining the long-term health of the resultant individual. They may also provide diagnostic markers for endometrial receptivity tests needed for improving current assisted reproductive technologies.

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