Mass spectrometry analysis of extracellular vesicle proteins

Thesis of doctoral (Ph.D.) dissertation

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Introduction

Recently a new type of intercellular communication has been recognized, that cells can communicate *via* the secretion of extracellular vesicles. These nanometer sized sub-cellular structures are surrounded by a phospholipid bilayer and enclose different molecular components (e.g. proteins, mRNA, miRNA) of the donor cell. Extracellular vesicles may influence the properties of the recipient cells in various ways. These extracellular vesicles play an extraordinary role in diverse biological and immunological processes (e.g. tumor pathogenesis, autoimmune diseases, antigen presentation to T-cells, immune-modulation, propagation of contagious agents, etc.)

Detection of proteins present in different types of vesicles is primarily performed by mass spectrometry, profiting from the excellent sensitivity and resolution of the method. However, in order to analyze proteins present in vesicles, first they need to be extracted. For the analysis of protein mixtures "bottom up" proteomics is usually the first choice. In this case the complex protein sample is enzymatically digested and the generated peptide fragments are separated and analyzed mainly by nanoUHPLC-MS(MS).

Thymus has a central role in immune cell development and induction of immune tolerance nevertheless proteomic data of thymic vesicles are not available. For this reason we focused our attention on the proteomic characterization of thymocyte-derived apoptotic bodies and microvesicles in order to gain an insight into the potential biological significance and functions of these sub-cellular structures.

Objectives

The main goal of my PhD work that was performed in collaboration with the Department of Genetics, Cell- and Immunobiology of the Semmelweis Unversity was to determine the proteins present in thymocyte derived microvesicles and apoptotic bodies. Furthermore we also wanted to learn about the role of these vesicles in the regulation of thymus function.

To perform these studies, first we had to solve some analytical challenges, and the limited amount of available sample demanded development of new methods and protocols, such as:

- method for the extraction of proteins not interfering with downstream LC/MS analysis
- miniaturized digestion protocol, which works well for small amounts of proteins in small sample volume and allow detection of minor components in protein mixtures

Methods

I applied three diverse methods for the extraction of proteins from the vesicles. The methods were the followings: *i)* use of surfactants, *ii)* gel electrophoresis and *iii)* freeze-thaw cycles. Best results could be achieved by the freeze-thaw method. The freeze-thaw method included freezing in liquid nitrogen (30 sec) 5 times and -20 °C freezer (1 hour) two times [1]. Each time thawing was carried out by sonication in a water bath (10min). The method successfully released the proteins from the vesicles enabling their digestion.

In the next step I compared three different in solution digestion protocols using a model sample (human plasma + beta-lactoglobulin (BLG) internal standard). Two of the protocols (protocol A and B) have been published previously, while the third protocol was the miniaturized digestion protocol [2] which I have developed during my PhD work. Details about the protocols are summarized in Table 1.

Table 1. The applied digestion protocols

		Protocol A	**Protocol B**	**"Mini" protocol**
Sample volume		**60 µL**	**12 µL**	**10 µL**
Sample components	**Plasma**	**0.04 µL**	**0.04 µL**	**0.004 µL**
	(Albumin	**30 pmol**	**30 pmol**	**3 pmol)**
	BLG	**20 pmol**	**20 pmol**	**2 pmol**
End concentration				
of the reagents	**RapiGest**	**0.16 %**	**0.08 %**	**0.008%**
	DTT	**5.3 mM**	**8.3 mM**	**2 mM**
	Iodoacetamide	**17.8 mM**	**10.5 mM**	**6.6 mM**
	NH_4HCO_3	**17.8 mM**	**52.6 mM**	**33 mM**
Trypsin: analyte ratio		**1:25**	**1:25**	**1:2.5**

„Mini protocol”: to 10 µL model sample (containing 2 µL 500 times diluted human plasma+2 pmol BLG in water) or microvesicle or apoptotic body extraction 1 µL reagent mixture (containing 0.13% RapiGest SF and 33 mM dithiothreitol) was added and kept at

60 °C for 30 min to achieve protein unfolding and reduction. Following reduction 3 μL reagent mixture containing 167 mM NH_4HCO_3 and 33 mM iodoacetamide was added to alkylate the sample which was kept for 30 min in the dark at room temperature. The alkylated samples were digested at 37 °C for 90 min with trypsin (0.5 μL, 4 μM). Note, in the case of the test mixture this corresponds to an enzyme: analyte ratio 1:2.5, which is ca. ten times higher than that used in Protocols A and B. The digestion was quenched by adding 0.5 μL formic acid (30 min at 37 °C); which also served to degrade the surfactant and to phase separate the hydrophobic part from the digested sample. The reaction product was centrifuged at 13500 rpm (corresponding to 17000 g) for 10 min, and the supernatant was transferred into a micro-vial for further analysis. Total volume of the resulting peptide mixture was 15 μL.

The LC–MS/(MS) analysis of the tryptic peptide mixtures was performed using a nanoflow UPLC system (nanoAcquity UPLC, Waters) coupled to a Q-TOF Premier mass spectrometer (Waters) equipped with nanoElectrospray source. The tryptic peptides were desalted online on a Symmetry C18 trap column (180 μm i.d.×20 mm, Waters), and then separated on a reverse phase analytical column (C18, 75 μm i.d. ×150 mm, 1.7 μm BEH300 particles, Waters). Proteins were identified by tandem mass spectrometry. Data were acquired in the data dependent acquisition mode (DDA) using 4 s cycles, consisting of a full scan spectrum (m/z: 400–1999) and MS/MS spectra of the three most abundant ions.

Data from the DDA experiments were processed using ProteinLynx Global Server v.2.3 (Waters). All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot 2.2) and X! Tandem (The GPM, thegpm.org; version 2007.01.01.1) softwares.

In case of extracellular vesicle samples Mascot was searched against SwissProt database with house mouse (*Mus musculus*) as taxonomy assuming digestion enzyme trypsin. One missed cleavage was allowed. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.15 Da and a parent ion tolerance of 50 ppm. Iodoacetamide derivative of cysteine was specified as a fixed modification. Oxidation of methionine and phosphorylation of serine, threonine and tyrosine were specified as variable modifications. Scaffold (version Scaffold_3_00_07, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides.

In case of human plasma samples the data was searched against SwissProt sequence database with human (*Homo sapiens*) as taxonomy using Mascot Server version 2.2 (Matrix

Science, London, UK). We used Mascot search to identify both miscleaved and non-specific peptide fragments (using semi-trypsin in the latter case). Proteins were identified according to two different criteria. 'Stringent' identification required identification of at least two peptide fragments, both with a Mascot ion score greater than 34 ($p<0.005$). In case of the 'lenient' criteria proteins with scores greater than 24 ($p<0.05$) were accepted, and identification of one peptide was deemed sufficient. Signal intensities were determined in chromatographic runs using single stage mass spectrometry. Performance of the developed "mini" protocol was evaluated using the following figures of merit: (a) the number of proteins identified, (b) comparison of signal intensities in the HPLC-MS chromatograms of selected peptide fragments.

The amount of histones present in apoptotic bodies and microvesicles were compared using a type of label-free quantification technique. Briefly, the average of the 3 most intensive peptide signals of detected protein were used as a measure of protein amount (in molar units); and were compared to the average of the 3 most intensive peptide signals of the BLG internal standard in a single stage MS run. As the concentration of the internal standard is known, quantitative estimation is possible.

Results

The literature on the mass spectrometry analysis of extracellular vesicles is modest regarding the extraction of proteins. I have compared three methods for the extraction of proteins from the vesicles. Using different concentrations of Triton-X 100 and SDS surfactants I couldn't identify proteins probably due to the ion supression caused by high amount of surfactants. In case of SDS gel electrophoresis followed by in gel digestion, which is the most widely used procedure in the literature, although proteins could be identified, the process consists of 46 steps and takes about 2 days to complete. Furthermore, extraction of digested peptides suffers from bias and the nanoUPLC-MS(MS) analysis of the high number of samples (one for each gel band) is also time-consuming. The best results were achieved by using freeze-thaw cycles both in terms of number of proteins identified and the time of sample processing.

My next aim was to develop a miniaturized tryptic digestion protocol that works well for small amounts of proteins using small volume of reagents. Tryptic digestion is a key step in the proteomic workflow. It is well described, and there are several protocols in use. In general these relate to the digestion of proteins in nmol quantities and sample volumes in the range of 100–500 μL. Meanwhile digestion of proteins present in low amounts (in pmol or fmol range) is not solved.

In order to identify small amounts of proteins in extracellular vesicles I intended to develop a method which works well

a) with small sample volumes

b) when the total protein amount is limited (< 5 pmol)

c) can detect minor components in protein mixtures

d) would yield semi-quantitative results.

For a test sample we have selected diluted plasma; which has one major (albumin) and a large number of well-known less abundant components. The test sample also contained BLG internal standard. Adding an internal standard protein has two advantages: first, it provides possibility for semi-quantitative determinations. Second, it may help avoiding (or at least reducing) problems due to irreversible adsorption of sample proteins to various surfaces, like the walls of the centrifuge tube or pipette tips. In such a case the presence of a large amount of standard protein (i.e. BLG) may help avoiding sample loss due to competitive adsorption. The total sample amount was fixed to 10 μL; containing ca. 3 pmol (200 ng)

albumin; various minor components in total amount of ca. 0.3 pmol; and 2 pmol BLG internal standard. Performing the steps of the digestion the sample volume increases only by 5 µL.

In the course of initial method optimization we have varied the sample and reagent volumes, the amount of denaturizing agent (ReapiGest), dithiothreitol and iodoacetamide. We have found that using a large amount of denaturizing agent has an adverse effect on identifying minor proteins, so we have reduced its concentration compared to existing protocols. We have varied the amount of dithiothreitol and iodoacetamide as well, but these parameters did not have a large effect on the results. Following initial method optimization, influence of the trypsin amount (enzyme:analyte ratio of 1:2.5, 1:5, and 1:20) and incubation time (90 and 180 min) was studied. These results indicated that higher trypsin to sample ratio is advantageous, especially for detecting minor proteins, so we have used a high trypsin/analyte ratio (1:2.5 molar ratio) and 90 min incubation time.

Results of the miniaturized protocol were compared to two other protocols, developed for digestion of larger sample amounts. We have also checked the results when the small amount of protein (5 pmol total protein content) was digested in a larger volume, the resulting mixture dried in a vacuum centrifuge; and reconstituted. The same amount of tryptic digest was injected onto the column in each case and the results clearly indicate that the "miniaturized" protocol is at least as efficient as the "standard" protocols.

Reproducibility of the „Mini" protocol was also checked, the RSD of peak areas were 8.3% for the selected peaks; and the number of proteins identified varied between 16 and 20. This accuracy is perfectly adequate for most proteomics studies, especially taking into account that in several cases 1 µL reagent was added to the sample.

Thymus is a central immune organ, which plays an important role in immune cell development and immune tolerance induction. Therefore we focused on the proteomic characterization of thymocyte-derived apoptotic bodies and microvesicles. Proteins were identified by tandem mass spectrometry according to stringent criteria presented in the literature. In this way 142 proteins were identified in apoptotic bodies and 195 proteins in microvesicles released by murine thymus cells. We found a strikingly high number of proteins shared by apoptotic bodies and microvesicles. The identified proteins included cytoskeletal (actin and tubulin), cytoskeletal binding-proteins (ezrin, moesin, cofilin 1), metabolic enzymes (GAPDH, alpha-enolase, malate dehydrogenase 1) and chaperones (T-complex protein subunits, hsp90). These proteins have also been detected previously in extracellular vesicles of various origins indicating the presence of common constituents in different types of vesicles.

The study has identified several proteins that suggest previously unidentified roles of these subcellular structures in regulating intra-thymic processes. We have detected several proteins that have been implicated as autoantigens in human autoimmune diseases. Both populations contained alpha enolase, a glycolytic enzyme that has been recently suggested to play a role in rheumatoid arthritis and other autoimmune diseases. Furthermore, we detected the presence of heat shock proteins implicated as autoantigens in atherosclerosis.

Another exciting finding of this study was the identification of key regulatory and signaling molecules in membrane vesicles, such as elongation factor 2 (E2F). E2F represents a family of transcription factors involved in cell cycle regulation and synthesis of DNA in mammalian cells, tumorigenesis, apoptosis and differentiation. LCK, a further regulatory molecule that we detected in thymic vesicles, is a tyrosine kinase that plays an essential role in T-cell receptor (TCR)-linked signal transduction pathway. Therefore it is a further molecule of outstanding importance for the selection and maturation of developing T-cell in the thymus and in mature T-cell function.

Our work has also revealed the presence of histones (H1-H4) in both apoptotic bodies and microvesicles. The presence and abundance of histone proteins in apoptotic bodies was not surprising given the mechanism by which apoptotic blebs are generated. However, unexpectedly we have identified strikingly high number of histone proteins also in microvesicles. Several quantitative estimates were made to compare the relative amount of histones in the samples. The relative amount of histones in apoptotic bodies was 20.3% of the total amount of proteins, while it was 6.1% in the case of microvesicles. This suggests that a major proportion of thymus derived microvesicles is possibly generated during apoptosis (and thus, may be referred to as apoptotic microvesicles).

Conclusions

1. I have developed a method for extracting the protein content of extracellular vesicles; which does not interfere with the downstream LC-MS analysis and has advantages compared to the generally used gel electrophoresis. The method applies freeze-thaw cycles and using it I could identify four times more proteins than by gel electrophoresis.
2. I developed a miniaturized digestion protocol ("Mini protocol"), which works well for small amounts of proteins in small sample volume and allows detection of minor components in protein mixtures. The results show that the developed miniaturized digestion protocol performs at least as well, possibly even better, than conventional protocols using large sample amounts. It has been demonstrated that the protocol is applicable even for the digestion of 100 fmol (10 ng) protein mixture. In this case minor components of the mixture present in 10-20 fmol amount can be detected by HPLC-MS/MS. The developed protocol enables the analysis of minute amount of samples and this allowed the examination of extracellular vesicles.
3. I have determined protein composition of thymocyte-derived extracellular proteins using the developed workflow. I have identified 142 proteins in apoptotic bodies and 195 proteins in microvesicles. Most of the identified proteins were detected in both type of vesicles. We have detected several proteins that have already been implicated as autoantigens in human autoimmune diseases. Another exciting finding of this study was the identification of key regulatory and signalling molecules in membrane vesicles, such as elongation factor 2.
4. Strikingly, both microvesicles and apoptotic bodies contained substantial amounts of different types of histones and their relative amount was determined using "label-free quantification". Identified histones were grouped into histone families and the relative amount of these families was also estimated. Major proportion of thymus derived microvesicles is possibly generated during apoptosis (and thus, may be referred to as apoptotic microvesicles).

Summary

In my PhD work, in collaboration with the Department of Genetics, Cell- and Immunobiology of the Semmelweis University, I have determined the protein composition of thymocyte-derived microvesicles and apoptotic bodies using a nanoLC-MS/MS technique. The presence and abundance of proteins with high immunological relevance within thymocyte-derived apoptotic bodies and microvesicles suggest that these subcellular structures may substantially modulate T-cell maturation processes within the thymus [1].

In order to achieve these results, I had to solve a number of analytical problems related to the analysis of minute amounts of protein mixtures; such as those present in extracellular vesicles. I have developed a method for extracting the protein content of extracellular vesicles, which does not interfere with LC-MS analysis. The method is based on freeze-thaw cycles and using this method I have identified four times more proteins than with gel electrophoresis commonly used method in the literature. Next, I have developed a miniaturized tryptic digestion protocol. The protocol works well for small amounts of proteins using a small volume of reagents and is capable of detecting minor proteins present in complex biological mixtures [2]. The developed miniaturized digestion protocol proved to be robust and well suited for mass spectrometry based proteomic applications. The developed workflow has been applied for the determination of proteins present in mouse thymocyte-derived extracellular vesicles. I have identified 142 and 195 proteins respectively, in apoptotic bodies and microvesicles. Among these I could detect autoantigens and important regulatory and signaling molecules.

Publications related to the thesis

1. **Lilla Turiák**, Petra Misják, Tamás G Szabó, Borbála Aradi, Krisztina Pálóczi, Oliver Ozohanics, László Drahos, Ágnes Kittel, András Falus, Edit I Buzás, Károly Vékey:
Proteomic characterization of thymocyte-derived microvesicles and apoptotic bodies in BALB/c mice.
Journal of Proteomics, 74 (2011) 942-947.
IF: 5.074

2. **Lilla Turiák**, Oliver Ozohanics, Fabio Marino, László Drahos, Károly Vékey:
Digestion protocol for small protein amounts for nano-HPLC-MS(MS) analysis
Journal of Proteomics, 74 (2011) 2025-2033.
IF: 5.074

Other publications

3. Oliver Ozohanics, **Lilla Turiák**, László Drahos, Károly Vékey:
Comparison of glycopeptide/glycoprotein enrichment techniques
Rapid Communications in Mass Spectrometry, 26 (2012) 215-17.
IF: 2.846

4. Judit Doczi, **Lilla Turiak**, Szilvia Vajda, Miklos Mandi, Beata Töröcsik, Akos A. Gerencser, Gergely Kiss, Csaba Konràd, Vera Adam-Vizi, Christos Chinopoulos:
Complex contribution of cyclophilin D to Ca2+-induced permeability transition in brain mitochondria, with relation to the bioenergetic state
Journal of Biological Chemistry, 286 (2011) 6345-6353.
IF: 5.328

5. Christos Chinopoulos, Akos A. Gerencser, Miklos Mandi, Katalin Mathe, Beata Töröcsik, Judit Doczi, **Lilla Turiak**, Gergely Kiss, Csaba Konràd, Szilvia Vajda, Viktoria Vereczki, Richard J. Oh, and Vera Adam-Vizi:
Forward operation of adenine nucleotide translocase during F0F1-ATPase reversal: critical role of matrix substrate-level phosphorylation
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