Final report

#111958 "Studies on the biomedical applications of extracellular vesicles" OTKA project

(2015-01-01 - 2019-08-31)

Background

Extracellular vesicles (EVs) are recently recognized subcellular structures released by all cells in an evolutionary conserved manner. The goal of this OTKA project was to gain insight into the potential biomedical applications of EVs by primarily focusing on two distinct types of diseases: the autoimmune inflammatory rheumatoid arthritis and pancreatic cancer. Because of the relative paucity of plasma samples available from patients with early, undifferentiated arthritis, during the course of this project we decided to focus on samples from patients with definite rheumatoid arthritis rather than undifferentiated arthritis.

Importantly, with the support of this OTKA grant, our Research Group has also carried out numerous studies by which we contributed to develop improved and standardized methodological platforms for EV isolation or detection for biomedical applications. Benefiting from the lessons of these studies, we carried out specific projects that focused on EVs in rheumatoid arthritis and pancreatic cancer. Importantly, besides the above studies, our OTKA support also enabled us to extend our research to additional diseases such as colorectal cancer and preeclampsia.

Recently, we used *in silico* systems biology approaches to identify correlations between genes involved in EV biogenesis and human diseases. Using a knowledge fusion system, we investigated whether certain groups of proteins implicated in the biogenesis/release of EVs, were associated with diseases and phenotypes. In addition, we tested if these proteins were enriched in publicly available transcriptomic datasets using gene set enrichment analysis methods. The top 20 most relevant diseases based on the gene list of EV biogenesis and secretion included rheumatoid arthritis and different types of cancer. These data provided substantial justification to our selected diseases/disease groups in which we studied the role of EVs during the OTKA-funded period.

(***Systems biology approaches to investigating the roles of extracellular vesicles in human diseases. Gézsi A, Kovács Á, Visnovitz T, Buzás EI.Exp Mol Med. 2019 Mar 15;51(3):33.).***

1. Contribution to the EV field by improving isolation and detection of EVs

One of our fundamental observations with relevance to the EV field was that size-based subpopulations of EVs had differential sensitivity to different detergents. This study was carried out combining tunable resistive pulse sensing at different concentrations of four detergents. Small EVs secreted by different cell lines were found to be more resistant to detergents than medium or large sized EVs. SDS was more potent to lyse EVs than Triton X-100. EVs were relatively insensitive to Tween-20 and deoxycholate. These data have important implications to the use of detergent lysis for proving the vesicular nature of a particle (e.g. by flow cytometry) ***(Differential detergent sensitivity of extracellular vesicle subpopulations. Osteikoetxea X, Sódar B, Németh A, Szabó-Taylor K, Pálóczi K, Vukman KV, Tamási V, Balogh A, Kittel Á, Pállinger É, Buzás EI.Org Biomol Chem. 2015;13:9775-82.).***

In the next study we compared small EV isolation techniques from rat and human blood plasma (ultracentrifugation and size exclusion chromatography). We found that albumin was present in the preparations even after 1h ultracentrifugation at 4°C. If ultracentrifugation time was longer or the sedimentation distance was shorter at 4°C, or if ultracentrifugation was performed at 37°C, the yield of isolated small EVs increased along with the amount of the co-isolated albumin. Efficiency and purity did not differ if samples were further diluted. Size exclusion chromatography yielded in a minor fraction of small EVs without significant albumin content. ***(Baranyai T, Herczeg K, Onódi Z, Voszka I, Módos K, Marton N, Nagy G, Mäger I, Wood MJ, El Andaloussi S, Pálinkás Z, Kumar V, Nagy P, Kittel Á, Buzás EI, Ferdinandy P, Giricz Z. PLoS One. 2015;10:e0145686.)***

We also introduced a novel radiolabeling technic for the assessment of the *in vivo* biodistribution of EVs. We labelled red blood cell-derived EVs using the (99m)Tc-tricarbonyl complex. We demonstrated the capability of this labeling method for *in vivo* biodistribution studies in a murine model. We found accumulation of the intravenously injected labeled EVs predominantly in the liver and spleen. We found that only a minor fraction of the (99m)Tc label became detached from the EVs proving that (99m)Tc-tricarbonyl complex EV labelling is a suitable method for radiolabeling of EVs for *in vivo* studies **(*Radiolabeling of Extracellular Vesicles with (99m)Tc for Quantitative In Vivo Imaging Studies. Varga Z, Gyurkó I, Pálóczi K, Buzás EI, Horváth I, Hegedűs N, Máthé D, Szigeti K. Cancer Biother Radiopharm. 2016;31:168-73.*).**

Finally and most importantly, we have found a solution to fill a long existing gap in EV standardization. EVs are structures defined by their phospholipid bilayer membranes. In spite of the defining role of membrane lipids, current EV quantification is either based on determination of the protein content of an EV preparation or on the particle number in a given EV sample. These approaches are highly error-prone given that EV preparations are often contaminated by co-purified proteins. Furthermore, particle-based enumeration very often overestimates the EV count also detecting protein aggregates and lipoprotein particles beside EVs. Until now the detection of lipids and standardization based on the lipid content were prevented by the lack of a widely available method for total lipid determination. Here we developed an improved, simple total lipid assay applicable for the determination of the lipid content of a given EV preparation. Our group was the first to introduce the sulfo-phospho-vanillin (SPV) lipid assay to EV research. Here we significantly optimized our previous assay increasing its sensitivity by approximately one order of magnitude. Thus, we provided the EV field a quick, reliable and sensitive test that may fill an existing gap in EV standardization. This novel, optimized lipid assay is almost as sensitive and as easy as measuring proteins with a simple BCA test. Patent is pending. This work was awarded the 2019 Innovation Prize of Semmelweis University ***(An improved 96 well plate format lipid quantification assay for standardisation of experiments with extracellular vesicles. Visnovitz T, Osteikoetxea X, Sódar BW, Mihály J, Lőrincz P, Vukman KV, Tóth EÁ, Koncz A, Székács I, Horváth R, Varga Z, Buzás EI.J Extracell Vesicles. 2019;8:1565263).***

2. Characterization of the role of EV surface interactome in health and in rheumatoid arthritis

It has been ignored previously that EVs have relatively large surface compared to their volume. Given that they are present in high number in biological samples, collectively extracellular vesicles represent a uniquely large interactive surface area, which can establish contacts both with cells and with molecules in the extracellular microenvironment. We proposed that these interactions may be characteristic for EVs in in health and disease ***(Molecular interactions at the surface of extracellular vesicles. Buzás EI, Tóth EÁ, Sódar BW, Szabó-Taylor KÉ. Semin Immunopathol. 2018; 40:453-464***.).

One of the major potential biomedical applications of EV is the use of circulating extracellular vesicles as novel biomarkers. In our study we have demonstrated for the first time that by using flow cytometry, 90 minutes after a high fat meal, a robust population of EV-mimicking chylomicrons are detectable in blood plasma samples. Based on our observation, current guidelines of circulating EV detection suggest the use of fasting blood plasma *(Circ Res. 2017;120:1632-1648*). We found that the majority of circulating particles within the size range of EVs lacked common vesicular markers and were identified as predominantly low-density lipoprotein, LDL. We have found that LDL mimicked the characteristics of EVs and also co-purified with them. Based on biophysical properties of LDL, this finding was highly unexpected. Using several EV isolation and purification methods we could not prepare LDL-free EV samples from blood plasma or from platelet concentrates. Also surprisingly, we found a significant spontaneous association of LDL with isolated EVs upon *in vitro* mixing. Our data point to the importance of careful study design and data interpretation in biomedical studies using blood-derived EVs. Given that LDL is a major risk factor of cardiovascular diseases, the described association of LDL with EVs raised many exciting questions regarding EV and LDL uptake by cells and their role in disease development. This study attracted significant attention from the scientific community (>100 citations until now***) (Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection. Sódar BW, Kittel Á, Pálóczi K, Vukman KV, Osteikoetxea X, Szabó-Taylor K, Németh A, Sperlágh B, Baranyai T, Giricz Z, Wiener Z, Turiák L, Drahos L, Pállinger É, Vékey K, Ferdinandy P, Falus A, Buzás EI. Sci Rep. 2016;6:24316..***

Another unexpected finding of our group was that sustained exposure of cells to the fluoroquinolone antibiotic ciprofloxacin induced the release of small EVs with surface associated mitochondrial and chromosomal DNA as well as DNA-associated proteins. Ciprofloxacin is widely used in humans against bacterial infections as well as in cell cultures against Mycoplasma contamination. A label-free optical biosensor analysis revealed DNA-dependent binding of exosomes to fibronectin. DNA release on the surface of exosomes was not affected any further by cellular activation or apoptosis induction. These data are also important from the perspective of biomarker research. According to our data, antibiotics-induced, EV surface-associated DNA may modify the baseline levels of circulating DNA in various pathological conditions ***(Antibiotic-induced release of small extracellular vesicles (exosomes) with surface-associated DNA. Németh A, Orgovan N, Sódar BW, Osteikoetxea X, Pálóczi K, Szabó-Taylor KÉ, Vukman KV, Kittel Á, Turiák L, Wiener Z, Tóth S, Drahos L, Vékey K, Horvath R, Buzás EI. Sci Rep. 2017;7:8202).***

3. Studies on EVs in rheumatoid arthritis

We analyzed membrane surfaces of circulating monocytes from patients with rheumatoid arthritis, healthy controls and human monocyte cell lines (U937 and Thp1). For EV analysis, we combined thiol labeling with staining with fluorochrome-labelled antibodies to EV specific surface markers to exclude EV mimicking signals from thiol containing protein aggregates. We found a significant elevation of surface thiols on circulating monocytes in rheumatoid arthritis patients ($p<0.05$). Surprisingly, newly released EVs of isolated CD14+ cells from rheumatoid arthritis patients had reduced thiol levels compared with healthy subjects ($p<0.01$). We also detected an overoxidized form of peroxiredoxin in EV-enriched preparations from blood plasma. These data support a role of EVs in the redox regulation of human monocytes, possibly representing an antioxidant mechanism (releasing of overoxidized membrane segments by EVs) ***(Monocyte activation drives preservation of membrane thiols by promoting release of oxidised membrane moieties via extracellular vesicles. Szabó-Taylor KÉ, Tóth EÁ, Balogh AM, Sódar BW, Kádár L, Pálóczi K, Fekete N, Németh A, Osteikoetxea X, Vukman KV, Holub M, Pállinger É, Nagy G, Winyard PG, Buzás EI. Free Radic Biol Med. 2017;108:56-65).***

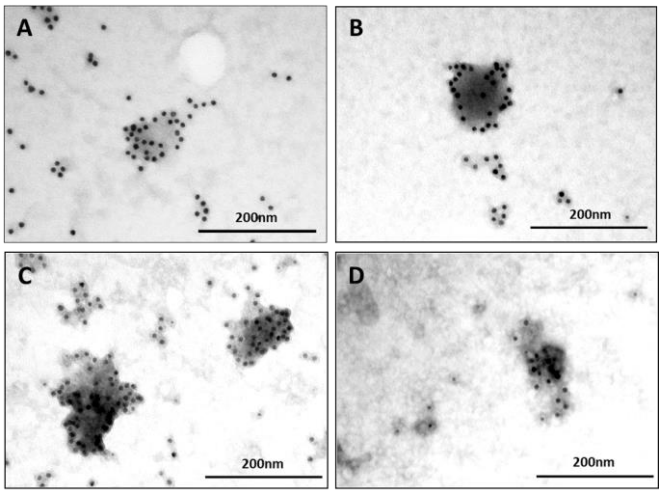
We also analyzed the role of circulating EVs in osteoclastogenesis. To this end, we isolated small EVs from blood plasma of patients with rheumatoid arthritis and healthy controls. In addition, patients with psoriatic arthritis were also included in this study. Both healthy and rheumatoid arthritis patient-derived small EVs strongly inhibited osteoclast differentiation. In contrast, psoriatic arthritis-derived small EVs had a stimulatory effect. When small EVs and CD14+ cells were interchanged between the three groups, only healthy and rheumatoid arthritis-derived small EVs inhibited the generation of osteoclasts in all groups, whereas psoriatic arthritis-derived small EVs did not mediate this effect. Our data suggest that blood-derived small EVs are potent regulators of human osteoclastogenesis having differential effector function in distinct inflammatory arthritides. ***Extracellular vesicles regulate the human osteoclastogenesis: divergent roles in discrete inflammatory arthropathies. Marton N, Kovács OT, Baricza E, Kittel Á, Győri D, Mócsai A, Meier FMP, Goodyear CS, McInnes IB, Buzás EI, Nagy G.Cell Mol Life Sci. 2017 Oct;74(19):3599-3611).***

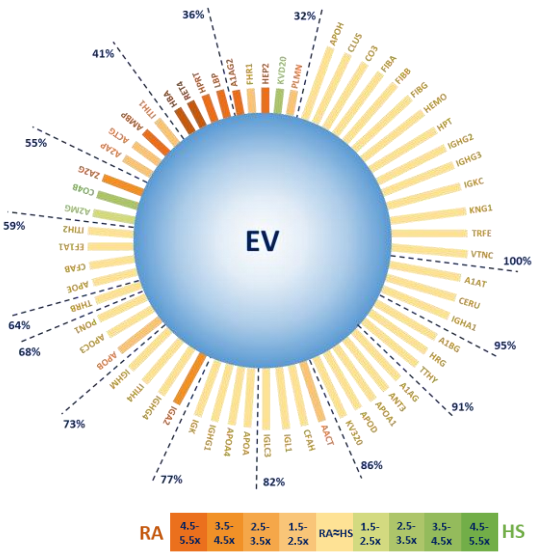
We hypothesized that similarly to the artificial nanoparticles, circulating EVs may have surface interactions with plasma proteins. Thus, we proposed that upon incubation of nascent EVs with blood plasma samples, EVs might serve as probes/baits that concentrate on their surface a disease-specific set of interacting proteins (protein corona). To test this hypothesis, we used MS (in collaboration with Lilla Turiak, MTA TTK). We isolated nascent EV "probes/baits" from serum-free conditioned media of THP-1 cells, and confirmed the presence of EVs by flow cytometry (annexin V-FITC labeling and Triton X-100 lysis). The nascent EVs were incubated with 0.5 mL platelet- and EV-free plasma samples from patients with rheumatoid arthritis and healthy controls for 30 minutes. This was followed by three washes and/or size exclusion chromatography and/or density gradient ultracentrifugation before the plasma protein-coated EVs were submitted to MS analysis. Our data enabled us to confirmed protein corona formation on the surface of nascent EVs.

The interaction between EVs and plasma proteins was strongly suggested by the difference in the number of proteins identified by MS in nascent EVs and 'plasma-incubated and washed' EVs (in samples standardized for protein content): 59.50±14.85 proteins were identified collectively in the 'nascent EVs only' plus the 'plasma only' samples, while 94.5±27.8 proteins were detected in the 'plasma-immersed and washed' EV samples ($p=0.0001$).

A significant interaction of the detected corona proteins was found using a free online database (https://string-db.org/) ($p<10^{-16}$). We also found an increased density of EVs with "protein corona" as compared to nascent EV controls. To investigate whether the protein corona formation was dependent on the cellular source EVs, we compared the surface protein interactome of washed platelet (PLT)-derived EVs and THP1-derived EVs. Our data supported the existence of formation of EV type-specific, common EV and diseases-specific protein coronas. To validate the interactions between EVs and plasma proteins detected with MS, we used immungold TEM analysis. On the surface of *ex vivo*, platelet–derived EVs we also demonstrated the co-localization of EV markers with plasma proteins using immuno-gold electron microscopy. These data give an entirely new understanding of the plasma protein "contaminants" of blood plasma-derived EV preparations. These data were presented as an oral presentation by Eszter Tóth at the Annual World Congress of ISEV (International Society for Extracellular Vesicles) in Kyoto, 2019. ***(Formation of a protein corona on the surface of extracellular vesicles in blood plasma. Eszter Tóth, Tamás Visnovitz, György Nagy, W Sódar Barbara, Anna Balogh, Lilla Turiák, László Drahos, Géza Jakab,Katalin Szabó-Taylor, Attila Bácsi, Edit I Buzás. Manuscript in preparation).***

Immuno-gold EM: A) alpha chain of fibrinogen (10 nm) B) the alpha chain of fibrinogen (10 nm) and Apo A1 (5 nm), C) haptoglobin (10 nm) and CD63 (5 nm), D) C3 (10 nm) and CD63 (5 nm).





Fold enrichment of EV-associated proteins from the plasma of rheumatoid arthritis patients (RA) and healthy subjects (HS)

4. Studies on EVs in pancreatic cancer, colorectal cancer and adrenocortical tumors

We assessed EVs from patients with chronic pancreatitis and pancreatic cancer in an attempt to extend the currently available list of biomarkers for pancreatic cancer. We assessed EVs both in the circulation and in the pancreatic juice of patients with chronic pancreatitis or adenocarcinoma. We also investigated EVs secreted by Capan-1 and MiaPaca-2 pancreatic cancer cell lines *in vitro*. EV concentration and size distribution were measured using tunable resistive pulse sensing. Morphology of EVs was assessed by transmission electron microscopy, and protein composition of EVs was analyzed by mass spectrometry (MS). The MS analysis of EV samples enabled a bias free assessment of the proteomic composition of EVs ***(Best practice of identification and proteomic analysis of extracellular vesicles in human health and disease. Sódar BW, Kovács Á, Visnovitz T, Pállinger É, Vékey K, Pocsfalvi G, Turiák L, Buzás EI. Expert Rev Proteomics. 2017 Dec;14(12):1073-1090.).***
We demonstrated for the first time the presence of EVs directly in human pancreatic juice samples. These EVs could be isolated for MS, and were surprisingly stable despite their presence within a lipase- and protease-rich body fluid. Importantly, we identified certain pancreatic juice EV-associated molecules (such as mucin 1, mucin 4, mucin 5ac, cystic fibrosis transmembrane conductance regulator protein, multidrug resistance protein 1) as possible candidate markers for pancreatic cancer. These molecules could also be detected in association with EVs secreted by the tested pancreatic cancer cell lines. Furthermore, we analyzed blood plasma-derived circulating EVs from patients with pancreatic cancer, chronic pancreatitis and healthy controls by MS. We found numerous proteins that were differentially present in EV preparations from patients and controls. By flow cytometry we demonstrated that mucin-1, mucin-4 and mucin-16 were also detectable on the surface of circulating EVs in the

blood plasma of some pancreatic cancer patients ***(Detection and proteomic characterization of extracellular vesicles in human pancreatic juice.Osteikoetxea X, Benke M, Rodriguez M, Pálóczi K, Sódar BW, Szvicsek Z, Szabó-Taylor K, Vukman KV, Kittel Á, Wiener Z, Vékey K, Harsányi L, Szűcs Á, Turiák L, Buzás EI. Biochem Biophys Res Commun. 2018 Apr 30;499(1):37-43.)***

Recently, we provided evidence for an *en bloc* transmission of MVB-like EV clusters through the plasma membrane. This MVB-like small EV clusters were detectable both in archived pathological human colorectal cancer samples *in situ* (in human tissues) and *in vitro*, where HT29 colorectal cancer cells also showed the release of similar structures as confirmed by immunohistochemistry and immune electron microscopy. The *en bloc* release of MVB-like small EV clusters may represent a novel mechanism of EV biogenesis. ***En bloc release of MVB-like small extracellular vesicle clusters by colorectal carcinoma cells. Valcz G*, Buzás EI*, Kittel Á, Krenács T, Visnovitz T, Spisák S, Török G, Homolya L, Zsigrai S, Kiszler G, Antalffy G, Pálóczi K, Szállási Z, Szabó V, Sebestyén A, Solymosi N, Kalmár A, Dede K, Lőrincz P, Tulassay Z, Igaz P, Molnár B.J Extracell Vesicles. 2019 Apr 8;8(1):1596668. *equal contribution)***

We also studied EV-associated miRNAs in adrenocortical malignancy where there is no blood marker for the preoperative diagnosis. EV-associated hsa-miR-483-5p was identified as a promising minimally invasive biomarker in the preoperative diagnosis of adrenocortical cancer (***Evaluation and diagnostic potential of circulating extracellular vesicle-associated microRNAs in adrenocortical tumors.Perge P, Butz H, Pezzani R, Bancos I, Nagy Z, Pálóczi K, Nyírő G, Decmann Á, Pap E, Luconi M, Mannelli M, Buzás EI, Tóth M, Boscaro M, Patócs A, Igaz P. Sci Rep. 2017 Jul 14;7(1):5474.).***

5. EVs in cardiovascular diseases

EVs are exciting novel players in cell-cell communication in cardiovascular diseases. They may provide an explanation for the development of vascular comorbidities in autoimmune diseases (such as rheumatoid arthritis) ***(Mechanisms of vascular comorbidity in autoimmune diseases. Nagy G, Németh N, Buzás EI. Curr Opin Rheumatol. 2018 Mar;30(2):197-206.).***
Interestingly, due to the previously underestimated heterogeneity of EVs, they can play either beneficial or detrimental roles in cardiovascular pathologies ***(Extracellular vesicles in cardiovascular disease: are they Jedi or Sith? Osteikoetxea X, Németh A, Sódar BW, Vukman KV, Buzás EI.J Physiol. 2016 Jun 1;594(11):2881-94.).***
Although EV biology primarily focuses on the biological effects of small EVs, medium sized EVs were also shown to play various experimentally proven roles in vascular homeostasis and diseases ***(Microvesicles in vascular homeostasis and diseases. Position Paper of the European Society of Cardiology (ESC) Working Group on Atherosclerosis and Vascular Biology.Ridger VC, Boulanger CM, Angelillo-Scherrer A, Badimon L, Blanc-Brude O, Bochaton-Piallat ML, Boilard E, Buzas EI, Caporali A, Dignat-George F, Evans PC, Lacroix R, Lutgens E, Ketelhuth DFJ, Nieuwland R, Toti F, Tunon J, Weber C, Hoefer IE. Thromb Haemost. 2017 Jun 28;117(7):1296-1316.).***

Recently, we contributed to a position paper on EVs of published by the Working Group on Cellular Biology of the Heart of the European Society of Cardiology ***(Extracellular vesicles in diagnostics and therapy of the ischaemic heart: Position Paper from the Working Group on Cellular Biology of the Heart of the European Society of Cardiology. Sluijter JPG, Davidson SM, Boulanger CM, Buzás EI, de Kleijn DPV, Engel FB, Giricz Z, Hausenloy DJ, Kishore R, Lecour S, Leor J, Madonna R, Perrino C, Prunier F, Sahoo S, Schiffelers RM, Schulz R, Van Laake LW, Ytrehus K, Ferdinandy P. Cardiovasc Res. 2018 Jan 1;114(1):19-34).***

6. EVs in preeclampsia

We hypothesized that preeclampsia-associated EVs induced functional and phenotypic alterations of monocytes. An altered phagocytosis-related molecular pattern was found on medium sized preeclampsia-associated EVs including an elevated CD47 "don't eat me" signal and a decreased exofacial phosphatidylserine "eat-me" signal. These EVs induced significantly lower chemotaxis and cell motility but accelerated cell adhesion of THP-1 cells suggesting that circulating EVs may play a role in the pathogenesis of preeclampsia. ***The impact of circulating preeclampsia-associated extracellular vesicles on the migratory activity and phenotype of THP-1 monocytic cells. Kovács ÁF, Láng O, Turiák L, Ács A, Kőhidai L, Fekete N, Alasztics B, Mészáros T, Buzás EI, Rigó J Jr, Pállinger É. Sci Rep. 2018 Apr 3;8(1):5426.).***

We have shown that recombinant HSPE1 promoted human Treg cell differentiation *in vitro.* We also demonstrated that EV-associated HSPE1 induced Treg development. Single-cell transcriptomics has enabled identification of 7 Treg cell subtypes. Our data indicated that HSPE1 and CAPG could serve as markers for identification of Treg subtypes. Furthermore, trophoblast-derived medium sized EV-associated HSPE1 and the miRNA cargo proved to have an important role in Treg cell expansion *in vitro*. ***Unravelling the Role of Trophoblastic-Derived Extracellular Vesicles in Regulatory T Cell Differentiation. Kovács ÁF, Fekete N, Turiák L, Ács A, Kőhidai L, Buzás EI, Pállinger É. Int J Mol Sci. 2019;20. pii: E3457.).***