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Improved Mass Spectrometric Proteomic Profiling of the Secretome of Rat Vascular Endothelial Cells

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Abstract

Serum albumin contamination of cells cultured in vitro significantly impedes the mass spectrometric analysis of proteins secreted by the cells. Here we report a novel washing and culturing technique for rat vascular endothelial cells that considerably reduces the concentration of the commonly used additive for cell culture, bovine serum albumin (BSA), in the secretome of these cells. Cells are rinsed stringently and cultured for 24 h in serum-free media without appreciably impeding cell growth or viability. The percentage of BSA scans identified by tandem mass spectrometry (LC-MS/MS) in stringently rinsed cells (average 13.2%) was significantly lower than either the moderately rinsed or no rinse cell treatments (average 35.2% and 45.2% respectively). Furthermore, the stringent wash treatment allowed the confident identification of a larger portion of the secretome of rat endothelial cells by LC-MS/MS.

Keywords

secretome; serum removal; liquid chromatography mass spectrometry; rat endothelial cell

Introduction

Endothelial cells and other cell types cultured in vitro are usually grown in media supplemented with high levels of bovine serum to achieve optimal growth. Serum-supplemented media can produce difficulties in the downstream analysis of these cells because of masking effects of highly abundant serum proteins. This is especially true when trying to investigate proteins secreted into the media. Secreted proteins likely play a direct role in the control and regulation of many biological processes such as growth and development, cell signaling, adhesion and binding, and apoptosis. The secretome is of particular importance as secreted proteins are potential biomarkers for diseases such as cancer and cardiovascular disease. The analysis of a cell's secretome could be valuable in disease diagnosis and prognosis, and thus, interest in investigating secreted proteins has greatly increased in recent years.^{1,2}

Although genomic techniques have been used to indirectly analyze the secretome, proteomic methods allow the direct examination of proteins and therefore are the method of choice in this study.³ Mass spectrometry is commonly used in proteomic profiling experiments to identify intact proteins based on molecular weight or by peptide sequencing using MS/MS

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Supporting Information Available: Complete list of all the proteins identified with more than one scan in the stringent rinsed cells. This material is available free of charge via the Internet at <http://pubs.acs.org>.

fragmentation. Direct secretome analysis by mass spectrometry of cells grown in vitro is complicated by serum proteins such as bovine serum albumin (BSA).⁴ Although serum-free growth media have been developed for endothelial cells, optimal growth and viability is only achieved in serum-supplemented media.^{5,6} The high quantities of albumin and other serum proteins in serum-supplemented media often mask the lower abundance proteins secreted by endothelial cells, concealing their identification by mass spectrometry given the low dynamic range of most MS/MS analyzers. The low dynamic range observed during peptide fragmentation is due to the limited amount of scans the MS/MS analyzer can perform as ions are trapped and fragmented. Current methods for serum removal involve techniques such as metabolic labeling of the secretome to differentiate it from serum proteins, binding of serum albumin to specially designed resins to remove serum proteins from secreted proteins, and removal of serum by washes.⁴ Current methods such as metabolic labeling are either time-consuming or, in the case of the use of resins designed to remove serum, risk the unintentional removal of important nonserum proteins. There is also a balance between serum albumin removal by washing and cell survival, as stringent washes can damage or kill the cells prior to secretome analysis.

In this study, we investigated the use of novel washing methods to reduce the concentration of the serum protein, BSA, in a rat endothelial secretome in preparation for subsequent MS analysis.

Experimental Section

Isolation and Culture of Rat Endothelial Cells

All animal protocols were approved by the Medical College of Wisconsin (MCW) Institutional Animal Care and Use Committee. All chemicals were obtained from Sigma (St. Louis, MO) unless noted.

Male Spague-Dawley rats from Harlan (7 weeks of age) were anesthetized by intraperitoneal injection of sodium pentobarbital (0.1 mL/100 g). The lower legs and abdomen were shaved and cleaned. Using sterile instruments, the sub-femoral artery and vein were isolated and extracted from the leg. The vessels were digested in 0.2% collagenase type 1 in Krebs solution (in mM: NaCl 120, KCl 4.7, CaCl₂ 3.0, MgCl₂ 1.43, NaHCO₃ 25, KH₂PO₄ 1.17, glucose 11, and EDTA 0.03) at 37 °C for 1 h.

The digests were centrifuged at 500 rcf for 10 min. The resulting pellet was resuspended in complete media (RPMI 1640 (Cellgro), 20% fetal bovine serum, 1% 100× antibiotic cocktail from Sigma (penicillin G, 10,000 units/mL, streptomycin sulfate 10 mg/mL and 25 µg/mL amphotericin B), 0.4% gentamicin µg/mL (Invitrogen, Grand Island, NY)) and plated in disposable 100-mm diameter culture dishes from Corning (Corning, NY). Four days after isolation, the media was changed to MEM media (MEM D-valine powder with L-glutamine (US Biological, Swampscott, MA), 20% fetal bovine serum, 1% antibiotic cocktail from Sigma (described above), 0.4% gentamicin to prevent potential fibroblast contamination. Cells were maintained on this media for 1 week and then returned to complete media and grown to confluence. All cells were passaged three times prior to treatment.

Rinsing and Secretome Extraction

Three different rinsing techniques were compared. In the first group, plates were not rinsed; the media was changed from complete media to “serum-free media” (RPMI 1640). The first group was designated the no rinse treatment. The second sample, moderate rinsing treatment, was rinsed two times with 3 mL of serum free media. Finally, the third sample, stringent rinsing treatment, was rinsed two times with 10 mL of Dubelcco’s phosphate buffered saline with calcium and magnesium (DPBS) and one time with 10 mL of serum-free media. Cells were

maintained on serum-free media for 24 h before media samples were collected. Media from each condition was concentrated using a vivaspin tube PES 5000 MWCO (Satorius, Geottingen, Germany). There were a total of four trials for each treatment.

Protein Digestion

For each of the three rinsing techniques, no rinse, moderate, and stringent, 300 μ g of secreted protein was reduced with 10 mM dithiothreitol (DTT) at 37 °C for 30 min. Samples were then incubated without light in 25 mM iodoacetamide (IAA) at 37 °C for 45 min. After reduction and alkylation, samples were precipitated with 4 $\times$ (v/v) ice-cold acetone and incubated on ice for 2 h. Precipitated proteins were resuspended in 500 mM ammonium bicarbonate and digested with 2 μ g trypsin (Promega, Madison, WI) overnight at 37 °C. Digestions were stopped with the addition of 1 μ L of 1% (v/v) formic acid and desalted with modified C₁₈ Zip-Tips (Millipore, Billerica, MA).

Mass Spectrometry

Mass spectral analysis was carried out on a ThermoFinnigan LCQ Deca XP plus ion trap mass spectrometer interfaced with a Surveyor LC system through an RP microcapillary column (100 μ m i.d. packed with 10 cm of 5 μ m C₁₈ RP particles (Phenomenex, Cheshire, UK)). Solvent A was 5% (v/v) acetonitrile in 0.1% (v/v) formic acid and solvent B was 95% (v/v) acetonitrile in 0.1% (v/v) formic acid. The protein digest injected onto the microcapillary column was resolved at the rate of 1 μ L/min, by the following gradient conditions: 0–30 min 0–5% B, 30–180 min 5–35% B, 180–240 min 35–65% B, 240–250 min 65–100% B, 250–260 min 100% B, 260–300 min 100% A.

The MS data obtained was interpreted using SEQUEST against uniprot_sprot.fasta database for protein identification.⁷ Peptides were accepted as being positively identified if they passed the following criteria: charge +1, Xcorr of 1.8, charge +2, Xcorr of 2.0, and charge +3, Xcorr of 2.2. Any peptide identified with an Xcorr of less than 3 was manually verified by examining the fragmentation patterns.

To determine which proteins were identified with the most confidence, we calculated a score for each protein that assesses the coverage of the protein in mass spectrometry:

$$\frac{\# \text{ of unique peptides} \times (\text{number of scans}/10) \times \text{highest Xcorr}}{\text{number of fractions the protein occurs in}}$$

By assigning proteins that are identified with two or more unique peptides a higher score than proteins identified with the same peptide in multiple scans, small peptide and small molecule signal effects are reduced. Likewise, the use of the highest Xcorr value discounts scans with low probability identification. The normalization to the number of fractions the protein occurs in reduces the impact of contaminants or common components such as trypsin, because common contaminants are usually found in most or all fractions whereas most peptides elute in only one or a few fractions.

Results

Averages of 106, 90, and 225 putative secreted proteins were identified by mass spectrometry in the no rinse, moderate rinse, and stringent rinse treatments, respectively (Table 1).

The percentage of contaminant BSA scans identified by mass spectrometry in the stringent wash (average 13.2%) was much lower than either the moderate or no rinse treatments (average 35.2 and 45.2%, respectively, Figure 1). The decrease in BSA scans identified in the stringent

wash treatment compared to the no rinse control treatment was statistically significant (p -value = 0.0052). The decrease in BSA scans in the moderate rinsed treatment was not significant compared to the no rinse control.

The decrease in BSA scans in the stringently rinsed cells was coupled with an increase in non-BSA proteins that were confidently identified with more than one scan (Table 2 and Figure 2). The stringently washed cells had almost two times more non-BSA proteins identified with more than one scan than the no rinse control when normalized to total protein identified per mass spectrometry run. The increase protein identification in stringently rinsed cells compared to the no rinse control and moderate rinsed cells was significant (p -value = 0.00158, single factor ANOVA). The moderate rinsed cells showed no significant increase in non-BSA proteins identified with more than one scan when compared to the no rinse control.

The four most confidently identified non-BSA proteins based on the score calculated as described in the methods section were Collagen alpha chain precursor, Actin, SPARC precursor (Secreted Protein Acidic and Rich in Cysteine), also known as Osteonectin, and Thrombospondin 1 precursor. (For a complete list of all the proteins identified with more than one scan in the stringent rinsed cells, see Supplementary Table 1, Supporting Information).

Discussion

Proteins secreted by endothelial cells are difficult to study in vitro due to contamination of the secretome by serum proteins such as BSA that are critical components of most cell culture media. We have developed a novel rinsing and culturing technique to remove serum proteins from the secretome prior to analysis by mass spectrometry.

As shown in the data above, it is important to adequately rinse the endothelial cells to remove serum proteins. The moderately rinsed cells, rinsed twice with 3 mL of serum-free media, showed no significant reduction in BSA compared to the no rinse control cells whereas the stringently rinsed cells, rinsed two times with 10 mL of DPBS and once with 10 mL of serum-free media did show a significant reduction in BSA peptides identified by mass spectrometry when compared to the no rinse control cells. Serum proteins such as BSA are notorious for binding to many surfaces including membrane and the plastic plates cells are grown in. It is possible that the moderate rinsing technique was not vigorous enough to remove BSA from the cellular surface and Petri plates and this is why we saw no significant reduction of serum proteins in the moderate rinse but did see significant reduction of BSA in the stringently washed cells.

The reduction of BSA peptides detected by mass spectrometry in the stringently washed cells was coupled with an increase in non-BSA proteins that were positively identified with more than one scan and more than one peptide per protein. This is an important result as proteins that are detected only once with one peptide in a given mass spectrometry run are not identified with high confidence. The more scans and peptides positively identified from a given protein, the more confidence there is in the protein's correct identification and the higher the score was based on the equation given in the methods section. Thus, we had a much higher degree of confidence in the proteins recognized as being secreted from the cultured endothelial cells that were stringently washed. In the moderate and no rinse control treatments, few non-BSA proteins were detected with more than one scan. Therefore, the moderate and no rinse control treatments would not be adequate rinsing techniques to use in experiments aimed at categorizing a given cell's secretome by mass spectrometry.

To determine whether the increase in non-BSA proteins in rinsed cell treatments is a reflection of increased lysis of cells, we determined the percentage of cytosolic and nuclear proteins identified in each treatment for the four replicates. No significant differences in the number of

cellular proteins were observed between the treatments. In all treatments, the percentage of cytoplasmic and nuclear proteins was 10 and 9%, respectively (data not shown). No increase in the percentage of cytoplasmic or nuclear proteins in the stringently washed cells was observed. These results strongly suggest that the increased identification of non-BSA peptides by mass spectrometry in the stringently washed samples is due to the reduction of BSA in the sample and not an increase of other nonsecreted proteins from lysed cells.

We also tried to remove BSA from mass spectrometry runs of the three rinsing treatments by including an exclusion list during our mass spectrometry running conditions. Data-dependent analysis was carried out with and without reject masses for BSA peptides for the same sample mixture to compare the interference of BSA peptides for protein identification in the two cases. Reject mass lists were created by selecting the masses of BSA peptides identified from the MS run without any mass exclusion list. We found no significant difference between any of the three rinsing treatments run with exclusion lists and without exclusion lists (data not shown). Thus, the use of an exclusion list does not correct or compensate for the experimental difficulties caused by BSA in the sample. The stringent method developed in this study was a balance between BSA removal and cell survival.

Finally, our novel stringent rinsing technique allowed us to reduce the amount of BSA sequenced by mass spectrometry and allowed us to more accurately identify the secretome of rat endothelial cells. One of the most commonly detected non-BSA protein identified in our mass spectrometry runs was the SPARC precursor (secreted protein acidic and rich in cysteine), also known as osteonectin. The protein seems to be instrumental in cellular growth by its interactions with cytokines, and the extracellular matrix and has been found to mainly be expressed in cells that are experiencing morphogenesis.^{8,9} The stringent rinsing technique developed in this paper can be used in the future to identify more proteins of interest secreted from endothelial cells and could even be used to compare the difference in the secretome from healthy and diseased endothelial cell states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Percentage of BSA scans versus total scans identified by mass spectrometry. Only the stringent rinse showed significant decrease in BSA percent compared to the no rinse control.

Figure 2.

Three treatments were concentrated and proteins separated by SDS PAGE (8%). Each treatment (20 μ g) was loaded on the gel. The gel was stained with Coomassie to demonstrate how the different rinsing techniques removed BSA (69 kDa, arrow on left) and increased identification of secretome proteins.

Table 1

Summary of Mass Spectrometry Results from BSA Removal Rinses

Total non-BSA proteins identified					
sample	trial 1	trial 2	trial 3	trial 4	average (standard error)
no rinse	63	187	117	54	106 ± 35.17
moderate rinse	78	97	123	57	90 ± 16.21
stringent rinse	176	183	288	248	225 ± 31.01

Total non-BSA proteins identified with > 1 scan					
sample	trial 1	trial 2	trial 3	trial 4	average (standard error)
no rinse	10	30	20	8	17 ± 5.85
moderate rinse	14	25	23	12	19 ± 3.73
stringent rinse	48	56	68	67	60 ± 5.51

Table 2Percentage of Proteins with Hits ≥ 1 versus Total Proteins in Sample

sample	trial 1	trial 2	trial 3	trial 4	average (standard error)
no rinse	17.19	16.49	17.80	16.36	17 ± 0.38
moderate rinse	18.99	26.53	19.35	22.41	22 ± 2.02
stringent rinse	27.68	30.98	31.49	27.31	29 ± 1.25