The analysis of pre-analytical and analytical factors that affect the measurement of cell-derived extracellular vesicles

Doctoral Theses

Bence György, MD

Semmelweis University
PhD School of Molecular Medical Sciences

Dissertation Supervisor:	Dr. Buzás Edit, Professor
Opponents:	Dr. Szekanecz Zoltán, Professor, Dr. Szeberényi Júlia, Ph.D.
Comprehensive exam	
Director:	Dr. Gergely Péter, Professor
Members:	Dr. Dérfalvi Beáta, Ph.D Dr. Miklós Katalin, Ph.D

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INTRODUCTION

Extracellular vesicles are cell-derived structures, limited by a phospholipid bilayer. Their size is variable with diameters ranging from 30 nm to 5 μm. Extracellular vesicles could be classified based on their biogenesis: vesicles formed upon exocytosis of multivesicular bodies are called *exosomes* (their size is 30-100 nm), vesicles derived from the plasma membranes are termed as *microvesicles* (their size is 100-1000 nm) and membranous structures formed during apoptosis are the so-called *apoptotic bodies* (their size is 800 nm-5 μm). The formation of extracellular vesicles is an evolutionally conserved process; all types of cells (both pro- and eukaryotes) were shown to secrete such structures into the extracellular environment, thereby forming a dynamic „extracellular vesicular compartment" in the intercellular space. Extracellular vesicles are involved in intercellular communication (they may transfer proteins or nucleic acids between cells), represent a novel alternative secretory pathway and have several potent biological functions (induction of blood clotting, antigen presentation, bacteriostatic effect etc.). Extracellular vesicle research is not only important in a cell biology perspective but also has relevant implications in biomedical sciences. Tumor-derived vesicles were shown to be able to transfer oncogenic proteins and RNA to normal cells, thereby transforming the recipient cells into malignant cells. Furthermore they might also educate bone-marrow progenitor cells toward a pro-metastatic phenotype. Based on these findings, the removal of vesicles from the blood plasma of patients with cancer might have therapeutic advantages.

Importantly, extracellular vesicles could be used as novel biomarkers of various diseases. Vesicle counts and composition might reflect cellular state of the donor cell. Tumor-derived extracellular vesicles are detectable in the blood plasma of patients suffering from cancer, and they represent a highly specific biomarker that could be used for screening (e.g. in prostate cancer). Vesicle counts were also shown to associate with autoimmune, cardiovascular and numerous other diseases.

Furthermore, extracellular vesicles might also represent novel drug delivery vehicles. Similarly to liposomes, small compounds could be encapsulated in vesicles. On the other hand, by exploiting their nucleic acid transporting capacity, they might be used as novel vectors in gene therapy. As these vectors are derived from the same species or even from the

same individual, it could by hypothesized that they are highly effective, without eliciting any immune response. Most interestingly, viruses were shown to bud into vesicular structures, thereby exploiting the exosomal shuttle machinery. The combination of vesicles and viruses in gene delivery might revolutionize gene therapeutic applications.

Extracellular vesicle field is relatively new, and there are several confounding factors that hinder correct analysis of vesicles. Vesicle classification, standard protocols for isolation and characterization are still a matter of debate. Vesicle analysis in biological fluids is confounded by other contaminating factors of similar size. During our study, we focused on the microvesicle fraction and by applying several methods; we turn the attention on some important confounding factors for the first time.

AIMS

- To set-up methods for microvesicle analysis
- The comprehensive analysis of the size distributions of microvesicles derived from different biological fluids
- The analysis of pre-analytical variables that affect the measurement of microvesicles, with focus on the use of various anticoagulants during blood collection
- The assessment of analytical variables that affect the measurement of microvesicles, with focus on the confounding presence of protein aggregates and immune complexes
- The analysis of factors that affect the isolation of microvesicles, with focus on non-vesicular particles that co-sediment with microvesicles
- Determination of synovial fluid microvesicular signatures in patients with osteoarthritis, rheumatoid arthritis and juvenile idiopathic arthritis

METHODS

Patients and samples

Microvesicles were analyzed from blood plasma, synovial fluid and from CCRF-CEM cell culture supernatants. We studied healthy control samples, and samples from patients with rheumatoid arthritis, osteoarthritis and juvenile idiopathic arthritis. We isolated platelet-free plasma using standard international protocols. Cells were also depleted from synovial fluid samples before analysis.

Isolation of microvesicles

Microvesicles were isolated using differential centrifugation. Briefly, after pelleting cells and platelets, samples were filtered through an 800 nm pore-sized filter. Then, microvesicles were pelleted using 20,500g for 60 minutes. The pellet was resuspended in PBS and vesicles were washed once or twice. Finally, microvesicles were resuspended in a buffer required for downstream analysis.

Determination of the size distribution of microvesicles

We determined the size of microvesicles using numerous methods simultaneously. Transmission electron microscopy (TEM) was used to visualize vesicles, atomic force microscopy was used to reconstruct the surface of microvesicles. The diameters of microvesicles were determined based on TEM and AFM images using the ImageJ software. We also aimed at analyzing vesicles in their native environment, i.e. in suspension. First, we performed dynamic light scattering (DLS) analysis on diluted biological fluids and on isolated microvesicle samples. The disadvantage of DLS analysis is that this method is only capable of analyzing total scattered light and particles could not be analyzed individually. Therefore we selected alternative methods to analyze vesicles on a particle-by-particle basis: the

nanoparticle tracking analysis (NTA), which is also based on light scattering and the Izon qNano, a method based on impedance.

Label-free optical methods

Label-free optical methods enabled us to detect the adhesion of microvesicles to functionalized surfaces. Grating coupled interferometry (GCI, Róbert Horváth et al.) was used to assess the adhesion of CCRF-CEM microvesicles to extracellular matrix proteins and quartz crystal microbalance technology (Attana AB, Stockholm, Sweden) was used to detect the adhesion of platelet-derived microvesicles to anti-CD41a antibody coated surfaces.

Flow cytometry

Flow cytometry is the most frequently applied method for the assessment of microvesicles. Originally, flow cytometers were developed to analyze cells, however, smaller vesicular structures are also detectable using specific amplification and gating settings. Flow cytometry settings were determined by our group, but later we applied the standard protocol of the International Society on Thrombosis and Haemostasis. The two protocols gave comparable results. Microvesicles were labeled annexin V (a phosphatidyl-binding protein) and antibodies against membrane proteins. Microvesicle concentration was determined using counting beads. To exclude non-vesicular particles (immune complexes and protein aggregates) during flow cytometry, we applied differential detergens lysis. By applying a detergent at low concentration, we could successfully discriminate vesicular events from non-vesicular particles, as vesicles were more sensitive to detergens lysis compared to protein aggregates and immune complexes.

Pre-analytical factors that affect the assessment of microvesicles

Microvesicle count is influenced by many pre-analytical variables, practically all factors from sample collection until analysis could alter vesicle counts dramatically. In our study, we analyzed the effect of anticoagulants used during blood collection. Blood was drawn into 6 blood collection tubes, containing different anticoagulants (citrate, ACD, CPDA,

CTAD, EDTA, heparin). Microvesicle numbers were assessed using flow cytometry and phsoshpatidylserine concentration was determined by a thrombin generation assay (Zymuphen). We also examined the extent of ***ex vivo*** (i.e. in the blood collection tube) vesicle formation in tubes with different anticoagulants. To induce *ex vivo* vesiculation, we applied a 60 minutes 37 °C degree incubation and gentle agitation.

Analytical factors that affect the assessment of microvesicles

We also analyzed whether protein aggregates and immune complexes confounded the assessment of microvesicles. First, we generated artificial immune complexes by mixing antigens with specific antibodies (lactoferrin-anti-lactoferrin, ovalbumin-anti-ovalbumin, IgM-anti-IgM), and we also isolated natural immune complexes from rheumatoid arthritis synovial fluids using anti-IgG and anti-IgM agarose columns. The size distribution of immune complexes were recorded using AFM, DLS and NTA. Furthermore, we also analyzed, whether immune complexes were detectable by flow cytometry and confounded the assessment of microvesicles. The protein aggregates and immune complex contaminants in microvesicle preparations were analyzed by mass spectrometry, fluorescent microscopy and immune-TEM.

Microvesicular signatures in joint diseases

Patients with rheumatoid arthritis (n=8), juvenile idiopathic arthritis (n=10) and osteoarthritis (n=8) were included in these experiments. By applying the differential detergent lysis T-cell ($CD3^+$, $CD4^+$, $CD8^+$), B-cell ($CD19^+$), platelet ($CD41^+$) and monocyte-derived vesicles were counted.

Statistical analysis

For statistical analysis SPSS 15.0 (IBM Corporation) and SigmaStat 11.0 (Systat Software, Inc., San Jose CA, USA) softwares were used. To test if a distribution is normal, we applied one sample Kolmogorov-Smirnov test. We used t-test or Mann-Whitney test to compare two groups and one-way ANOVA or Kruskal Wallis ANOVA to compare multiple groups. As a post hoc test we used Tukey or Mann-Whitney test with Bonferroni correction.

For pairwise comparison we used paired t-test or Signed Rank test. To analyze correlations, we used Spearman or Pearson correlations.

RESULTS

Set-up of methods for microvesicle analysis

Microvesicles derived from blood plasma and synovial fluid were visualized by AFM and TEM. Based on these images, the determined mean size of microvesicles was around 150 and 250 nm in diameter (range 80-400 nm). Using DLS and Izon qNano, we obtained similar results. By contrast, NTA detected numerous particles in the microvesicle preparations below 100 nm, presumably corresponding to protein aggregates. Microvesicles were successfully detected and phenotyped by flow cytometry using our or the ISTH standard protocol. GCI confirmed the binding of CCRF-CEM vesicles to laminin, whereas the QCM technology demonstrated the binding of platelet-derived microvesicles to a surface coated with anti-CD41a antibody.

Pre-analytical factors that affect the assessment of microvesicles

In this study we confirmed that the applied anticoagulant during blood collection is a crucial factor in the assessment of microvesicles and it significantly affects microvesicle levels measured by flow cytometry and Zymuphen assay (ANOVA, $p<0,001$). The microvesicle counts were the highest in tubes with heparin (Signed Rank test, $p<0,05$), and lowest in tubes with ACD. Agitation and 37 °C incubation significantly increased microvesicles counts in conventionally used citrate tubes ($p<0,05$ in both cases, paired t-test). Strikingly this *ex vivo* elevation was minimal in ACD tubes. Next we analyzed ACD components (citric acid and dextrose) separately on the inhibition of *ex vivo* vesiculation, and we found that citric acid was the active component. Based on our results, microvesicle counts in ACD tubes represent in vivo counts better as artificial vesiculation in the blood collection tube is highly reduced. The *in vivo* elevation of microvesicle counts was not affected in ACD tubes (e.g. in pregnancy, Mann-Whitney test, $p<0,01$).

Analytical factors that affect the assessment of microvesicles

We observed originally that indirect staining of microvesicles results in high number of positive events within the microvesicle gate during flow cytometry, in all samples. This might indicate immune complex formation. The analyzed natural and artificial immune complexes by AFM, DLS and NTA revealed that indeed, immune complexes have overlapping size distribution with microvesicles (80-400 nm). Therefore it is not surprising that the isolated natural and artificial immune complexes give signals within the microvesicle gate, measured by flow cytometry. The next step was to discriminate between immune complexes and microvesicles: we successfully demonstrated that the application of a detergent at low concentration (e.g. 0,05% Triton X-100) disintegrates microvesicles without affecting immune complexes. Using Triton X-100, we confirmed the confounding effect of immune complexes on microvesicle assessment, as if non-vesicular events are not excluded, vesicle counts could be overestimated. Furthermore, self-aggregation of the detection antibodies might also lead to false positive results.

Based on similar size, immune complexes might also co-sediment with microvesicles. Using fluorescent microscopy and immune-TEM, we confirmed this hypothesis analyzing microvesicle preparations isolated from synovial fluid of patients with rheumatoid arthritis. We also demonstrated by DLS and flow cytometry that immune complexes could be pelleted using the same protocols, as for microvesicles. Therefore immune complexes potentially contaminate microvesicle preparations. Mass spectrometry analysis of rheumatoid arthritis, osteoarthritis and juvenile idiopathic arthritis synovial fluid-derived microvesicles revealed that these preparations contain non-vesicular proteins (plasma proteins, immune complex proteins, complement proteins etc.) among classical vesicular ones (actin, heat shock proteins, alpha-enolase, HLA-I etc.). These data indicate that the specific isolation of vesicles is crucial in order to study their biological effects.

Microvesicles as biomarkers of joint diseases

Using the differential detergent lysis, we identified characteristic microvesicular signatures in various joint disorders. Synovial $CD3^+$ and $CD8^+$ vesicle counts were significantly elevated in patients with rheumatoid arthritis compared to osteoarthritis (Mann-Whitney test, after Bonferroni correction, p=0,027 and p=0,009). In patients with juvenile idiopathic arthritis, B-cell-derived microvesicle counts were significantly lower compared to

rheumatoid arthritis and osteoarthritis (Mann-Whitney test, after Bonferroni correction, p=0,009, and p=0,004, respectively).

CONCLUSIONS

- The methods used in our study are suitable for the detection of microvesicles (TEM, AFM, DLS, flow cytometry, NTA, Izon qNano and label-free optical methods)
- The isolated microvesicles were characterized by the same size distribution, measured by different methods (80-400 nm, mean microvesicle size 150-200 nm)
- We propose that the use of ACD tubes for microvesicle assessment is superior compared to conventional citrate tubes, as low pH inhibits artificial, *ex vivo* vesicle formation, whereas *in vivo* elevated microvesicle count are also detectable
- Microvesicles share biophysical properties with immune complexes (size, light scattering and sedimentation) therefore immune complexes and protein aggregates confound the assessment of vesicles and might also contaminate microvesicle preparations
- Differential detergent lysis could be advised to differentiate between vesicles and non-vesicle structures during routine flow cytometry measurements
- Using the differential detergent lysis, we successfully identified characteristic microvesicular signatures in joint diseases; $CD8^+$ T-cell derived vesicles were significantly elevated in patients with rheumatoid arthritis

MY PUBLICATIONS

Publications related to the Doctoral Theses:

1. **György B**, Szabó TG, Turiák L, Wright M, Herczeg P, Lédeczi Z, Kittel A, Polgár A, Tóth K, Dérfalvi B, Zelenák G, Böröcz I, Carr B, Nagy G, Vékey K, Gay S, Falus A, Buzás EI. (2012) Improved flow cytometric assessment reveals distinct microvesicle (cell-derived microparticle) signatures in joint diseases. **PLoS One**. 7: e49726

2. **György B**, Szabó TG, Pásztói M, Pál Z, Misják P, Aradi B, László V, Pállinger E, Pap E, Kittel A, Nagy G, Falus A, Buzás EI. (2011) Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. **Cell Mol Life Sci**. 68: 2667-88.

3. **György B**, Módos K, Pállinger E, Pálóczi K, Pásztói M, Misják P, Deli MA, Sipos A, Szalai A, Voszka I, Polgár A, Tóth K, Csete M, Nagy G, Gay S, Falus A, Kittel A, Buzás EI. (2011) Detection and isolation of cell-derived microparticles are compromised by protein complexes due to shared biophysical parameters. **Blood**.117: e39-48.

Publications unrelated to the Doctoral Theses:

1. Baka Zs, **Gyorgy B**, Géher P, Buzás EI, Falus A and Nagy G. (2012) Citrullination under physiological and pathological conditions. **Joint Bone Spine**, 79: 431-6.

2. Pásztói M, Nagy G, Géher P, Lakatos T, Tóth K, Wellinger K, Pócza P, **György B**, Holub MC, Kittel A, Pálóczy K, Mazán M, Nyirkos P, Falus A, Buzas EI. (2009) Gene expression and activity of cartilage degrading glycosidases in human rheumatoid arthritis and osteoarthritis synovial fibroblasts. **Arthritis Res Ther**. 11: R68.

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5. Buzás EI*, **György B***, Pásztói M, Jelinek I, Falus A, Gabius HJ. (2006) Carbohydrate recognition systems in autoimmunity. **Autoimmunity.** 39: 691-704. ***megosztott első szerző**

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