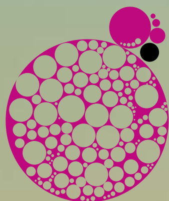

6th GEIVEX Symposium

Santiago de Compostela

October 26-28, 2022

ISBN: 978-84-09-44895-1
D.L.: C 1755-2022

ABSTRACT BOOK AND PROGRAM

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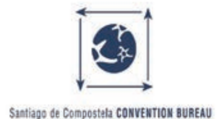

Symposium Technical secretariat:

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Dear colleague,

Since our last symposium back in 2019, and besides these difficult times in the context of COVID19 health crisis, at GEIVEX we are excited to announce the **6th International GEIVEX symposium** that will be held on 26-28th of October 2022 in Santiago de Compostela, Spain. GEIVEX is a young, but already consolidated, non-profit organization which exists to promote research, innovation, and knowledge exchange in the field of Extracellular Vesicles (EVs). Our purpose is to advance personalized medicine by developing new tools and biomarkers with high social impact to generate benefits and equality in global health.

On this occasion, based on the successful previous experience at Granada GEIVEX symposium in 2019, we have also organized a Hands-on workshop on the pre-meeting day (October 25th) under the scope **"Hands on exploring methodologies for EV translational research and biomarker detection"**. We are very aware that this type of activity is very welcomed by EVs researchers and a great opportunity for the companies to promote state-of-the art technologies for EV analysis. Thus, this demonstration will be focus in EV-specialised equipment of interest for EV researchers, both in the laboratory and at translational level for the clinical practice. We believe that the combination of basic and clinical research, like in many other fields, is the basis of translational EVs analysis leading to future approaches for tackling disease. This 6th International Symposium will focus on novel concepts and clinical applications of EVs paying special attention to methodology for the identification of biomarkers and therapy targets. In order to make this symposium even more exciting, to achieve productive discussions and maintain the excellent standards, we are working on a selected list of high-quality keynote speakers for each session that we believe will fulfil EVs researcher expectations.

Beyond all, this 6th International Symposium in 2022 will provide an important opportunity to create and strengthen partnerships between professionals dedicated to EVs research, from both basic and clinical fields and to meet renowned speakers in the field. The chosen venue will be the Auditorium of the Faculty of Medicine of Santiago de Compostela University (USC), with all the modern facilities for conferences and located at the heart of the Santiago de Compostela historic old town. Compostela is a World Heritage Site by UNESCO thanks to its monumental beauty, extraordinary conservation and for being the goal of a millenary pilgrimage route: El Camino de Santiago (the Way of St. James). The venue is located beside Praza do Obradoiro that holds the Cathedral of Santiago de Compostela, the Prazas Quintana, Inmaculada and Praterías surrounded by the main historical streets of the city with plenty of cafés, restaurants, and charming hotels.

The Faculty of Medicine, holding the venue Auditorium, is a historic building recently refurbished with its own Cafeteria and Restaurant, offering a relaxed and open environment for interaction between attendees and high visibility for sponsors.

We look forward to your active participation in the meeting that will additionally let you enjoy an exceptional location.

María Pardo-Pérez
On behalf of the Local Organizing Committee

María Yáñez-Mó
GEIVEX President

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Tuesday October 25th

"Hands-on exploring methodologies for EV translational research and biomarker detection"

8:30-9:00h Registration

- ✓ 9:00-9:30 TIPS &TRICKS OF EV ISOLATION AND HANDLING
  Marcella Franquesa, REMAR Group, Institut Germans Trias i Pujol (Campus Can Ruti), Badalona (Barcelona)
- ✓ 9:30-10:00 TECHNICAL ASPECTS OF SORTING OF EVS BY FLOW CYTOMETRY
  Oscar Fornas Carreño, UPF-CRG Flow Cytometry Unit, Barcelona
- ✓ 10:00-10:30 EXTRACELLULAR VESICLES IN THE CLINICS. TOWARDS EV-NO-INVASIVE TESTING
  Atocha Romero, Hospital Universitario Puerta Hierro Majadahonda
- ✓ 10:30-11:00 NEW APPROACHES FOR THE USE OF EVs IN LIQUID BIOPSY
  Hector Peinado, CNIO, Madrid
- ✓ 11:00-11:30 COMBINING DIVERSE METHODOLOGIES USING EVs AS TROJAN HORSES FOR CANCER THERAPY AND DIAGNOSIS AND COVID-19
  Pilar Martín-Duque, Universidad de Zaragoza

11:30-12h Coffee break

SPONSORS TALKS 15 min +5 min questions

- 12:00-12:15 Nasas Biotech: Lorena Alonso Alconada
  ExoGAG: A NEW TECHNOLOGY FOR EXTRACELLULAR VESICLES ISOLATION COMPATIBLE WITH MULTIPLE OMICS

- 12:20-12:35 NanoFCM: Rob Tempest
  THE NANOANALYZER: COMBINING FLOW CYTOMETRY & PARTICLE ANALYSIS TO MEET THE CHALLENGES OF THE NANO-SCALE

- 12:40-12:55 Iesmat: Francisco López/Sergio Ganarul
  NANOPARTICLE TRACKING ANALYSIS (NTA) - MEASUREMENT OF MICROVESICLE SIZE AND CONCENTRATION

- 13:00-13:15 Izasa: Aitor Gonzalez Granja
  INNOVATIVE SOLUTIONS ON THE DIRECT CHARACTERIZATION OF EXTRACELLULAR VESICLES

115-15h Lunch

Practical sessions: 15:00-18:30h

Wednesday 26th October

8:00 Registration

8:45 Welcome remarks

Institutional welcome

Welcome by the Local Organizing Committee.

GEIVEX president

9:15 OPENING LECTURE:

MARIA ELENA BOTTAZZI, Baylor College of Medicine, Houston

"Past, Present and Future of Neglected Tropical Disease Vaccines: How the COVID-19 experience will accelerate their development "

Chair: Antonio Marcilla (Universitat de Valencia, Valencia).

10:30 SESSION I: EVS IN INFECTION AND IMMUNITY

Chairs: Lydia Álvarez-Erviti (Center for Biomedical Research of La Rioja (CIBIR), Logroño) and Mar Valés-Gómez (CNB-CSIC, Madrid).

10:30- OC1. Dynamin-like proteins are required for secretion of extracellular vesicles and virulence of Mycobacterium tuberculosis. **Vivian Salgueiro Toledo (Universidad Autónoma de Madrid)**
10:40- OC2. Exploring the role of Extracellular Vesicles from the liver flukes Fasciola hepatica and Dicrocoelium dendriticum in the parasite-host communication. **Christian Miquel Sánchez López (Universidad de Valencia)**
10:50- OC3. Functional characterization of P. vivax genes preferentially expressed in the human bone marrow and spleen and the role of EVs in these hemopoietic tissues. **Alberto Ayllón (ISGLOBAL – Institut de Salut Global de Barcelona)**
11:00- OC4. Two cargo proteins of Fasciola hepatica EVs show different effects on hepatic stellate cells and alter macrophages cytokine profile. **Aranzazu González (Universidad de Valencia)**
11:10- OC5. Proteomics characterization and functional analysis of circulating extracellular vesicles in Chagas disease patients with different clinical manifestations.**Carmen Fernández Becerra (ISGLOBAL – Institut de Salut Global de Barcelona)**

11:30 Coffee break

12:00 SESSION II: LIQUID BIOPSY AND BIOMARKERS

Chairs: Héctor Peinado (CNIO, Madrid) and Jose Antonio López-Guerrero (IVO, Valencia)

12:00- OC6. Plasma extracellular small RNA biosignature identifies early symptomatology in Huntington´s Disease. **Marina Herrero Lorenzo (Universitat de Barcelona)**
12:10- OC7. EXOGAG, the new method for the isolation of Extracellular Vesicles and Glycoproteins, unmasks biomarkers and a new molecular mechanism in kidney disease. **Marta Vizoso González (Instituto de Investigación Sanitaria de Santiago de Compostela – IDIS)**
12:20- OC8. Non-coding RNA profile in liquid biopsy associated with systemic lupus erythematosus activity, relevance of exosomal fraction. **Olga Martínez Arroyo (INCLIVA Biomedical Research Institute, Valencia)**
12:30- OC9. From urinary EVs to clinical testing: the road of a new biomarker for renal fibrosis in kidney transplanted patients. **Marta Clos Sansalvador (IGTP)**
12:40- OC10. Circulating extracellular vesicles carry metabolic dysregulation and obesity-associated comorbidities risk biomarkers with application to the clinical practice. **Nerea Lago Baameiro (Instituto de Investigación Sanitaria de Santiago de Compostela –IDIS)**
12:50- OC11. Proteomic analysis of urinary extracellular vesicles reveals sub-clinical cardiorenal risk with added value to urine analysis. **Miriam Anfaiha Sánchez (IIS – Fundación Jiménez Díaz)**

13:15 Lunch

14:15 SPONSORED PRESENTATIONS

Chair: Héctor Peinado (CNIO, Madrid)

Solmeglas:
"Colocalization Measurements – next Generation of NTA"
Izon:
"Scalable Isolation & Purification of Extracellular Vesicles: An Izon Approach"
NanoFCM:
"The nanoanalyzer: combining flow cytometry & particle analysis to meet the challenges of the nano-scale"
Izasa:
"Innovative solutions on the direct characterization of extracellular vesicles"

15:20 SESSION III. EVs IN CANCER

Chairs: Francesc Borrás (Institut Germans Trias i Pujol, Badalona) and Atocha Romero (FIB Hospital Universitario Puerta de Hierro, Madrid)

15:20- OC12. Effects of MAPK inhibitors targeted therapy on metastatic melanoma cell surface, soluble and EV-associated immune modulating NKG2D-ligands. Carmen Campos Silva (Spanish National Center of Biotechnology)
15:30- OC13. Study and validation of the metabolic signature of extracellular vesicles as a prognostic and predictive marker in pancreatic cancer. Pilar Espiau Romera (IIS Aragón)
15:40- OC14. The extracellular vesicle senescence-associated secretory phenotype (evSASP) in the intercellular communication during cancer chemotherapy. Valentín Estévez Souto (Health Research Institute of Santiago de Compostela)
15:50- OC15. Extracellular vesicles versus metalloprotease cleavage: a novel regulatory mechanism for NKG2D-Ligands. Silvia López Borrego (National Centre of Biotechnology – CNB)
16:00- OC16. Relevance of melanoma-derived EV-DNA in tumor progression. Enrique Bastón de la Fuente (Spanish National Cancer Research Centre)
16:10- OC17. Cancer intercellular communication: glycans as mediators of cellular targeting and reprograming. Daniela Freitas (i3S, University of Porto)
16:20- OC18. The role of glycosylated extracellular vesicles in modulating the phenotypic behaviour of recipient gastric cancer cells. Càtia Alexandra Coelho Ramos (Instituto de Investigaçao en Saúde – i3S)
16:30- OC19. Differential protein and glycan packaging into extracellular vesicles by 3D gastric cancer cells. Álvaro Martins (Instituto de Investigaçao e Innovaçao em Saúde – i3S Associaçao)

16:45 Coffee break

17:15 FLASH POSTERS PRESENTATIONS and POSTER WALK

Chair: María Yáñez-Mó (UAM, IIS-IP, Madrid)

FLASH POSTERS:

FUNDAMENTAL EVS BIOLOGY

P1. Extracellular vesicles from the third stage larvae of Anisakis pegreffii: investigating possible proteins implicated in the host-parasite adaptation and pathogenesis. **María Leticia Palomba (Tucsia University)**
P2. Candida albicans secretes different EVs according to its morphology. An active and assembled 20S proteasome complex is only secreted in hyphal EVs. **Raquel Martínez López (Universidad Complutense de Madrid)**
P3. Proteomics of porcine seminal plasma: inside and outside of extracellular vesicles. **Isabel Barranco (University of Bolognia)**

EVs IN CANCER

P4. Ferroptosis transmission by small extracellular vesicles in epithelial ovarian cancer cells. **Carmen Alarcón Veleiro (CICA - INIBIC – Universidade da Coruña)**
P5. Identification of exosome modulators in 3d breast cáncer models using exoscreen and cell painting technologies. **Esther Masiá Sanchís (CIPF)**

EVs IN OTHER DISEASES

P6. Divergent contributions of extracelular vesicle subpopulations to Huntington's Disease Neuropathology. **Rocío Pérez González (Instituto de Investigación Sanitaria y Biomédica de Alicante (ISABIAL) / Instituto de Neurociencias**
P7. Extracellular vesicles released by adipose or hair follicle mesenchymal stem cells induce neuroprotection and modulate neuroinflammation in primary cell cultures. **Rosa María Hernández (University of the Basque Country (UPV / EHU)**
P8. Gamma catenin containing extracellular vesicles are detected in multiple pathologies. **Alberto Benito Martín (Universidad Alfonso X El Sabio)**

LIQUID BIOPSY AND BIOMARKERS

P9. Monitorization of response in multiple myeloma patients treated with the anti-BCMA drug conjugate Belantamab mafodotin through circulating extracellular vesicles". **Mercedes Garayoa (Cancer Research Center – IBMCC (CSIC-USAL) // Institute of Biomedical Research of Salamanco (IBSAL)**

P10. Combined exosomal and plasma non-coding RNA signature associated with urinary albumin excretion in hypertension. **Ana Ortega Gutiérrez (Biomedical Research Institute of Hospital Clínico de Valencia INCLIVA)**
P11. Implementation of an interdisciplinary core facility for the study of extracelular vesicles at the national Hospital of Paraplegics. **Virginia Vila del Sol (Hospital Nacional de Parapléjicos – SESCAM)**
P12. Intelectin-1, epithelial and protector marker on cardiovascular disease, is identified in extracellular microvesicles of neutrophils. **David Sánchez López (Instituto de Investigación Sanitaria de Santiago de Compostela - IDIS)**
P13. Isolation and characterization of extracellular vesicles from fluidic samples in the context of Mycobacterium tuberculosis infection. **Laura Lerma Martínez (Universidad Autónoma de Madrid)**
P14. Secondary Focal Segmental Glomerulosclerosis (FSGS) biomarker discovery from urinary Extracellular Vesicles (uEV). **Sergio García García (IGTP)**

ADVANCES IN EV METHODOLOGY

P15. Co-isolation of highly abundant soluble proteins in exosome samples trips biomarker discovery up. **Sara Vázquez Mera (Facultade de Bioloxía – Universidade de Santiago de Compostela)**
P16. Comparison of techniques for the isolation of VP40 virus-like particles and extracellular vesicles. **Rocío Mellid Carballal (CiMUS – USC)**
P17..High-throughput EV purification from biological fluids for diagnostic application. **Nora Olszok (Evotec)**
P18. Optimization of an RNA isolation method for EV-Based Non-invasive Testing of ALK fusions in lung cancer patients. **Lucía Robado de Lope (Instituto de Investigación Sanitaria Hospital Universitario Puerta del Hierro)**
P19. Proof of concept of using a membrane sensing peptide for sEVs affinity-based isolation. **Joaquín Morales (Universidad Autónoma de Madrid (UAM)**
P20. Standardization of an ELISA test for direct CD73 functional evaluation of MSC-derived extracellular vesicles. **Miriam Moron Font (IGTP)**
P21. Tangential flow filtration as a proper alternative for extracellular vesicles isolation. **Clara García Vallicrosa (CIC bioGUNE)**
P22. The Oxylipin Profile of Human Milk Extracellular Vesicles. **Julia Kuligowski (Health Research Institute La Fe)**

EVs IN INFECTION AND IMMUNITY

P23. Bone marrow-on-a-chip for the study of EVs in malaria cryptic infections. **Nuria Sima (ISGlobal – Institut de Salut Global de Barcelona)**
P24. Characterization of extracellular vesicles secreted by thymic regulatory T cells (thyTreg-EVs). **Marta Fernández Castillo (Instituto de Investigación Sanitaria Gregorio Marañón)**
P25. Comparison of the proteomic profile of plasma-derived extracellular vesicles isolated by different methods from patients with leishmaniasis. **Ana María Torres García (Instituto de Salud Carlos III)**
P26. Detecting and diagnosing the liver stage of Plasmodium infection in circulating extracellular vesicles. **Bárbara Teixeira (Instituto de Medicina Molecular – Joao Lobo Antunes)**
P27. Proteomics analyses of Extracellular Vesicles from peritoneal exudates reveal the elicited inflammatory/autoimmune response in the chronic graft versus host disease lupus model. **Mercedes Zubiaur Marcos (IPBLN-CSIC)**

EVs-BASED THERAPY AND TRANSLATIONAL MEDICINE

P28. Comparative study of pEVs isolated from different platelet sources in wound healing. **Andreu Miquel Amengual Tugores (Universitat de les Illes Balears)**
P29. Exogenously loading monocytes extracellular vesicles with mRNA. **Cristina Fornaguera Puigvert (Institut Químic de Sarrià (IQS), Universitat Ramon Llull (URL)**
P30. Exosomal miRNAs changes following cancer therapy-induced cardiotoxicity. **Marta Vilar Sánchez (CiMUS, IDIS)**
P31. Extracellular vesicles from the conjunctiva: Isolation, characterization, and antioxidant effect. **Ismael Romero Castillo (Universidad de Valladolid - IOBA)**
P32. Funcionalization of MSC-EVs with quercetin as a potential senolytic treatment for aterosclerosis. **Fátima Ainara González Moro (Universidad Autónoma de Madrid – UAM)**
P33. In vivo loading of recombinant proteins into extracellular vesicles for the treatment of lysosomal storage diseases. **Diego Baranda Martínez-Abascal (Vall D´Hebron Institute of Research)**
P34. Nanoengineering exosomes with nanoparticles. **Marina Martínez Cruz (Universidad de Zaragoza)**
P35. Nanoparticles-like vesicles from food and their impact on S. Aureus and E. Coli growth. **Mari Cruz Manzanaque López (Universidad de Valencia)**

P36. Pasteurization influences on honey vesicle-like nanoparticles and its biological activity. **Mari Cruz Manzanaque López (Universidad de Valencia)**
P37. Pomegranate-derived extracellular vesicles as therapeutic nanocarriers of exogenous miRNAs. **María del Carmen López de las Hazas Mingo (Fundación IMDEA Alimentación)**
P38. Therapeutical use of EVs derived from platelets and from mesenchymal stromal cells: a comparative miRNA study. **María Antonia Forteza Genestra (Group of Cell Therapy and Tissue Engineering, Research Institute on Health Sciences-IUNICS)**
P39. The role of Extracellular-vesicles in the development of an mRNA-based Chagas disease vaccine candidates. **Cristina Poveda (Baylor College of Medicine)**

Thursday 27th October

9:00 SESSION IV. FUNDAMENTAL EVs BIOLOGY

Chairs: Antonio Marcilla (Universitat de Valencia, Valencia) and Hernando del Portillo (ISGlobal (Barcelona) + IGTP (Badalona)

9:00- OC20. A GWC screen to unravel new molecular mechanisms involved in EV uptake. **Víctor Toribio Serrano (Universidad Autónoma de Madrid)**
9:10- OC21. SASP mediated by sEV as senomorphic target. **Sergio Lucio Gallego (CICA-INIBIC – Universidade da Coruña)**
9:20- OC22. Morphological characterization of porcine seminal extracellular vesicles by Cryo-electron microscopy (Cryo-EM). **Ana Parra (Universidad de Murcia)**
9:30- OC23. Identification of the Wnt Signal Peptide that directs secretion on Extracellular Vesicles. **Uxía Gurriarán (OHRI)**

10:00: MORNING LECTURE:

PHIL FELGNER, University of California

"150 years of vaccine science"

Chair: Hernando del Portillo, ISGlobal (Barcelona) + IGTP (Badalona)

11:15 Coffee break

11:45 SESSION V. ADVANCES IN EV METHODOLOGY

Chairs: Maria J Vicent (CIPF, Valencia) and Juan Manuel Falcón-Pérez (CICbioGUNE, Derio)

11:45- OC24. Optimization of size exclusion chromatography for extracellular vesicle isolation. **Beatriz Benayas López (Universidad Autónoma de Madrid – UAM)**
11:55- OC25. Comparison of Novel Combined Biophysical Approach and Traditional Physical Protocols for Isolating Small Extracellular Vesicles from Goat Milk. **María Isabel González Gutiérrez (Fundación para la Investigación del Hospital Gregorio Marañón de Madrid)**
12:05- OC26. Nanosystems based on silver sulphide nanoparticles encapsulated in goat milk small extracellular vesicles for their use as probe in optical imaging. **Elena Aguilera Jiménez (Fundación para la Investigación del Hospital Gregorio Marañón de Madrid)**

12:15- OC27. Engineering Milk Small Extracellular Vesicles by Click Chemistry. Desiré Herreros Pérez (Fundación para la Investigación del Hospital Gregorio Marañón de Madrid)
12:25- OC28. Large-scale Serum-free production of Extracellular Vesicles (EVs) from immortalized Wharton's jelly Mesenchymal Stromal Cells (iWJ-MSC) using hollow fiber bioreactors. Sergio García García (IGTP)
12:35- OC29. Design of a large-scale and GMP-compliant MSC-EV production process for clinical testing. Marta Montguió Tortajada (German Trias I Pujol Research Institute – IGTP)

13:00 Lunch

14:00 AFTERNOON LECTURE:

LOLA FERNÁNDEZ MESSINA, Universidad Complutense de Madrid, Spain.

"Unravelling the role of EV-shuttled miRNAs as immune modulators"

Chair: Marcella Franquesa (Institut Germans Trias i Pujol, Badalona).

15:15 SESSION VI. EVS-BASED THERAPY AND TRANSLATIONAL MEDICINE

Chairs: Marcella Franquesa (Institut Germans Trias i Pujol, Badalona) and Mercedes Zubiaur (Instituto de Parasitología y Biomedicina "López-Neyra" CSIC-Universidad de Granada)

15:15- OC30. Platelet-derived extracellular vesicles show therapeutic potential on a 3D tendon disease model. Manuel Gómez Florit (IdISBa – Instituto de Investigación Sanitaria de las Isles Balears)
15:25- OC31. Effect of miR-21 into mesenchymal stem cells -derived extracellular vesicles behaviour on inflamm-aging. María del Carmen Arufe Gonda (UDC – INIBIC – CICA)
15:35- OC32. Manipulation of microRNA distribution with EXO- and CELLmotifs as a novel approach against atherosclerosis. Rubén García Martín (Joslin Diabetes Center, Centro Nacional de Biotecnología)
15:45- OC33. Placental MSCs and their exosomes as vehicles for the Na/I symporter (hNIS): A new agent for gene therapy and diagnostic. Pilar Martín Duque (Universidad de Zaragoza)

15:55- OC34. Design of glycolipid-functionalized extracellular vesicles for their selective targeting to dendritic cells. **Jordi Guixeras Carreras (Institut Químic de Sarriá – IQS)**
16:05- OC35. Enhanced production of immunomodulative MSC-EV by 3D bioreactor culture in chemically-defined medium. **Yvan Courageux (IGTP)**
16:15- OC36. Prevention of alpha-synuclein pathology in gut and cord by shRNA-MC delivered by RVG-extracellular vesicles. **María Izco Gaviria (Fundación Rioja Salud)**

16:40 Coffee break

17:10 GEIVEX activities and general assembly.

Presentations from GEIVEX mobility fellows. Networking with ISEV and other National Societies, the MOVE initiative.

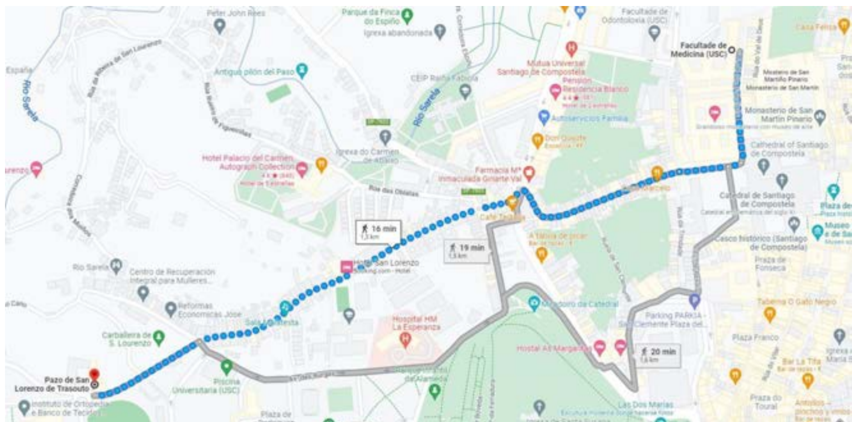
21:00 Symposium dinner

Pazo de San Lorenzo

Robleda de San Lorenzo s/n

15705 Santiago de Compostela

A Coruña - España



Friday 28th October

9:15 CLOSING LECTURE

MIGUEL LÓPEZ, Universidade de Santiago de Compostela (USC)

"sEVs-mediated targeting of hypothalamic AMPK: a new hope for the treatment of obesity"

Chair: María Pardo (IDIS, Santiago de Compostela)

10:30- SESSION VII. EVs IN OTHER COMPLEX DISEASES

Chairs: María Pardo (IDIS, Santiago de Compostela) and Gloria Álvarez-Llamas (IIS-Fundación Jiménez Díaz, Madrid)

10:30- OC37. UHPLC-MS/MS-based Metabolomics reveals differences on Extracellular Vesicles secreted by obese hepatocytes, and their effects on adipocyte metabolism. **María Azparren Angulo (CICbioGUNE)**
10:40- OC38. Cortico-striatal activation via physical training modulates the proteomic content of striatal extracellular vesicles in a model of Huntington's disease. **Julia Solana Balaguer (Universitat de Barcelona)**
10:50- OC39. Saturated fatty acid-enriched extracellular vesicles mediate communication between liver cells in metabolic associated fatty liver disease. **Irma García Martínez (IIBM – CSIC – UAM)**
11:00- OC40. Functional role of extracellular vesicles in the communication of obese adipose tissue with the liver, and their involvement in the establishment and maintenance of human hepatic steatosis. **Tamara Camino (Instituto de Investigación Sanitaria de Santiago de Compostela)**

11:15 Coffee break

11:45 ROUND TABLE DEBATE:

"GEIVEX: the road from basic research to translation and impact"

Hernando del Portillo

ICREA Research Professor – ISGlobal (Barcelona) + IGTP (Badalona)

María J. Vicent

Centro de Investigación Príncipe Felipe (CIPF) Valencia

Mª José Alonso

Universidad de Santiago de Compostela (USC), Santiago de Compostela

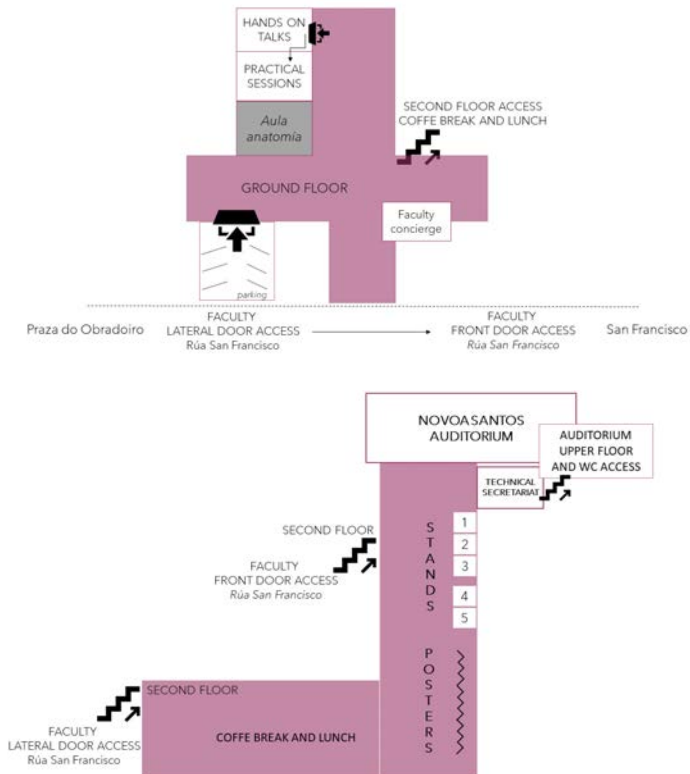
María de la Fuente

Instituto de Investigación Sanitaria de Santiago de Compostela (IDIS), Santiago de Compostela

13:00 Awards and closing remarks

THE VENUE

The meeting will take place at the Medicine Faculty Auditorium from the University Campus Vida. This recently renovated venue offers a large auditorium with 400 seats and a hall in which poster sessions, coffee breaks and sponsors stand will take place to ensure high visibility of the different activities.



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ORAL

OC1. Dynamin-like proteins are required for secretion of extracellular vesicles and virulence of Mycobacterium tuberculosis

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Keywords: Extracellular vesicles, vesicle biogenesis, *Mycobacterium tuberculosis*, Dynamin-like proteins, pathology.

Extracellular vesicles (EVs) are membrane-bound nanostructures released by eukaryotic and prokaryotic cells to communicate with their environment. *Mycobacterium tuberculosis*, the etiological agent of human tuberculosis (TB), actively secretes EVs in culture and during infection and can carry protein, lipids, lipoproteins and siderophores. *In vitro* studies have suggested that mycobacterial EVs (MEVs) contribute to nutrient acquisition, toxin delivery, and immune modulation, indicating a potential role in the TB pathogenesis. Our preliminary studies pointed to a specific class of proteins, the Dynamin-like proteins (DLPs) as key players in vesicle biogenesis. This superfamily of proteins comprise large GTPases that are involved in membrane fusion and fission. *M. tuberculosis* harbors two DLPs so-called IniA and IniC, in this work, using a DLP mutants, we show that both dynamins, IniA and IniC, are essential factors for MEVs secretion and virulence in *M. tuberculosis.* Furthermore, the release of EVs for *M. tuberculosis* confined within macrophages allow the communication with uninfected host cells and stimulate an inflammatory response that promotes bacterial proliferation and host pathology. These findings significantly advance our understanding of mycobacterial EVs biogenesis and its contribution to TB pathogenesis and highlight the potential of the mycobacterial dynamin-like proteins as drug targets to increase host resistance to TB.

OC2. Exploring the role of Extracellular Vesicles from the liver flukes *Fasciola hepatica* and *Dicrocoelium dendriticum* in the parasite-host communication.

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Keywords: *Fasciola hepatica, Dicrocoelium dendriticum, Liver, Macrophages, Extracellular Vesicles*

Introduction

The trematodes *Fasciola hepatica* and *Dicrocoelium dendriticum* can coexist in the liver and bile ducts of their mammalian hosts, including humans. Parasites pathology is mainly associated with liver damage, caused by the migration of the flukes, their excretory/secretory products (ESP), and local inflammation. The manipulation of the host response is mostly due to the release of ESP at the host-parasite interface, which include extracellular vesicles (EVs). These EVs may modulate different immune cells as well as liver cells in the host.

Objective

Study the effects from EVs released by *F. hepatica* (FhEVs) and *D. dendriticum* (DdEVs) on human macrophages, hepatic stellate cells (HSC) and hepatocytes *in vitro*.

Methods

Parasites were obtained and cultured for 4h. EVs were obtained by differential centrifugation and Size Exclusion Chromatography (SEC), and characterized by NTA, TEM and immunogold labeling (EVtrack ID EV220006). THP1-XBLUE™-CD14 macrophages, LX-2 and HepG2 cells were treated with EVs from both parasites either separately or in transwell assays for different times, and cells were analyzed through Label-free quantitative proteomics. Cell responses were monitored by RT-qPCR, ELISA and Western Blotting. MTT assays were performed to assess EVs cytotoxicity.

Results

EVs showed different effect depending on the parasite. In macrophages, FhEVs have an anti- inflammatory action downregulating NF-κβ expression and promoting the secretion of regulatory cytokines. In contrast, DdEVs seem to have pro-inflammatory properties via NF-κβ, with TNF-α and IL-8 induction. We also identified proteins that might be involved in FhEVs–macrophage interaction. In HSC, proteomic analyses revealed that no common proteins were up- or down-regulated upon FhEVs or DdEVs treatment. FhEVs upregulated fibrinogenic proteins involved in extracellular matrix remodeling, whereas DdEVs mainly affected proteins involved in the ROS response, inflammation and apoptosis. Cell viability assays showed that DdEVs reduced the viability of HSC and Hepatocytes, whereas FhEVs only reduced LX-2 viability.

Conclusion

Our data confirm different behavior of FhEVs and DdEVs when interacting with host cells, this maybe related with their different migration pattern in the host. We also demonstrated macrophages as key regulators of the inflammatory response produced by parasite helminths EVs. Target proteins have been identified as candidates for mediating the anti-inflammatory response.

Funding

Supported by the Agencia Estatal de Investigación, Ministerio de Ciencia e Innovación, Spain (Grant PID2019-105713GB-I00/AEI /10.13039/501100011033), and Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Valencia, Spain (Grant PROMETEO/2020/071). C.M.S-L is the recipient of a predoctoral fellowship (PRE2020-092458), by AEI /10.13039/501100011033. A. G-A is the recipient of a predoctoral fellowship (CPI-20-353), by Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Valencia, Spain (PROMETEO/2020/071). The research group is part of "Red Traslacional para la Aplicación Clínica de Vesículas Extracelulares, Tentacles", (RED2018-102411-T, Agencia Estatal de Investigación, Spain).

OC3. Functional characterization of *P. vivax* genes preferentially expressed in the human bone marrow and spleen and the role of EVs in these hemopoietic tissues.

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Keywords: *P. vivax*, CRISPR/Cas9, bone-marrow, spleen, extracellular vesicles

Introduction

Plasmodium vivax is the most widely distributed human malaria parasite with over 7 million clinical cases in the world. Noticeably, the bone marrow (BM) and the spleen have recently emerged as cryptic erythrocytic niches of natural infections where the largest parasite biomass is hidden. Of interest, we previously demonstrated that infection induced BM dyserythropoiesis altering RNA profiles related to erythropoiesis and identified *P. vivax* spleen-dependent genes associated with cytoadhesion. Last, we have shown that circulating extracellular vesicles (EVs) from natural infections contain parasite proteins and act as intercellular communicators facilitating cytoadherence of infected reticulocytes obtained from patients to human spleen fibroblasts (hSF).

Objective

The aim of this study is to functionally characterize spleen-dependent genes and selected genes from the parasite upregulated in the BM through CRISPR-Cas9 editing and to determine the role of EVs in these tissues.

Methods & Results

Initially, we performed global transcriptional analysis of BM samples. Bioinformatics analysis revealed a list of genes preferentially expressed in BM as compared with peripheral blood. We used a computational approach to select a list of spleen-dependent genes based in our data of splenectomised monkeys. Due to the lack of in vitro culture system for *P. vivax*, we have generated a plasmid-construct to express those genes in culturable *P. falciparum* using the CRISPR-Cas9. A transgenic line expressing a hypothetical spleen-dependent protein was generated and confirmed through genomic integration, transcript identification, protein expression by western blot, confocal immunofluorescence microscopy and single cloning. EVs from patients facilitated the binding of such transgenic line to hSF. Other transgenic lines are being generated. Last, to study the role of EVs as intercellular communicators, we performed single-cell transcriptomics of hSF and CD34+ cells after uptake of plasma-derived EVs from *P. vivax* patients. Notably, genes related to erythropoiesis and adhesins were differentially expressed.

Conclusion

The functional characterization of *P. vivax* BM and spleen ligands will enable us to gain better understanding about the physiopathology of the parasite and the parasite's tropism towards these reticulocyte-rich tissues. Moreover, studies on the role of EVs in these tissues will also unveil molecular insights on the formation of these cryptic erythrocytic niches, a major challenge towards elimination of this human malaria parasite.

OC4. Two cargo proteins of *Fasciola hepatica* EVs show different effects on hepatic stellate cells and alter macrophages cytokine profile

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Keywords:

Introduction

The trematode *Fasciola hepatica* is the causative agent of liver fluke disease in mammalian species. Modulation of host immunity is partly due to the release of extracellular vesicles (EVs) that can be internalized by host cells.

The **objective** of this study is to identify two proteins present in *F. hepatica* derived EVs and examine their effects on hepatic stellate cells (HSC) and macrophages, which are key cell types involved in the parasite interaction with the host.

Methods

Enolase and Fh16.5 proteins were produced in *Escherichia coli* M15, and purified with a Ni-NTA kit (Qiagen). Proteins were added to cultures of LX-2 hepatic stellate cell line (SCC064, Merck), and THP-1-XBlue™-CD14 macrophages (InvivoGen). Cell pellets were analyzed through Label-free quantitative proteomics and Western Blotting. EVs from treated cells were purified using Size Exclusion Chromatography (SEC), and characterized by NTA, TEM and proteomic profiling (EVtrack ID EV220011). MTT assays were used to assess cytotoxicity. Anti-inflammatory effects were assayed on macrophages using the Quanti-blue™ reagent (InvivoGen). The presence of TNF-α, IL-1β, TGF-β and IL-6 in EVs was determined by ELISA.

Results

Enolase and Fh16.5 tegumentary protein showed no cytotoxicity, but only enolase showed anti-inflammatory properties in LPS-stimulated macrophages. Both proteins altered the proteomic profile in both cell types and their secreted EVs. Regarding LX-2 cells, proteins involved in fibrosis and extracellular matrix remodeling were different. In the case of THP-1 cells, the levels of proteins involved in inflammation, cell proliferation and migration were affected. LX-2 EVs showed higher particle concentrations after treatment with Fh16.5. THP-1 EVs showed different cytokine levels after treatments, as Enolase reduced their content in IL-6, TNF-α and IL-1β.

Conclusion

Enolase and Fh16.5 tegumentary protein from *F. hepatica* EVs have anti-inflammatory effects *in vitro* and alter the proteomic profile of HSCs and macrophages, suggesting their role *in vivo* modulating fibrosis.

Funding

Supported by the Agencia Estatal de Investigación, Ministerio de Ciencia e Innovación, Spain (Grant number PID2019-105713GB-I00/AEI/10.13039/501100011033), and Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Valencia, Spain (Grant PROMETEO/2020/071). C.M.S-L is the recipient of a predoctoral fellowship (PRE2020-092458) funded by AEI /10.13039/501100011033. A. G-A is the recipient of a predoctoral fellowship (CPI-20-353) funded by Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Valencia, Spain (PROMETEO/2020/071). Part of "Red Traslacional para la Aplicación Clínica de Vesículas Extracelulares, Tentacles", (RED2018-102411-T, Agencia Estatal de Investigación, Spain).

OC5. Proteomics characterization and functional analysis of circulating extracellular vesicles in chagas disease patients with different clinical manifestations

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Keywords: Chagas disease, *T.* cruz*i*, Extracellular vesicles, biomarkers, Functional assays

Introduction

Chagas disease caused by *Trypanosoma cruzi* is one of the most prevalent parasitic infections in Latin America and responsible for millions of yearly clinical cases. Moreover, in recent years, the migratory movements worldwide have contributed to the emergence of this neglected tropical disease outside endemic regions, especially in USA and southern Europe. One of the main clinical problems in chronic Chagas disease (CCD) is the lack of effective biomarkers for therapeutic response and disease outcome during infection.

Objective

The main objective of this study is to use extracellular vesicles (EVs) for identifying novel biomarkers in CCD, specifically in the context of therapeutic response and disease prognosis during the chronic infection.

Methods

Human cohorts using in this study: 1) CCD patients at indeterminate phase of the disease (Barcelona, Spain) and 2) CCD patients with cardiac manifestations (Cochabamba, Bolivia). Plasma samples were collected before and at the end of treatment. As negative controls, patients with cardiac complications not related to Chagas disease and healthy donors were also included. EVs were enriched by size exclusion chromatography (SEC) or immunocapture. Characterization was done by NTA, WB and protein mass spectrometry. For functional assays HUVEC and THP1 cells were incubated with EVs.

Results

Mass spectrometry analysis of affinity-captured (CD9, CD81 and CD63)-EVs identified the presence of *T. cruzi* proteins in circulating EVs from both cohorts, *albeit* variably. Of interest, the total number of parasite proteins was higher in the indeterminate group as compared to the group with cardiac manifestations. Moreover, some parasite proteins from the cardiac group disappeared after drug treatment and others were associated with either group. To get molecular insights, EVs from these groups as well as from in vitro culture *T. cruzi* parasites grown in two different types of cardiac cells were used in functional assays of HUVEC (angiogenesis) and TPH1 (immune modulation) cells.

Conclusion

Results will be presented and discussed in the context of new biomarkers for assessing disease prognosis, for evaluating the recovery of chronic patients after treatment and for unveiling EV-mediated intercellular communication in Chagas disease patients.

Acknowledgements
To the patients and healthy donors that participated of this study. We are particularly grateful to Hernando A del Portillo for helpful discussions and to Marc Nicolau, Cameron
Ellis, Nieves Martinez and Carme Subirà for technical support.

OC6. Plasma extracellular small RNA biosignature identifies early symptomatology in Huntington´s Disease.

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Keywords: Small RNA, plasma, biomarker, premanifest, Huntington´s disease

Despite the advances in the understanding of Huntington's disease (HD), no disease-modifying treatments exist, and therapeutic development and HD-clinical trials continue to fail. Major efforts are focused on the assessment of measurable outcomes for optimal therapeutic response. Recent insights on HD described small non-coding RNA (sRNA) as key players in the disease. The profiling of extracellular sRNAs (exRNA), found in body fluids as freely circulating, associated to protein-complexes, and/or encapsulated in extracellular vesicles (EVs), supposes a promising approach for defining non-invasive biomarkers.

Using an optimized method for plasma sub-fractionation and EVs purification by Size-exclusion chromatography (SEC) and Ultrafiltration (UF), we explored sRNA content in EVs and Non-EVs compartments, providing a deep exRNA analysis and offering a complementary source of valuable information.

Characterization of plasma-EVs from three different cohorts, including healthy controls, premanifest HD, and manifest HD, revealed no differences in size and morphology of EVs. Using SeqCluster bioinformatic tool for sRNA annotation and quantification, we highlighted that most differentially expressed (DE) sRNAs in HD-EVs are downregulated in comparison to Control-EVs, with many changes occurring at premanifest stages. In addition, those DE sRNAs in EVs showed a strong correlation with cognitive symptoms. Otherwise, in the Non-EVs compartment, miRNAs appeared upregulated in HD patients. Selected sRNAs showing the most differential profile between groups were validated by RT-qPCR in additional samples as potential future biomarkers for HD.

In conclusion, these findings suggest that EVs and Non-EVs plasma compartments offer complementary and valuable information, reflecting early clinical and pathological changes in HD patients. The deregulation of exRNAs could constitute a biosignature for the progression of HD, improving the sensitivity of protein-based biomarkers at the premanifest stage.

This work was supported by the grant PID2020-113953RB-I00 funded by the Spanish Ministry of Science and Innovation/ Spanish State Research Agency/ 10.13039/501100011033. It was also supported by a 2021 Human Biology Project from the Huntington's Disease Society of America (HDSA). The predoctoral contract is supported by a FPI grant from the same Spanish Ministry.

OC7. EXOGAG, the new method for the isolation of Extracellular Vesicles and Glycoproteins, unmasks biomarkers and a new molecular mechanism in kidney disease.

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Keywords : Polycystic Kidney Disease, Biomarkers, Urine, Glycosaminoglycans

Glycosaminoglycans (GAGs) are large polysaccharides that interact through glycosidic bonds with proteins and lipids, forming the extracellular matrix; or with secreted proteins, such as uromodulin. Glycosylation is altered in pathologies, as cancer or kidney diseases.

GAGs are also present in extracellular vesicles (VEs), nanometric structures delimited by a lipid bilayer that cells release and whose charge (RNA/miRNA, DNA and proteins) is essential in intercellular communication.

Our group has developed a method for GAG, glycoproteins and VEs isolation in any biological sample, called EXOGAG (commercialized by Nasas Biotech), which led us to identify and characterize new signalling mechanisms, and identify new prognostic/diagnosis biomarkers, for example, in polycystic kidney disease (PKD).

Urine samples have been collected from patients genetically diagnosed with type I and II PKD at different stages of the disease. Using EXOGAG, GAG-glycoprotein-VEs complex has been isolated and characterized by different proteomic techniques (Western Blot, mass spectrometry), image characterization (electron microscopy, immunofluorescence) and vesicular component analysis (ExoView® or NanoTracking Analysis®).

EXOGAG has allowed us to identify a new biomarkers in urine (in protection) in PKD patients, which are altered in disease progression, even anticipating currently used kidney damage markers. The characterization of these complexes has led us to discover signalling mechanisms between the different segments of the nephron, and whose function is altered in different pathologies, including polycystic kidney disease.

This new method for isolating the fraction associated with GAG in urine samples has allowed us to identify prognostic/diagnostic biomarkers of kidney diseases, based on glycoprotein and vesicular profile. Likewise, it has led us to identify new signalling mechanisms of the nephron, which opens a new field for a better understanding of renal pathophysiology. These results uncovered the potential as a method of EVs isolation for its use in the research of new cellular communication pathways or cellular mechanisms.

OC8. Non-coding RNA profile in liquid biopsy associated with systemic lupus erythematosus activity, relevance of exosomal fraction

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Keywords: Systemic lupus erythematosus; Plasma exosomes; microRNAs; Biomarkers; Biological pathway

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease where the exosomes have a pathogenic role throughout their cargos, especially non-coding RNA (ncRNA).

OBJECTIVE

The aim of this study is to identify a representative circulating ncRNA profile, with relevance in plasma exosomes, associated to SLE activity.
Methods: Plasma samples were obtained from 96 SLE patients and 25 healthy controls to isolate EXO-P by differential ultracentrifugation. RNA was extracted from both EXO-P and plasma and ncRNAs were identified using high-throughput SmallRNA sequencing analysis. Then, the more representative ncRNA biotype was validated in a higher SLE cohort by qPCR and biological pathways regulated were identified by Over-Representation Analysis (ORA).

RESULTS

MicroRNAs (miRNAs) were the most representative ncRNA biotype in both biofluids (plasma and P-EXO), followed by lncRNA, piRNA and Y-RNAs. In addition, plasma fraction had a higher diversity of differentially expressed ncRNA biotypes than exosomal fraction. Analysing only miRNAs in SLE, the biofluid specificity was observed, being the majority of miRNAs up-regulated in exosomes and down-regulated in plasma, and only 1.2 % were common in both fractions. Focusing on miRNAs to compare both biofluids, we validated 12 miRNAs with highest or lowest fold changes in exosome fraction, miR-15a-5p, miR-27b-3p, miR-101-3p, miR-140-3p, miR-143-3p, miR-144-3p and miR-144-5p miRNA are significantly increased in EXO-P with regard to plasma, except hsa-miR-140-3p that is decreased, and miR-222-3p not showed differences. In addition, all exosomal miRNAs levels were different between SLE groups. In addition, all validated exosomal miRNAs had different levels between SLE groups and miR-15a-5p, miR-144-3p, miR-144-5p, miR-27b-5p and miR-101-3p showed a significant ROC curve for SLE discrimination (AUC > 0.6). Finally, ORA by KEGG and GO databases showed that the exosomal miRNA profile regulates mainly signaling pathways implicated in inflammatory processes (MAPK, VEGF, p53…) and targets involved in the DNA transcription activation or repression.

CONCLUSIONS

There is a biofluid specific miRNA profile associated with SLE, with few common miRNAs. Exosomal miRNAs showed differences between SLE groups, and a statistical power for SLE discrimination. Finally, the identified signaling pathways involved in inflammatory and immunity processes regulated for these exosomal miRNAs, could be used as potential targets.

Acknowledgements: This research was funded by "Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III" [PI18/01405, PI19/01796 and PI21/00249, CD18/00166 (Sara Borrell) FI20/00096 (PFIS) and the European Regional Development Fund (ERDF) and FSE.

OC9. From urinary EVs to clinical testing: the road of a new biomarker for renal fibrosis in kidney transplanted patients.

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Keywords: Urinary extracellular vesicles, renal fibrosis, biomarker, non-invasive, nephrology

Renal fibrosis (RF) is the main responsible of Chronic Kidney Disease progression through silent and gradual accumulation of extracellular matrix, and is strongly associated with worsening renal function, especially but not exclusively, after kidney transplantation. Current clinical analytical parameters do not reflect fibrotic lesions and the "gold standard" for RF diagnosis is renal biopsy (RB), an invasive procedure with major limitations precluding optimal patient monitoring.

During the last years, a plethora of non-invasive urine-extracellular vesicles (uEV)-derived biomarkers have been described to overcome biopsy limitations, with little progression to the clinics. This may be due, in part, to the still limited applicability to clinical practice of biomarker discovery used techniques such as proteomics.

Some years ago, using proteomic approaches, our group identified vitronectin (VTN) in uEV as a potential biomarker for RF in transplanted patients. In those experiments, SEC-enriched uEVs from a total of 23 patients were studied in a discovery phase (shotgun proteomics) and another cohort of 37 patients were studied in a verification phase (targeted proteomics). This observation was further validated at a pilot scale using a clinical-applicable process, such as ELISA testing.

This pilot study confirmed an increased VTN level in urine samples from transplanted patients with high levels of fibrosis (biopsy proven), and thus further supported the results observed by the proteomic analysis.

Recently we have extended these observations to a total of 71 transplanted patients. Our results point to VTN monitoring as a potential biomarker of RF in renal-transplanted patients of over 3 years of evolution. Interestingly, VTN expression highly correlates to RF compared to increased serum Creatinine or Proteinuria, the gold-standard parameters for biochemical monitoring of patients.

Overall, our results show the potential of VTN as a non-invasive biomarker for RF monitoring, and further demonstrated the use of uEV as a feasible platform for the discovery of clinical-applicable biomarkers.

Acknowledgements: This work was supported by Instituto Carlos III project PI19/00837, integrated in the National R + D + I and funded by the ISCIII and the European Regional Development Fund. MCS is supported by a PFIS grant FI20/00021.

OC10. Circulating extracellular vesicles carry metabolic dysregulation and obesity- associated comorbidities risk biomarkers with application to the clinical practice

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Keywords: Extracellular vesicles, adipose tissue, circulating biomarkers, Exoview, plasma, obesity.

Introduction

Extracellularar vesicles (EVs) have now become a key factor in adipose tissue (AT) communication contributing to obesity-associated metabolic dysregulation. Recently, our group has analysed the proteome of EVs released by subcutaneous (SAT) and visceral (VAT) human adipose tissue explants from plasma of obese patients, as well as those released by obese and insulin-resistant adipocytes grown in vitro. This allowed to identify a panel of obesity- associated biomarkers that correlate with the type of adiposity, inflammation, and insulin resistance.

Objective

To stablish a global obesity panel of specific biomarkers of metabolic alteration at different metabolic homeostasis implicated organs to aid clinical practice, and secondly, study inter-organ EVs crosstalk in vitro functional analysis to find innovative pharmacological targets and treatments.

Methods

Different techniques for monitoring plasma EVs were tested and optimised: serial ultracentrifugation, Size Exclusion Cromatography (SEC) with immunodetection, flow cytometry (Apogee) and Single Particle Interferometric Reflectance Imaging Sensor (SP-IRIS)/Exoview, a new equipment based on functionalised chips coated by capture antibodies (tetraspanins or proteins of interest).

Results

All tested methodologies allowed the isolation of circulating EVs, however, only flow cytometry and Exoview appear viable or reproducible for liquid biopsy. Ultracentrifugation and SEC proved to be suitable for further validation with immunodetection, but this would not be feasible in translational level. Although it could be applied in clinical practice, calibrated Apogee flow cytometer detected only EVs larger than 200 nm, thereby losing information of smaller EVs. Nevertheless, Exoview allowed us to capture EVs from just 1µl of plasma obtaining data on vesicle size, quantity, and also, individual vesicle analysis, getting quantitative values of the tetraspanin profile (CD9/CD63/CD81) and AT-specific proteins including their co-localization with our biomarkers of interest. We are currently developing custom ExoFlex chips using AT-specific capture antibodies to enrich plasma samples with AT vesicles; in addition, we co-localize and quantify proteins from our obesity biomarker panel in these vesicles. The resulting data is being correlated with clinical data and tracked in the same patients before and during weight loss intervention (bariatric surgery/diet/exercise).

Conclusions

Analysis of AT biomarkers at the circulating level could be useful for monitoring metabolic dysregulation, adiposity type and inflammation status in obesity; as well as for assessing the efficacy of treatment interventions, and monitoring associated comorbidities, and for this, Exoview could be the feasible methodology to bring into EVs biomarkers to clinical practice.

Acknowledgements
This work was funded by the Instituto de Salud Carlos III-FEDER (PI19/00305). Lago Baameiro N is funded by a Xunta de Galicia GAIN 2021 Predoctoral Contract and Camino T is funded by a FPU17 contract (Ministerio de Educación, Cultura y Deporte) and by the FPU2021 stay.

OC11. Proteomic analysis of urinary extracellular vesicles reveals sub-clinical cardiorenal risk with added value to urine analysis

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Keywords: Urinary extracellular vesicles, proteomics, chronic kidney disease, cardiorenal risk, biomarker

Introduction

High albuminuria (urinary albumin to creatinine ratio (ACR)>30mg/g) is an indicator of cardiovascular and renal risk. There are also clinical evidences of early cardiorenal risk in normoalbuminuric subjects (ACR<30mg/g) within the high-normal range (ACR=10-30mg/g), however, they are out of clinical surveillance.

Objective: We aimed to molecularly stratify the normoalbuminuria condition and identify alterations in urinary extracellular vesicles (uEVs) which may aid in assessing individual risk while revealing pathophysiological processes behind early albuminuria development.

Methods

Hypertensive patients within the normoalbuminuria range were classified based on their ACR values as control (C) (ACR<10 mg/g) and high-normal (HN) (ACR=10-30 mg/g) (n=10). uEVs were isolated by ultracentrifugation and characterized by Western blot, nanoparticle tracking analysis and electron microscopy. uEVs proteins were analyzed by isobaric-labeling (TMT) and LC-MS/MS. Systems biology analysis was carried out and coordinated protein responses were evaluated. Identified proteins and functional categories were considered significant if p-value<0.05. Additionally, a comparative analysis of the significantly altered uEVs' proteins was performed with previous data from our laboratory in urine from the same subjects' cohort [1]. Targeted analysis by immunohistochemistry was performed in human kidney tissue (n=9) for those proteins showing altered levels in uEVs between C and HN groups and no variation in urine, to evaluate in situ kidney alterations and the diagnostic potential of uEVs.

Results

A total of 480 proteins and 263 functional categories were found significantly varied in HN subjects compared to C. A subset of 48 proteins, previously detected in urine without group alteration, showed significantly altered abundance in uEVs from HN *vs.* C subjects reflecting impaired homeostasis, coagulation and lipid metabolism deregulation. These proteins of early damage were identified in tubular and glomerular components of human kidney.

Conclusion: uEVs constitute a valuable source in diagnosis of renal diseases, implementing the information available from urine. uEVs proteins evidence early cardiorenal damage within the normoalbuminuria condition paving the way towards a personalized medicine in the control of cardiorenal risk.

Acknowledgments

This work was supported by the Instituto de Salud Carlos III co-supported by FEDER grants (PI16/01334, PI20/01103, FI21/00128), REDinREN, RICORS 2040, Fundación SENEFRO/SEN, and Comunidad Autónoma de Madrid (PEJ-2020-AI/BMD-17899, PEJD-2019-PRE/BMD-16992, 2018-T2/BMD-11561).

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OC12. Effects of MAPK inhibitors targeted therapy on metastatic melanoma cell surface, soluble and EV-associated immune modulating NKG2D-ligands

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Keywords: NKG2D-ligands, metastatic melanoma, targeted therapy, MAPK inhibitors, immunomodulation

Introduction

Metastatic cutaneous melanoma is a type of skin cancer with very low overall survival rate, due to short-term drug resistance. We described previously that the BRAF-inhibitor (BRAFi) vemurafenib, a commonly used targeted therapy for metastatic melanoma with the $BRAF^{V600E}$ mutation, could lead to immune evasion mechanisms by decreasing the immune activating NKG2D-ligands *in vitro*. The use of an inhibitor of MEK (a kinase downstream of BRAF in the MAPK signalling pathway) combined with BRAFi can delay the development of drug resistance in many patients.

Objective

The main objective of this work is to study the effect of MAPK inhibitors (MAPKi) combination therapy on the NKG2D system and, hence, on tumour recognition.

Methods and Results

Here we demonstrate that inhibition of MAPK signaling using trametinib (MEKi), and the combination of BRAFi with MEKi *in vitro*, causes downmodulation of NKG2D-ligands in melanoma cells, together with other NK activating ligands, which may impair NK cell recognition and eventually contribute to drug resistance and immune evasion. We also studied the level of regulation at which the expression of these ligands was affected. NKG2D-ligands can be released by proteolytic cleavage and in Extracellular Vesicles (EVs). *In vitro* treatment with MAPK inhibitors also decreased soluble and EV-associated NKG2D-L in most of the melanoma cell lines used. In addition to the *in vitro* studies, changes on the levels of soluble and EV-associated NKG2D-ligands were detected by immunoassays in sera from melanoma patients during treatment with targeted therapies.

Conclusion

Our results evidence a down-modulation of NKG2D-L induced by MAPKi in metastatic melanoma cells *in vitro* and suggest a possible related effect in patients. Based on these observations, the suitability for combination strategies of MAPKi with different immunotherapies will be discussed, as well as the possibility of using soluble and EV-NKG2D-L as prognostic or predictive factors in patient biological fluids.

Acknowledgements

Ricardo Jara-Acevedo and Alexandra Beneitez-Martínez for the anti-tetraspanin antibodies. Sheila López-Cobo for protocol optimization and advice. Estíbaliz Alegre for help with patient sample selection.

OC13. Study and validation of the metabolic signature of extracellular vesicles as a prognostic and predictive marker in pancreatic cancer

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Keywords: PDAC, tumor metabolism, patient stratification, EVs.

Introduction
Pancreatic ductal adenocarcinoma (PDAC) is an extremely lethal disease caused by late diagnosis, aggressiveness and lack of effective therapies. Based on its intrinsic heterogeneity, patient stratification models have been correlated according to molecular and metabolic subtypes: the lipogenic subtype is correlated with a classical molecular signature, whereas glycolytic tumors are associated with a highly aggressive basal profile. Due to their properties, extracellular vesicles (EVs) are useful as an indicator of PDAC tumor progression in liquid biopsies. In addition, the specific content of EVs may provide a unique molecular signature indicative of the metabolic status of the tumor.

Objective
Identification of the metabolic signature of EVs in liquid biopsies for the proper stratification of pancreatic cancer patients into metabolic subtypes.

Materials
Primary cultures of PDAC patient-derived xenograft models (PDXs), stablished cell lines and PDXs tumor pieces ex vivo.

Methods
Quantitative PCR (qPCR). Western Blot. *EVs isolation techniques: Ultracentrifugation, Ultrafiltration, Immunoprecipitation, CD9-affinity column, Size exclusion chromatography (SEC). Nanoparticle Tracking Analysis (NTA). Metabolomic analysis. Statistical analysis*: ANOVA and Kruskal-Wallis-H test.

Results
Starting from a previously defined metabolic signature in PDAC, we have classified both PDXs and established cell lines into glycolytic and lipogenic subgroups. The chemosensitivity profile of PDAC cell lines differs according to their metabolic phenotype, with the glycolytic subtype being more resistant to Gemcitabine. Moreover, we are comparing different EV isolation techniques corresponding to glycolytic and lipogenic metabolic profiles, to subsequently characterize enzyme and metabolite content. Although a commercial isolation kit didn´t allow us to obtain enough EVs, we were successful by using ultracentrifugation, the combination of ultrafiltration and immunoprecipitation techniques, CD9 affinity column, and SEC from both supernatants of tumor cells in culture and blood extracted from mice with orthotopic pancreatic tumors. We have classified exosome isolation techniques according to their specificity, quantity and downstream applications, based on our own results. Interestingly, enzymes and metabolites useful for the proper classification into metabolic subtypes can be detected in EVs.

Conclusion
While the results are preliminary, they open the possibility of achieving a better clinical management of this deadly disease.

OC14. The extracellular vesicle senescence-associated secretory phenotype (evSASP) in the intercellular communication during cancer chemotherapy

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Keywords: Cellular senescence, paracrine signalling, evSASP, intercellular communication

Introduction

Cellular senescence is a stable cell cycle arrest triggered by multiple stimuli. Senescent cells show a specific secretory phenotype known as SASP, which is composed by a soluble fraction (mainly formed by cytokines, chemokines and metalloproteases) and an extracellular vesicle fraction (evSASP). EV biogenesis and release is upregulated during cellular senescence, which could be related to paracrine signaling. On the other hand, senescent cells can induce paracrine senescence in neighboring cells, but the main factor that is mediating this process is still unknown.

Objective

We hypothesized that the evSASP could be the main mediator of the paracrine senescence induction.

Methods

To shed light on the role of the evSASP inducing paracrine senescence we performed conditioned media (CM) experiments in the context of chemotherapy-induced senescence in a lung cancer model. Then, we used ultracentrifugation and size exclusion chromatography methodologies to dissect the evSASP from the soluble fraction to treat recipient cells. Moreover, we blocked the biogenesis of EVs by using GW4869, a specific neutral sphingomyelinase inhibitor, to produce a CM with reduced levels of EVs to check if depletion of EVs could correlate with the alleviation of paracrine senescence.

Results

We found that CM from senescent cells could induce paracrine senescence in recipient cells, and that the paracrine senescence induction is alleviated when senescent cells cannot produce EVs due to the GW4869 treatment. In addition, we found that EVs isolated by SEC could induce paracrine senescence.

Conclusion

We demonstrated a key role of evSASP in the context of paracrine senescence induction

Acknowledgements

We want to thank all our collaborators and the funding agencies

OC15. Extracellular vesicles versus metalloprotease cleavage: a novel regulatory mechanism for NKG2D- Ligands

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National Centre for Biotechnology (CNB)

Keywords: MICA; cell trafficking; MMPs; MPIs; EVs

The system of the activating immune receptor NKG2D and its ligands is crucial for the recognition of tumours and virus-infected cells. NKG2D-ligands (NKG2D-L), which belong to two genetic families, MICA/B and ULBPs, follow different routes of cell trafficking, leading to their expression either at the cell surface on stressed cells, or as soluble proteins, after the cleavage by matrix metalloproteases (MMPs) or associated to extracellular vesicles (EVs). The release of NKG2D-L constitutes an immune evasion mechanism that can help tumour progression. Thus, MMPs could play a critical role regulating NKG2D-L expression. In fact, MMP dysregulation has been long studied in cancer biology and MMP inhibitors (MPIs) were tried as anti-cancer treatments. Previous studies from our laboratory have suggested that ULBP2, usually cleaved very efficiently by MMPs, was released in EVs when metalloprotease cleavage was inhibited. Up until now, MICA*008 is the only MICA allele consistently found associated to EVs. Here, using both CHO (Chinese hamster ovary) transfectant cell lines and cells endogenously expressing MICA, we demonstrate that several MICA alleles (MICA*002, *009 and *011) can also be recruited into EVs after *in vitro* administration of MPIs. We also studied MICA shedding after metalloprotease inhibition, which resulted in an increase in soluble levels, only after long-term exposures to MPIs (24 hours). Probably this reflects the longer time required by MICA to be recruited into EVs. We further explored the effect on NK cells of EV-enriched preparations from cells transfected with the different MICA alleles. We observed NKG2D downmodulation on NK cells incubated with EVs from MICA*002, *009 and *011 transfectants, but not with EVs from the parental cell line after MPI treatment. These data suggest that MMP inhibition can deeply affect immune regulation. All these observations provide evidence that protease dysregulation can direct several MICA alleles to EVs, affecting tumour evasion mechanisms. Our data also suggest a trafficking pathway connection between EV recruitment and proteolytic cleavage by MMPs. Whether this could be a general cell biology pathway is unknown, however, several other proteins can also be released by these two mechanisms.

Acknowledgements

KTS was a PhD studen of Research and Innovation (ANID-Chile) FONDECYT (1221031), and was awarded with a visiting scholarship (ANID fellowship; 21171812); SLB is a registered PhD student at Universidad Autónoma de Madrid and receives funding from the Spanish Ministry of Universities, being recipient of a FPU fellowship (FPU20/07300).

Work was supported by grants to MVG from the Spanish Ministry of Science and Innovation (MCIU/AEI/FEDER, EU) RTI2018-093569-B-I00 and RED2018-102411-T (Translational Network for clinical application of EV, Tentacles).

OC16. Relevance of melanoma-derived EV-DNA in tumor progression

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Keywords: Melanoma, Metastasis, Nucleic Acids, DNA Damage, Liquid Biopsy

Introduction

Extracellular vesicles (EVs) have emerged as a novel communication mechanism that tumor cells use for influencing their surrounding microenvironment, promoting cancer progression and metastasis. It has been shown that nucleic acids (NAs) can be shed into EVs and these EVs-NAs display sequences representing the entire genome and the mutational profile of tumour cells. Nevertheless, the functional consequences of this cargo have been poorly investigated.

Objectives

In this project have analyzed the amount of DNA shed in EVs (EV-DNA) and genome instability levels in a panel of low and high metastatic melanoma cell lines. Moreover, we are studying the functional consequences of the horizontal transfer of EV-DNA in recipient cells.

Methods

We have isolated sEVs and lEVs from different melanoma models by differential ultracentrifugation and analyzed both DNA and RNA content. We have measured the basal genomic instability of these cells by immunofluorescence in a High Content Screening Opera analyzing the phosphorylation of γH2AX. In order to explore the influence of EVs on recipient cells, melanocytes and human foreskin fibroblasts were treated with melanoma-derived sEVs and DNA Damage-related pathways were measured by immunofluorescence. In addition, we have engineered melanoma models to express CD9-, CD63- and H2B-GFP as well as performed experiments labelling EV-DNA to analyze the horizontal transfer between cells.

Results

We found that intrinsic genomic instability is reflected extrinsically in the amount of DNA secreted in sEVs. In addition, we have observed co-localization of micronuclei with CD63 and CD9 positive regions suggesting enucleation of DNA in EVs. Moreover, EV-shed DNA can be horizontally transferred by EVs between tumor and stromal cells. Thus, we are currently analyzing the nature of the sequences represented in EVs and the main effects in recipient cells. Finally, we have observed an increase the activation of several pathways related to EV-DNA uptake after treatment with melanoma-derived sEVs.

Conclusion

Our results suggest that genomic instability is correlated with DNA secretion in melanoma-derived EVs and metastatic potential. Additionally, uptake and horizontal transfer of EV-DNA can induce DNA damage responses. We postulate that melanoma-derived EV-DNA may promote alterations in tumor-surrounding cells, and that their effects could favor oncogenic transformation and cancer progression.

Acknowledgements

LABAE19027PEIN Fundación AECC, Beca Leonardo 2021 Fundación BBVA

OC17. Cancer intercellular communication: glycans as mediators of cellular targeting and reprograming

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Keywords: Extracellular Vesicles; Glycosylation; Gastric Cancer; Metastasis; Biomarkers

Introduction

Extracellular vesicles (EVs) are a heterogeneous group of secreted particles involved in intercellular communication, which carry large repertoire of molecules, including glycoconjugates [1,2]. The synthesis of truncated *O*-glycans is a common feature of gastric tumor cells associated with aggressiveness and poor-prognosis [3-4]. We previously described the role of these glycans in inducing increased aggressiveness *in vitro* and *in vivo*, by the activation of key signaling pathways [4], and its presence in gastric cancer cell-derived EVs [5]

Objective

We hypothesized that EVs carrying tumor-associated glycans are capable of reprograming the functional behavior of recipient cells. Furthermore, they hold the potential to be used as biomarkers in gastric cancer.

Methods

We evaluated the biological impact of differentially glycosylated EVs in recipient cell behavior and its biodistribution in mouse models. We have fully characterized the proteomic and glycoproteomic profile of these EVs.

Results

We were able to detect proteins displaying tumor-associated glycans in EVs isolated from gastric cancer cells and patient's plasma samples. Interestingly, EVs carrying different glycosylation profiles were able to induce behavioral changes in recipient cells and to drive specific organ tropism in mouse models. We have identified key cancer-related proteins with specific glycosylation being transported in cancer EVs.

Conclusion

Our work demonstrates the presence of truncated O-glycans in gastric cancer EVs isolated from patient's samples, highlighting their potential as circulating biomarkers. Furthermore, we detailed the impact of differentially glycosylated EVs in horizontal reprograming and intercellular communication *in vitro* and *in vivo*, suggesting that they may play a role during the gastric cancer metastatic process.

Acknowledgements

We acknowledge the Portuguese Foundation for Science and Technology (FCT) for funding the project EXPL/MED-ONC/1001/2021 and Bial Foundation and Ordem dos Médicos for the Maria de Sousa Award. Daniela Freitas acknowledges FCT under the stimulus of Scientific Employment, Individual Support (2020.04384.CEECIND).

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OC18. The role of glycosylated extracellular vesicles in modulating the phenotypic behaviour of recipient gastric cancer cells

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Keywords: Gastric cancer, glycosylation, glycans, migration, invasion

Introduction

Alterations of the glycosylation pathway are commonly found in gastric cancer cells and are associated with disease progression and patients' poor survival. Our group demonstrated that tumor- associated glycans could enhance cancer cell aggressiveness in vitro and in vivo by activating key signalling pathways. In addition, we were pioneer in detecting these glycans in gastric cancer extracellular vesicles (EVs).

Objective

In this study we aimed to explore the impact of EVs carrying different glycan profiles in reprogramming the phenotypic behaviour of recipient cells.

Methods

We isolated EVs from glycoengineered gastric cancer cell lines displaying different glycan profiles. EVs were isolated by ultracentrifugation and characterized by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). The presence of classic EV markers and specific glycans was evaluated by western blotting. The impact of EVs carrying different glycan profiles in modulating the phenotypic behaviour of recipient cells was evaluated by co-culture assays.

Results

EVs were successfully isolated from gastric cancer cells displaying different glycosylation patterns, which were also detected in isolated EVs. Of note, gastric cancer cells displaying tumor-associated glycans secreted larger amounts of EVs when compared to control cells. In addition, an enrichment of the tumour-related glycans was observed in EVs when compared to the parental cells and we were able to detect it at the EV membrane. Interestingly, we observed that EVs carrying these glycans were able to induce increased migration and invasion in different gastric cancer recipient cell lines, in contrast to control EVs.

Conclusion

We have demonstrated that EVs carrying different glycan profiles could reprogram the phenotypic behaviour of recipient cells. We are further evaluating other functional and molecular features occurring in recipient cells to disclose the mechanism by which EV glycosylation plays a role in cancer progression, which holds the potential for the development of novel targeted therapies.

Acknowledgements

We thank to the Portuguese Foundation for Science and Technology (FCT) that funded the projects PTDC/MEC-ONC/0491/2021 and EXPL/MED-ONC/1001/2021 and Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (FEDER) for the project Norte-01-0145-FEDER- 000051- "Cancer Research on Therapy Resistance: From Basic Mechanisms to Novel Targets", that supported the development of this work. Daniela Freitas acknowledges FCT under the stimulus of Scientific Employment, Individual Support (2020.04384.CEECIND). Cátia Ramos acknowledges FCT and i3S for the Ph.D. research scholarship (UI/BD/152090/2021).

OC19. Differential protein and glycan packaging into extracellular vesicles by 3D gastric cancer cells

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Keywords: Gastric cancer, Glycosylation, Proteomics, Extracellular vesicles.

Introduction

Alterations in the glycosylation machinery are common events associated with cancer progression that lead to the synthesis of aberrant glycan structures. These are involved in cancer hallmarks affecting patient prognosis and survival. Extracellular vesicles (EVs) shed by cancer cells carry several functional molecules, such as miRNAs, proteins, and glycans, holding modulatory capacity on the recipient cell to promote tumor progression. Our group has previously identified the presence of aberrant glycan structures in EVs released by cancer cell models. Current 3D cell culture methods have been shown to better mimic cancer in vivo phenotypes compared to monolayer cell culture. However, it remains to be clarified the effects of 3D cell culture conditions on the EV glycosylation molecular features.

Objective

The aim of this study is to evaluate the glycosylation profile and the proteomic content of EVs derived from established glycoengineered cancer cell models by applying different cell culture methodologies, namely 3D and 2D monolayer cell culture.

Methods

EVs were isolated by Ultracentrifugation followed by size exclusion chromatography from 2D and 3D conditions were characterized, and their glycosylation profile was assessed using specific glycan- binding monoclonal antibodies, and lectins. Moreover, the EV proteome was analyzed using mass spectrometry.

Results

Interestingly, our results revealed that 3D cellular architecture not only promoted EV secretion but also led to specific distinct glycosignatures of the derived EVs, for instance, affecting the expression of the tumor-associated glycan STn, the branched and bisecting N-glycans, and the level of fucosylated glycans.

Conclusion

This study highlights the importance of cell culture methodologies when studying EVs and their glycosylation profile as well as offering insight on glycosylation alterations found in EVs derived from 3D cell culture that resemble the tumor microenvironment.

Acknowledgements

This work is a result of the projects PTDC/MEC-ONC/0491/2021 and EXPL/MED- ONC/1001/2021 funded by national Funds through the Portuguese Foundation for Science and Technology (FCT). DF acknowledges FCT under the stimulus of Scientific Employment, Individual Support (2020.04384. CEECIND). Á M.M acknowledges FCT under the PhD scholarship (2020.05359.BD).

OC20. A GWC screen to unravel new molecular mechanisms involved in EV uptake

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Keywords: Genome-Wide CRISPR, EV uptake, flow cytometry

The outstanding potential of Extracellular Vesicles (EVs) in medicine deserves a detailed study of the molecular aspects regulating their incorporation into target cells.
We have performed a Genome Wide CRISPR screen by flow cytometry sorting, to identify possible molecular candidates that may regulate EV uptake.

We have used a GWC cell library in K562 cells, which includes 10 sgRNAs for each gene of the whole human genome, plus negative controls, summing up a total of 250,000 individual sgRNAs. Total EVs were isolated from SKMEL147 melanoma cell line by ultracentrifugation at 100000g. EVs were characterized by NTA, Electron Microscopy, Western blot and confocal microscopy. EVs were stained with Alexa633-C5-Maleimide, a fluorescent compound able to covalently bind to sulphur residues of proteins and glycans, and the unbound compound was removed by passing the sample through a ExoSpinColumns (3000MW). For each assay 3.6×10^{12} of stained EVs were used. 500×10^{6} cells were used per assay in order to have a 2000x coverage.

After two hours of incubation with cells, the cell culture was washed and analysed with a flow cytometer for cellular sorting. 5% high and low fluorescence populations were sorted.

Total genomic DNA was obtained, and sgRNAs sequences amplified, adding Illumina adaptors as overhangs, to perform NGS. The results point out to the COMMD and the WASH complex as possible candidates among others. Quantitative EV uptake assays previously developed in the laboratory, based on Renilla Luciferase, will be used to validate the hits found in the Genome Wide CRISPR screen.

OC21. SASP mediated by sEV as senomorphic target

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Introduction

Cells have the capacity to modulate the microenvironment through secreted molecules and factors like interleukins, cytokines, chemokines, extracellular matrix proteins, etc. Pathological process like cancer or natural process like ageing can modify this microenvironment. The ageing modifications are related with the increasing number of senescent cells. Cellular senescence is a process characterized by a cell cycle arrest, an increased β-galactosidase activity and a secretome that acquires a specific phenotype. This **senescence- associated secretory phenotype** known as **SASP** leads the microenvironment to a more pro- inflammatory state triggering with time age-related diseases. SASP has the capacity of paracrine senescence transmission. **Small extracellular vesicles (sEV)** are an important part of SASP. The regulation of the sEV biogenesis has a high potential to develop **senomorphics**, drugs that modulate SASP, to treat age-related diseases such as type II diabetes, cardiovascular diseases among others.

Objective

Find a proteomic signature of the SASP mediated by sEV to reveal pathways associated with the SASP senescence transmission through sEV.

Methodology

In this study we knock-down in mesenchymal stem cells *RELA* or *RAB27A,* genes implicated in the paracrine senescence and sEV biogenesis respectively, using CRISPR-Cas9 methodology. We compared the paracrine senescence transmission through sEV in these cells with proliferation and β-galactosidase activity assays after the treatment with senescent or non- senescent sEV. Finally, we perform the shot-gun technique Tandem Mass Tag (TMT) Systems (10-plex) to identify, quantify and compare the proteome of senescent cells with the knock- down senescent ones.

Results

The paracrine senescence transmission in the *RELA* and *RAB27A* knock-downs was inhibited. The quantitative and comparative proteomic analysis identified 4099 proteins, which of 25 were differentially regulated by the SASP mediated by sEV. These proteins are involved in the Golgi traffic and network.

Conclusion

This study provides evidence that Golgi traffic and transport are involved in the SASP mediated by sEV. This data will be useful to design new therapeutic strategies or support e actual ones against age-related diseases.

OC22. Morphological characterization of porcine seminal extracellular vesicles by Cryo-electron microscopy (Cryo-EM)

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Keywords: Cryo-microscopy; Extracellular vesicles; Morphology, Porcine; Seminal plasma.

Porcine seminal plasma (SP) contains a large number of extracellular vesicles (EVs) that are still poorly examined. This study aimed to morphologically characterize porcine seminal EVs using Cryo-EM, a technique that provides high spatial resolution and the ability to visualize EVs in their native form. SP samples (n: 6) were collected from ejaculates of fertile boars. Seminal EVs (sEVs) were isolated by a procedure combining serial centrifugations, ultrafiltration and size exclusion chromatography. For Cyo-EM analysis, 3-5 µl of sEV-samples were placed on glow-discharge grids that were frozen at -180 °C by immersion in liquid ethane. The grids were visualized with a Jeol JEM- 2200FS/CR EM operated at 200 kV. Images (n: 44) were analyzed using Image J software and sEV morphology was recorded according to size (largest diameter, nm), shape (round, oval or elongated), electron density (gray-scale), complexity (single or multiple) and the presence or absence of surface proliferations. A total of 394 particles were visualized, of which 347 (88.07%) were surrounded by a well-defined membrane and therefore considered EVs. The size of sEVs ranged from 32.56 nm to 735.89 nm. The sEVs were divided into two subtypes according to their size, termed small sEVs (S- sEVs, <200 nm) and large sEVs (L-sEVs, >200 nm). There were more ($P<0.001$) S- sEVs (80.40%, 279/347) than L-sEVs (19.60%, 68/347). Small sEVs were more ($P<0.001$) rounded (254/279, 91.03% vs 17/68, 25.00%) and less ($P<0.001$) electrodense (127.80±1.30 vs 104.50±3.01) than L-sEVs. More S-sEVs than L-sEVs showed ($P<0.001$) external protrusions (34.41%, 96/279 vs 13.23%, 9/68). In conclusion, the population of porcine seminal EVs was heterogeneous in terms of size, shape, electron density, and surface proliferations, which would indicate that they would have different origin cells, cargo and target cells. The study was funded by the Spanish Ministry of Science and Innovation (MCIN/AEI/10.13039/501100011033, PID2020- 113493RB-I00).

OC23. Identification of the Wnt Signal Peptide that directs secretion on Extracellular Vesicles

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Keywords: EVs, Wnt7a, Coatomer, Signal Peptide, Duchenne Muscular Dystrophy

Introduction

Wnt proteins are a family of secreted glycoproteins that govern developmental and regenerative processes, as well as pathological conditions. Our group found that, upon a muscle injury, Wnt7a expression is upregulated in regenerating myofibers. Moreover, intramuscular injection of Wnt7a into dystrophic mice restores muscle function. However, the high hydrophobicity of Wnt7a impedes its systemic delivery to treat all muscles. Despite the relative insolubility of Wnt7a caused by the palmitoylation required for Frizzled receptor biding, Wnt proteins actively participate in long-range paracrine signaling between Wnt-producing cells and distal recipient cells. These observations raise the controversial question of how long-range Wnt signals are regulated. We discovered that Wnt7a is highly secreted on small Extracellular Vesicles (EVs) following muscle injury to stimulate distal regeneration.

Objective

Our goal is to decipher the specific mechanism of Wnt7a-EVs secretion to manufacture highly efficient Wnt7a-EVs for systemic treatment of Duchene Muscular Dystrophy (DMD).

Methods

Using Tangential Flow Filtration, we standardized a Wnt purification protocol to isolate Wnt EVs without contamination by other sources of Wnt secretion. Structure-function analysis, BioID experiments and *in silico* interaction modeling were performed to elucidate the specific mechanism that regulates Wnt-EVs secretion.

Results

We identified the signal sequence in Wnt7a, termed the Extracellular Vesicle Signal Peptide (ESP), which directs EVs secretion, and revealed that palmitoylation or n-terminal signal peptide are not required for Wnt7a EV secretion. ESP binds specifically to Coatomer proteins through a positively charged motif to direct trafficking of Wnt to EVs. The positively charged motif and mechanism is conserved among Wnts.

Conclusions

Our studies identify the signal peptide that traffics Wnt cargo to EVs surface and elucidate a novel mechanism that facilitates long-range Wnt signaling. These results suggest that systemic delivery of Wnt7a loaded on EVs represents a potential therapy for DMD. Moreover, the use of the ESP to direct the display of cargo proteins on the surface of EVs opens the door for multiple therapeutic applications. We anticipate that our discovery will be a starting point for more sophisticated delivery systems, as well as establish the fundamental knowledge for Wnt secretion in pathological contexts.

OC24. Optimization of size exclusion chromatography for extracellular vesicle isolation

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Keywords: EVs purification, size exclusion chromatography, conditioned medium

Extracellular vesicle (EV) purification from biofluids or even from conditioned media is one of the main difficulties in the field. Multiple isolation methods have been described, but none of them can efficiently purify EVs from all contaminants present in a complex biofluid sample. Size exclusion chromatography (SEC) is a promising method, as it has been demonstrated its ability to remove soluble protein and even protein aggregates from samples. However, due to the similar size between extracellular vesicles and some lipoprotein subpopulations, SEC cannot efficiently remove all of them from plasma samples or even from conditioned medium from cell culture.

In collaboration with Agarose Bead Technologies, we are working in improving size exclusion chromatography for extracellular vesicle isolation from conditioned media and biofluid samples.

A melanoma cell line (SKMEL-147) was cultured during 5 days in presence of 5% FBS, previously EV depleted. Cell debris and apoptotic bodies were eliminated from conditioned medium before concentration of the sample. 500ul of concentrated conditioned medium were used for SEC. We collected 25 fractions of 500ul that were lately analyzed by dot blot, nanodrop and transmission electron microscopy (TEM) in order to study the separation of EVs, lipoproteins (LPPs) and soluble protein obtained with each agarose tested. First, we tested BCL agaroses, which are the most usually employed for EV isolation. 2BCL was more efficient at separating EVs from lipoproteins, whereas 4BCL recovered higher EV yields. Then we tested combined columns with 2BCL, 4BCL and 6BCL, but the combination did not improved the results when compared with simple columns.

We tested agaroses that differ in agarose bead size and crosslinking level. Both parameters affected EV isolation yields and separation from LPPs. An agarose with a smaller bead size rendered an almost perfect separation between EVs and LPPs, with a similar recovery yield of EVs.

Finally, we tested this small bead agarose with a plasma sample, in which LPP greatly outnumber EVs. With this sample, a small fraction of LPPs were present on EV fractions but most of the LPP were again separated.

In conclusion, we have found an agarose that can more efficiently isolate EVs from conditioned media in comparison with the agaroses usually employed, without significant LPPs or protein contamination. Future experiments will be directed to optimize the workflow for its use in clinical scenarios.

OC25. Comparison of Novel Combined Biophysical Approach and Traditional Physical Protocols for Isolating Small Extracellular Vesicles from Goat Milk

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Keywords: Goat milk, milk extracellular vesicles, isolation methods, differential ultracentrifugation

Introduction

Goat milk small extracellular vesicles (GoMi-sEVs) are emerging as novel diagnostic or drug delivery platforms, due to their physicochemical features and biological role related to inflammatory response. However, heterogeneity of this milk source and lack of standardized isolation protocols point the need to develop new methodologies that allow collecting stable, pure and homogeneous GoMi-sEVs.

Objective

Optimization of a novel biophysical isolation approach based on combining well-known physical isolation methods (differential ultracentrifugation, dUC, and size exclusion chromatography, SEC) and the biological treatment of milk with microbial rennet. This method was compared with two already published protocols developed for the isolation of other milk sEVs.

Methods

Goat milk was centrifuged (4 steps of 5000, 13000, 35000 and 100000 G) for removing fat, cellular debris and large vesicles. Casein was mainly eliminated by precipitation after treatment of milk with microbial rennet (0.5% v/v at 37ºC). Additional SEC was performed for purity improvement. In order to confirm the successful of our approach, GoMi-sEVs were also isolated by two other physical protocols, originally designed for the collection of sEVs from other milk sources. On one hand, following Gao´s protocol (*Pediatric surgery international*, 2019), goat milk was twice centrifuged (2000 and 12000 G) and defatted supernatant were passed by 0.22µm PES membrane filter. GoMI-sEVs were precipitated at 100000 G. On the other hand, Izumi protocol (*Circulating MicroRNA*, 2013) was also applied by centrifuging goat milk at 1200 G and twice 21500 G. Supernatant was gradually filtered by 0.65, 0.45 and 0.22µm PES membrane filter, and GoMI-sEVs were precipitated at 100000 G. Isolated vesicles from the three methodologies were characterized and compared by Transmission Electron Microscopy (TEM), Coomassie assay, Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA), Western Blot and Zeta Potential.

Results

GoMi-sEVs isolated following our biophysical optimized protocol were positive for CD81 and TSG101, showed negatively surface charge (-23.93±2.10 mV), high purity, homogeneity (PdI:0.08) and exhibited consistent size measurements between NTA (125.30±5.60 nm) and DLS (128.14±4.13 nm). NTA also revealed a high particle concentration ($6.56\pm2.25\cdot10^{11}$ particles/mL), corresponding to 3.77±0.81 mg/mL of protein content. In contrast, TEM showed aggregation and residual protein contamination in samples isolated following the Gao and Izumi protocols. In both cases, samples were negative for CD81 biomarker and presented lower surface charge than the vesicles isolated by the biophysical approach, probably due to the residual proteins attachment to the vesicles membrane. Regarding the morphology, GoMi-sEVs from Gao and Izumi protocols presented higher size than the vesicles isolated by our optimized procedure, measured by DLS and NTA, and also less particle concentration although exhibiting large amount of protein content due to the presence of impurities.

Conclusions

We present a novel isolation protocol for the collection of pure and stable sEVs from goat milk, by combining physical and biological techniques. The success of this methodology was validated through the comparison of the physicochemical properties of vesicles isolated from this method and two others approaches design for the collection of bovine and human milk extracellular vesicles.

OC26. Nanosystems based on silver sulphide nanoparticles encapsulated in goat milk small extracellular vesicles for their use as probe in optical imaging.

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Keywords: Small extracellular vesicles, silver sulphide nanoparticles, near-infrared, optical imaging.

Introduction

In the last years small extracellular vesicles (SEVs) are becoming a potential delivery system (DS), due to their liposome-like structure, robustness and pharmacokinetic properties. At this point, SEVs represent a wonderful "Trojan horse" for smaller nanoparticles (NPs) or molecules to improve their pharmacokinetic properties *in vivo* after their exogenous administration. Through the encapsulation of smaller NPs in natural SEVs, we are able to increase their circulation life and optimize the uptake and accumulation in target tissues and organs, based on the biological role of these SEVs in oncological and inflammatory processes. One of the emerging NPs used for optical imaging are silver sulphide (Ag2S) NPs, due to their fluorescence emission properties within the second near-infrared region (NIR-II) spectrum (1000-1400 nm), in which self-fluorescence of tissues is reduced to a minimum. However, as synthetic nanosystems of small size, they might be rejected, engulf or excreted rapidly by the organism.

Objective

This work presents the chemical optimization of the encapsulation of NIR-II Ag2S NPs into the SEVs structure, taking advantage of the properties of these vesicles. Different tests included different surface charges (positive, negative and neutral) and different sizes (~30nm and ~10nm) of those Ag2S NPs. Further in vivo evaluation of the novel Ag2S-SEVs nanoconjugates was performed to assess their pharmacokinetic profile.

Methods

Ag2S NPs were synthesized by thermal decomposition of the silver-sulfur precursor (AgDDTC). The final product was treated by sonication, transferred to water and functionalized with different HS-PEG (MW = 750 $g \cdot mol^{-1}$) polymers conferring different superficial charges (positive, negative and neutral). The encapsulation was performed at 37ºC in presence of the detergent saponine. 50µg of SEVs were used for each encapsulation of negative, neutral and positive NPs. Purification was performed with size exclusion columns. *In vivo* imaging evaluation was performed in a melanoma animal model (B16/F10) with an InGaAs camera coupled with an 850 nm longpass filter.

Results

The use of different Ag2S NPs confirmed a charge dependent effect in the encapsulation. The negative Ag2S NPs were unstable in encapsulation conditions and only positive and neutral NPs were encapsulated. In the case of SEVs-neutralNPs DLS showed a lower polydispersity index (0.379 for positives and 0.2 for neutral) and just one population, and TEM images showed low population of free NPs. In the case of SEVs-positiveNPs, although encapsulation was successful, there was a high presence of unencapsulated NPs (impurities). The *in vivo* assay confirmed the optical properties of the nanoconjugates although self-fluorescence from the hair of the mouse limited the quantification of the signal. Nevertheless, *ex vivo* imaging of the organs confirmed the hepato-biliar metabolism, typical for these EVs.

Conclusion

We have successfully carried out the encapsulation of positively and neutrally charged Ag2S NPs into milk SEVs. *In vivo* validation confirmed the optical properties of the new SEVs-NPs nanoconjugates.

OC27. Engineering Milk Small Extracellular Vesicles by Click Chemistry

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Keywords: Pretargeting, small extracellular vesicles, functionalization, drug delivery system, oncology

Introduction

The use of extracellular vesicles as drug delivery platforms, especially small extracellular vesicles (SEVs), has increased in recent years due to their amphiphilic structural properties and nanometric size. More effective therapy has emerged in recent years the so-called "EVs engineering" based on surface chemical modification has gained strength, improving their pharmacokinetics and their bioavailability in target cells. In this work, we present for the first time the use of "click chemistry" for the specific functionalization of SEVs with clickable fluorophores to selective redirection of these vesicles to target processes. Bioorthogonal Click Chemistry presents extraordinary advantages over other click reactions such as fast kinetic, high specificity, physiological conditions, and, most importantly, direct *in vivo* application due to the absence of toxic components such as copper.[1,2] In searching for potential *in vitro* and *in vivo* application of our approach for tumoral processes targeting, we have chosen as selective target the intercellular adhesion molecule-1, which is located on the cell surface (ICAM-1).

Objective

We present for the first time the chemical validation of the click chemistry reaction (Syd–DBCO-based) in SEV whit the fluorophore SCy5 and the further proof-of-concept in vitro validation as a potential approach for the specific targeting of cancer cells employing a-ICAM-1-DBCO/Syd-Exo-SCy5 system in a cervical carcinoma cell line.

Methods

For click reaction validation, 100µg of goat milk SEVs were isolated by ultracentrifugation and functionalized with Sydnone-NHS ester (Syd, 10µL, 333mM) overnight (ON) at RT and pH=8.5. After that, we added DBCO-SCy5 (25µL) and mixed it for 2h at RT. The purification was performed using PD-10 and Syd–DBCO-SEVs were characterized by HPLC, DLS, Fluorimetry, and TEM. For in vitro evaluation, 100µg of milk SEVs were directly fluorescent labeling with Sulfo-Cyanine5 (SCy5, 1µL, 16.9 mM) for 90 min at 4ºC and pH 8.5; and further functionalized with Syd (10µL, 333mM) for ON at RT. These SEVs were characterized by HPLC and NTA. On the other hand, 500 mg of a-ICAM-1 were functionalized with DBCO-PEG4-NHS (DBCO, 5 mL, 2.2 mM) for 4h at RT and pH=8.5 and characterized by HPLC and MALDI-TOF. To validate the potential of the DBCO-a-ICAM-1/Syd-SEVs system, Syd-SEVs-SCy5, and a-ICAM-1-DBCO were mixed for 15 min at RT and the final product was characterized by HPLC and NTA. Both products were purified by PD-10. In vitro assays were performed in ICAM-1 positive cell line (HeLa), measuring their fluorescence by flow cytometry and confocal imaging after click reaction.

Results

The chemical validation of the new click methodology was confirmed by the appearance of a bluish color in the sample due to the binding of the fluorophore. The physicochemical characterization of the functionalized SEVs by HPLC, NTA, DLS and TEM showed that our chemistry did not modify the morphology and size of the SEVs (158.4 nm and cup shape morphology). Subsequent *in vitro* validation confirmed the specific targeting of our vesicles in HeLa tumor cells by confocal microscopy and flow cytometry, with absence of binding in the case of vesicles without click reagents.

Conclusions

We have validated for the first time the use of click chemistry on SEVs for their surface enrichment with fluorophores and their specific targeting of HeLa tumor cells.

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OC28. Large-scale Serum-free production of Extracellular Vesicles (EVs) from immortalized Wharton's jelly Mesenchymal Stromal Cells (iWJ-MSC) using hollow fiber bioreactors

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Keywords: MSC-EV, 3D-culture, Serum-free EVs, Exosomes, Bioreactor

Background

Mesenchymal stromal Cells (MSC) have been extensively researched due to their immunomodulatory and antifibrotic functions, which are partially mediated by secretion products, with special interest on extracellular vesicles (EVs). MSC-EVs as therapeutic agents possess several advantages over cellular therapies and are proposed as therapeutic tools in a plethora of human inflammatory diseases. However, large scale high-throughput EV production is still a challenge.

Objective

In this work, we aim to create an immortalized MSC cell line to generate and purify MSC-EVs by size exclusion chromatography (SEC) in a scalable and clinically translational manner.

Methods

To generate immortalized MSC cell lines we transfected Wharton's Jelly MSC (iWJ- MSC) with hTERT. iWJ-MSC were cultured in 10% FBS αMEM medium in 2D cell culture flasks to analyse doubling time, stability and compare MSC characteristics. iWJ-MSC-EVs were isolated by SEC from 48h conditioned medium. Furthermore, we used hollow fiber bioreactors to 3D-culture immortalized MSC in synthetic xeno-free medium and isolate iWJ-MSC-EVs by SEC. The following EV parameters were analysed: tetraspanin and CD90 expression by bead- based flow cytometry, protein quantification by BCA, negative markers (calnexin) by dot blot, EV imaging by cryo-TEM, and T cell suppression assays to asses immunomodulatory potential. **Results:** iWJ-MSC were comparable in terms of phenotype to primary cultures while presenting unlimited proliferation capacity, up to 175 days of follow-up (maximum period followed up). iWJ-MSC-EVs from primary and immortalized cell cultures suppressed T cell proliferation, and did not differ in the EV features tested. Thirty million iWJ-MSC were cultured for 5 weeks in a hollow fibre bioreactor and glucose, ammonia, and LDH were monitored to confirm the metabolic activity and survival of the cells. We obtained 24h conditioned medium 5 days every week which was concentrated and frozen until EV isolation. Isolated bioreactor EVs were comparable to 2D culture-isolated EVs.

Conclusions

MSC-EVs can be generated and isolated from immortalized cell cultures with no significant alterations in standard cell and EV parameters. In addition, iWJ-MSC-EV production is scalable for downstream clinical application by culturing MSC in commercially available bioreactors with synthetic serum free culture media in a clinically translational manner.

Acknowledgements

This work was supported by Instituto Carlos III project PI20/00097, integrated in the National R + D + I and funded by the ISCIII and the European Regional Development Fund. SGG is supported by the Catalan Health department ("Departament de Salut") in receipt of a grant from PERIS-PIF-Salut (SLT017/20/000158), and MF is supported by ISCIII (MS19/00018), co-funded by ERDF/ESF, "Investing in your future".

OC29. Design of a large-scale and GMP-compliant MSC-EV production process for clinical testing

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Keywords: GMP, Mesenchymal stromal cells, Immunomodulation, Production optimization, Clinical-grade

Introduction

Mesenchymal stromal cell-derived extracellular vesicles (MSC-EV) have a growing clinical interest as a regenerative and immunomodulatory therapy alternative for a plethora of diseases. For that, large-scale production methods complying with good manufacturing practices (GMP) are needed. The adaptation of lab-scale methods to work in clean room facilities and setting QC points can be challenging.

Objective

To set up a clinical-grade MSC-EV production workflow.

Methods

We have designed a clinical-grade, GMP-compliant MSC-EV production process ensuring umbilical cord (Wharton's Jelly) MSC-EV stringent purification by tangential flow filtration (TFF) followed by size-exclusion chromatography (SEC) from the supernatant of clinical-grade MSC-WJ cultures, formerly a waste product. Cell growth, viability, phenotype and potency were monitored for each batch. We designed identity, purity and potency assays for MSC-WJ-EV batches, and analysed their reproducibility and consistency to establish GMP-compliant analytical methods. Safety and biodistribution was tested by intravenous (*i.v.*) administration in mice.

Results

We established acceptance ranges of process parameters and GMP-compliant protocols, and defined EV product elution in a semi-automated SEC elution process. Purified EV consistently expressed EV (CD9, CD63, CD81), MSC (CD44, CD90, CD105) and potency surrogate markers (CD24, CD59, CD73), while lacked CD31, CD45, HLA-ABC and HLA-DR, accordant to MSC-WJ phenotype. Size and morphology were confirmed by cryo-electron microscopy (median 130 nm (95% CI 142-151 nm) and EV protein and RNA yields were correlated to cell numbers and culture volumes. In terms of potency assays, we optimized T cell proliferation and CD73 enzymatic activity assays to check for immunomodulatory activity for each batch. After *i.v.* infusion in healthy mice, EV were found rapidly in lungs, then liver, kidney and spleen, but no signal was found in brain nor gonads. Increasing doses of MSC-WJ-EV proved safe even after repeated doses in mice, and no teratogenicity was observed. Efficacy studies are in process.

Conclusion

We have adapted the lab-scale process and protocols of MSC-WJ-EV production to a large- scale, GMP-compliant workflow with clean room-friendly equipment and materials. After validation, this platform can be scaled-up and assessed in relevant clinical testing.

Acknowledgements
MICINN (PID2019-110137RB-I00 and PRE2020-095357); Instituto de Salud Carlos III (ICI20/00135 and Red RICORS-TERAV RD21/0017/0022) as a part of the Plan Nacional de I+D+I, and co-funded by ISCIII-Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER) and AGAUR (2017-SGR-483).

OC30. Platelet-derived extracellular vesicles show therapeutic potential on a 3D tendon disease model

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Keywords: platelets; tendon; tissue engineering; regenerative medicine; tendinopathy

Introduction

Tendon diseases are common clinical problems that can dramatically affect the quality of life of individuals across the demographic spectrum. Current clinical approaches do not tackle the etiology of the disease, underlined by an unresolved inflammatory scenario, hypercellularity, neovascularization, and a dysregulation of native extracellular matrix (ECM) composition. Extracellular vesicles (EVs) are being considered as very attractive therapeutic agents to trigger repair/regenerative processes in injured tissues. Herein, the therapeutic potential of EVs derived from platelets was evaluated using an engineered 3D tendon disease in vitro model.

Methodology

First, bioengineered tendon disease models consisting of electrospun isotropic nanofibrous scaffolds coated with hydrogels encapsulating human tendon-derived cells (hTDCs) were produced. Platelet-derived EVs populations were isolated by centrifugation at different speed of platelet lysate. Then, EVs were added to tendon disease model culture media, and their influence in cells phenotype, ECM remodeling and cytokines expression was assessed over culture time.

Results

As expected, after 14 days of culture, the isotropic nanofibrous architecture induced a disease-like phenotype in hTDCs. Although EVs did not have a remarkable influence in hTDCs morphology, these were able to influence their biological response. Interestingly, the addition of platelet EVs reestablished the expression of tendon-related markers in diseased hTDCs and decreased the expression of osteogenic and fibrotic markers. Moreover, EVs increased the expression of different ECM components such as COL1A1 and COL6A1, and the expression of MMP-3, important factors in the balance between the synthesis and degradation of tendon ECM. Moreover, the presence of EVs was found to modulate the inflammatory response, as demonstrated by an increase in the mRNA levels of anti-inflammatory mediators, like IL-4, which might contribute to blunt the inflammatory processes occurring in damaged tissue.

Conclusions

Overall, we show that platelet-derived EVs have a positive influence on tendon cells cultured on a disease-like in vitro model, not only by increasing the expression of healthy tendon cells markers and promoting ECM remodeling, but also by increasing the expression of anti-inflammatory cytokines. The beneficial effects of these vesicles are worthy to be explored in further studies to provide more insights on how EVs interact with tendon cells, becoming a promising therapeutic tool for tendon injuries recovery.

OC31. Effect of miR-21 into mesenchymal stem cells -derived extracellular vesicles behaviour on inflamm-aging.

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Keywords: Extracelular vesicles, Mesenchymal stem cells, miR-21-5p, Syndecan-1.

Introduction

Extracelular vesicles (EV) which include exosomes and small EV, contain multiple bioactive molecules such as miRNA. Recent evidence demonstrated that EV are key players on cell-cell communication processes, influencing cellular activities and regulating the expression of target genes in the receptor cells.

Material and methods

Mesenchymal stem cells (MSC) from human umbilical cord stroma were stable modified using lentivirus transduction to inhibit miR-21-5p. Shotgun proteomic analysis was performed in the MSC-derived EV to check the effect of MSC-miR-21 inhibition in their protein cargo. We studied the paracrine effect of EV by culturing MSC-miR-21^{-} to which MSC-miR-21^{-}-derived EV (2×10^{7} EV) or MSC- miR-21^{+}-derived EV (2×10^{7} EV) were added. The effect of these EV on SASP expression was studied by RT-PCR.

Results

A total of 1.861 proteins were identified with at least a unique peptide and were able to establish the proteomic profile of miR21^{-} -EV vs unmodified EV. Syndecan-1 was the most decreased protein into miR21- extracelular vesicles. This result was orthogonally validated in mesenchymal stem cells miR-21^{-} by RT-PCR using Tagman probes. NTA revealed that MSC-derived EV production was decreased (25%) in MSC with its miR-21 inhibited relative to MSC-mimic-derived EV Syndecan-1 is involved into inflammation and EV production. The interaction between miR-21 and Syndecan-1 have been demonstrated through functional experiments through Synstatin, an inhibitor of Syndecan-1. MSC-miR21^{-}-derived EV were found to produce a statistically significant inhibitory effect on SASP expression and in the opposite way, these cells increased their SASP expression statistically significantly when were treated with MSC-miR-21^{+}-derived EV.

Conclusions

This work demonstrates the importance of miR-21 on the density of EV production and its role in senescence through Syndecan-1. Besides, all these results indicate that Syndecan-1 is a new target for anti-inflammatory therapy.

Acknowledgements

JM was funded by "Programa Talento Senior" GAIN-Xunta de Galicia. JFL was funded by Xunta de Galicia, grant number ED481D-2021-020. MCA and RBM have been funded by Instituto de Salud Carlos III through the project "PI20/00497" .Co-funded by European Regional Development Fund "A way to make Europe".

OC32. Manipulation of microRNA distribution with EXO- and CELLmotifs as a novel approach against atherosclerosis

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Keywords: microRNAs, EV sorting, atherosclerosis, cell communication

Introduction

Small extracellular vesicles (sEVs) have emerged as a novel mode of cellular communication in which microRNAs produced and released from one cell are taken up by cells at a distance where they can lead to changes in gene expression and cellular function. We recently identified short sequence motifs in microRNAs that promote their sorting into sEVs (EXOmotifs) or their retention in the producing cell (CELLmotifs). Interestingly, adding an EXOmotif to a microRNA can increase its delivery and ability to inhibit target genes in distant cells (Garcia-Martin et al, *Nature* 2022).

Objective

In this follow-up study, by using EXO- and CELL-motifs we aim to enhance microRNA sEV loading and delivery from an accessible donor tissue (i.e. adipose tissue) to a less accessible target tissue (i.e. the liver). This will be done in the context of atherosclerosis, which is the leading cause of cardiovascular disease and death worldwide, including Spain.

Methods

By in-silico target prediction tools and mimic transfection, we searched for the most effective microRNA to target Proprotein convertase subtilisin/kexin type 9 (PCSK9), a major regulator of LDL-receptor. In vivo experiments were performed by intravenous administration of an adenovirus vector to achieve microRNA overexpression. In vitro sEV-mediated communication was assessed by transwell systems.

Results

We identified a conserved microRNA, miR-763, with a potent targeting capacity on PCSK9, which was comparable to a current siRNA drug, Inclisiran, which has full-complementarity for its target. As a proof of its in vivo action, overexpression of this microRNA in the liver of mice leads to reduced PCSK9 levels and increased LDL-receptor. In order to boost sEV loading of miR-763 from adipose tissue, we incorporated EXOmotifs or removed a CELLmotif from its sequence. This manipulation of miR-763 sequence leads to enhanced loading of this microRNA in the sEV released by adipocytes. Lastly, hepatocytes coincubated with donor adipocytes overexpressing these new "sEV-prone" versions of miR-763 show a reduction of PCSK9 expression, indicating enhanced delivery at a distance.

Conclusion

These data suggest that artificial manipulation of microRNA sequence by adding or removing EXO- and CELLmotifs, respectively, might be a suitable strategy to enhance sEV-mediated microRNA delivery from one donor tissue to a target tissue to regulate a gene altered in disease.

Acknowledgements

This work was supported by the following grants: NIH-R01DK082659, DFG-GA2426/1-1 and FAPESP-2017/10179-9.

OC33. Placental MSCs and their exosomes as vehicles for the Na/I symporter (hNIS): A new agent for gene therapy and diagnostic.

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Keywords: Cancer, radiotherapy, imaging, metastasis, NIS

Introduction

The Na/ I symporter gene (hNIS) is expressed in the thyroid and allows the accumulation of iodine where it is expressed. Moreover, it is widely used (i) as a reporter gene for molecular imaging (when the positron emitter isotope is I^{124} for PET or Tc^{99} for SPECT) or (ii) as a therapeutic gene for cancer therapy, mediated by the accumulation of I^{131}. An unresolved challenge is how to direct this gene specifically to the tumoral area.

Objectives

As hNIS is expressed at the placental tissue (because it transfers iodine to the foetus from the maternal blood), and MSCs and their exosomes target tumoral tissues, *in this work we decided to study whether placental MSCs and their derivatives (exosomes) could reach tumoral areas, transferring NIS potential to diagnose and treate tumours at the time*.

Methods

We used a number of primary and metastatic models to check MSCs targeting to the tumoral areas, and IVIS, SPECT/CT, MRI and radiotherapy to test the NIS potential and its theragnostic effect.

Results

In this work we have checked whether placental MSCs (1) express hNIS endogenously and therefore transfers the imaging and therapeutic potentials when administered with radioactive iodine (2) are capable to reach the tumoral areas when they are intravenously injected due to the tumoral tissues extravasation, with great sucess. *Placental MSCs exosomes were capable to target even metastatic distal areas, transferring NIS potential. Therefore tumors could be diagnosed by PET and treated with radiotherapy almost at the same time.*

Conclusions

Our findings highlight the possibility of the use of endogenous NIS expression as a therapy and opening a wide range of new possibilities to treat and diagnose cancer in parallel.

Acknowledgements

This research was supported by Instituto de Salud Carlos III (ISCIII) (PI19/01007 and DTS21/00130).

OC34. Design of glycolipid-functionalized extracellular vesicles for their selective targeting to dendritic cells

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Keywords: Surface functionalization, glycolipids, dendritic cells targeting, vaccination.

Introduction

Current cancer therapies have reached a plateau because new drugs that follow the traditional treatment pathway still have severe systemic toxicity. Moreover, they are not able to cause higher cancer cell mortality due to the acquired resistances that tumours generate towards chemotherapy drugs. As an alternative, several studies have proposed immunotherapy, which involves activating the immune system selectively, so that it recognises tumours as threats to be killed and can thus fight these cancer cells. Of the different types of immunotherapies, therapeutic vaccines aim to awaken the patient's immune system. They are based on the administration of a tumour antigen once the disease has already appeared, with the intention of activating an immune response, especially of the cytotoxic lymphocytes that are responsible for eliminating tumour cells. Encoding antigens with nucleic acids is advantageous because of the self-adjuvant property of the genetic material and the ease of exchanging the encoded antigen without significantly changing the formulation. However, nucleic acids are very labile molecules and unable to penetrate cells. Therefore, they need a vector that protects them and transports them specifically to the target cells, the antigen presenting cells.

Objective

the aim of the present work is to develop new nano- vectors based on lipid systems that encapsulate the antigenic genetic material and are selectively vectorised towards the antigen presenting cells.

Methods

Of the various existing vectors, extracellular vesicles, nanosystems naturally released by all human cells that already contain genetic material and have intercellular communication functions through gene material and proteins transference, have been chosen and compared with liposomes as artificial lipid systems.

Results

It has been designed a new type of vectorization of extracellular vesicles, using cholesterol- and mannose-based glycolipids, which are expected to integrate into their lipid membrane and expose mannoses on the surface, so extracellular vesicles can be selectively targeted to antigen presenting cells. Two synthetic routes have been developed to obtain two glycolipids, which differ in the presence or absence of a linker between the two units. Then, the methodology of incorporation of these glycolipids into the vesicles has been established and their integration has been confirmed by confocal microscopy. Finally, their functionality has been studied by cell uptake assays, demonstrating a cell uptake efficiency of more than 50% when the glycolipids have been added to the extracellular vesicles.

Conclusions

this work confirms that extracellular vesicles selectively targeted to antigen presenting cells are promising nanosystems to become the nucleic acid- based therapeutic vaccines of the near future.

Acknowledgements

This work was supported by MINECO/FEDER (grants, SAF2015-64927-C2-2-R; RTI2018-094734-B-C22 and PID2019-104350RB-100).

OC35. Enhanced production of immunomodulative MSC-EV by 3D bioreactor culture in chemically-defined medium

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Keywords: Bioreactor, Immunomodulation, Production optimization, Chemically-defined

Introduction

There is a growing interest in the clinical therapeutic development of Mesenchymal Stromal Cell-derived Extracellular Vesicles (MSC-EV) due to their regenerative and immunomodulatory potential. For that, we aim for their large-scale production, focusing on 3D-culture. Nevertheless, we need to study if this method impacts MSC-EV functional activity.

Objective

This study aims to test 3D culture of Wharton's Jelly MSC (MSC-WJ) for a scaled-out production of MSC-WJ-EV.

Methods

We lead a comparative study of the production efficiency of EV derived from clinical-grade MSC- WJ cultured either in scaled-up 2D cell culture flasks or scaled-out in 3D microcarriers-based bioreactors using either human serum-supplemented (SER) or chemically-defined medium (CHEM). Cell growth, viability and phenotype were monitored for each condition and MSC-WJ-EV were purified by size exclusion chromatography (SEC) after concentration by ultrafiltration or tangential flow filtration. Next, MSC-WJ-EV were characterized in terms of identity, purity, morphology, yield and functional activity.

Results

MSC-WJ expressed a stable phenotype across the different culture settings. Cell recovery was lower in both CHEM conditions as well as in both 3D cultures, although aggregate formation difficulted cell harvest in the latter. Purified MSC-WJ-EV were recovered from each condition, and bead-based flow cytometry confirmed the stable expression of both EV (CD9, CD63, CD81) and MSC (CD44, CD73, CD90, CD105) markers in all conditions. A significant decrease in EV size was observed after 3D-CHEM culture with a delay in SEC and confirmed by cryo-electron microscopy (median 117nm (95%CI 111-120nm) in 2D SER and 100nm (95%CI 95-103nm) in 3D CHEM; $P<0.0001$). CHEM-based cultures yielded higher EV protein and RNA quantities relative to cell numbers and culture volumes. In terms of immunomodulatory activity, MSC-WJ-EV derived from 3D cultures showed greater and consistent inhibition of T cell proliferation as well as CD73 enzymatic activity (60% 2D *vs* 100% 3D positive batches).

Conclusion

While 2D and SER culture conditions yielded higher cell production, 3D-CHEM culture conditions allowed harvests of smaller-sized MSC-WJ-EV with superior immunomodulatory potential.

Acknowledgements

MICINN (PID2019-110137RB-I00 and PRE2020-095357); Instituto de Salud Carlos III (ICI20/00135 and Red RICORS-TERAV RD21/0017/0022) as a part of the Plan Nacional de I+D+I, and co-funded by ISCIII-Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER) and AGAUR (2017-SGR-483).

OC36. Prevention of alpha-synuclein pathology in gut and cord by shRNA-MC delivered by RVG-extracellular vesicles

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Keywords: Parkinson's disease, alpha-synuclein, gene therapy, RVG-exosomes, Enteric Nervous System

Introduction

The development of new therapies to slow down or halt Parkinson's disease (PD) progression is a healthcare priority. A key pathological feature of PD is the presence of alpha-synuclein aggregates and transmission of this pathology between neurons plays a central role in disease progression. Current data suggest that the neurodegenerative process starts in the enteric nervous system (ENS) and spreads via the vagus nerve to the lower brainstem, a process that precedes degeneration of the dopaminergic neurons. Consequently, strategies to decrease the expression of alpha-synuclein in the ENS and central nervous system (CNS) will be an attractive approach to prevent disease progression at pre-clinical stages. We designed shRNA minicircles (shRNA-MC), with the potential for prolonged effectiveness, and used RVG-extracellular vesicles (RVG-EVs) as vehicle for specific delivery into the CNS and ENS.

Objective

We aimed to assess the effect of shRNA-MC RVG-EVs to down-regulate alpha-synuclein expression in organs affected in pre-clinical stages of PD, as intestine and cord.

Methods

RVG-EVs were isolated from dendritic cells transfected to express the RVG-peptide and loaded with shRNA-MCs by electroporation. RVG-EVs containing shRNA-MC were injected intravenously in a progressive alpha-synuclein preformed fibrils mouse model. Alpha-synuclein down-regulation in cord and distal intestine was evaluated after 90 and 120 days by qPCR and Western blot.

Results

The treatment was able to reduce the alpha-synuclein mRNA and protein levels in cord and distal intestine.

Conclusion

Our results support the potential use of anti alpha-synuclein shRNA-MC RVG-EVs as a therapy to delay or halt the PD pathology progression.

OC37. UHPLC-MS/MS-based Metabolomics reveals differences on Extracellular Vesicles secreted by obese hepatocytes, and their effects on adipocyte metabolism

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Keywords: Metabolomics, EVs, Metabolic syndrome, Lipidomics, Liver

Introduction

The composition of extracellular vesicles (EVs) is altered in many pathological conditions, and their molecular content provides essential information on features of parent cells and mechanisms of crosstalk between cells and organs. Metabolic Syndrome (MetS) is a cluster of clinical manifestations including obesity, insulin resistance, dyslipidemia and hypertension that increases the risk of cardiovascular disease and type 2 diabetes mellitus. We investigated the crosstalk between liver and adipocytes by characterising EVs secreted by primary hepatocytes isolated from Zucker rat model, and studied the effect they have on 3T3-L1 adipocytes.

Methods

By using differential ultracentrifugation, density gradient, NTA, cryo-TEM, Western-blotting, Raman spectroscopy, proteomics and lipidomics we perform a deep molecular characterisation of EVs secreted by lean and obese primary hepatocytes. In addition by using radio labelling we analyse the bio distribution of these vesicles, and by using UHLPC-MS/MS metabolomics we study the effects that these hepatocytes-secreted vesicles produce on adipocytes metabolism.

Results

We found that steatotic hepatocytes secrete EVs with significantly reduced exosomal markers in comparison with their lean counterpart. Moreover, proteomic analysis revealed that those EVs reflect the metabolic state of the parent cell in that the majority of proteins upregulated relate to fat metabolism, fatty acid synthesis, glycolysis, and pentose phosphate pathway. Lipidomic analysis showed that EVs released by a fatty liver carry different lipid signature. Triacylglycerides, especially species having longer fatty acid chains, were observed enriched in obese compared to lean hepatocytes. In addition, hepatocytes-secreted EVs influenced lipolysis and insulin sensitivity in recipient 3T3-L1 adipocytes. Untargeted metabolomic analysis detected alterations in different adipocyte metabolic pathways in cells treated with hepatic EVs.

Summary/Conclusion

Our work showed that steatosis has a significant impact in the amount and composition of EVs secreted by hepatocytes. Moreover, our data point to the involvement of hepatic-EVs in the development of pathologies associated with Metabolic Syndrome.

OC38. Cortico-striatal activation via physical training modulates the proteomic content of striatal extracellular vesicles in a model of Huntington's disease

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Keywords: Extracellular vesicles, cortico-striatal activation, physical training, Huntington's disease, proteomics

Huntington's disease (HD) is a neurological disorder caused by a CAG expansion in the Huntingtin gene (*HTT*). HD pathology mostly affects to striatal medium spiny neurons and results in an altered cortico-striatal function. Recent studies report that exercise training and cortico-striatal stimulation attenuate the neuropathology in HD, resulting in an amelioration of some motor and cognitive functions. Extracellular vesicles (EVs) are released into the circulation during physical training as potential means for inter-tissue communication.

The aim of this study is to investigate whether the activation of the cortico-striatal pathway via physical training modulates the cross-talk between cells in the striatum via EVs, in the R6/1 mouse model of HD.

We stimulated the cortico-striatal pathway of the WT and R6/1 mice exposing them to rotarod motor training. One hour and a half after training, brains were dissected out and EVs were isolated from the striatal tissue by differential ultracentrifugation. Then, EVs were purified by size exclusion chromatography and EVs- enriched fractions per animal were pooled together. EVs were then characterized by nanoparticle tracking analysis and proteomics.

We observed alterations in the small exosome population in the R6/1 EVs when compared to WT EVs. Moreover, R6/1 EVs recapitulated some signaling and energy deficiencies already described in HD at the proteomic level. In R6/1 samples, physical training induced a restoration of EVs amount and protein content. Furthermore, training seems to modulate crucial pathways of metabolism and neurodegeneration.

All these data put striatal- derived EVs in the spotlight to understand the signaling and metabolic alterations in neurodegenerative diseases and physical exercise as a possible therapeutic approach.

This work was funded by a FPU grant from the Spanish Ministry of Science, Innovation and Universities (MICIU) (grant #FPU18/00194) and the MICINN project # PID2020-119236RB-I00.

OC39. Saturated fatty acid-enriched extracellular vesicles mediate communication between liver cells in metabolic associated fatty liver disease.

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Keywords: MAFLD, lipotoxicity, extracellular vesicles, inflammation, insulin resistance

Introduction

Metabolic associated fatty liver disease (MAFLD) is the most common chronic liver disease worldwide with no yet approved pharmacotherapy. Lipotoxicity triggers MAFLD progression due to accumulation of toxic lipids species as saturated free fatty acids (SFAs) in hepatocytes, and may activate pro-inflammatory pathways and release extracellular vesicles (EVs). Patients with MAFLD have also increased levels of circulating EVs. EVs have also been identified as messengers in the interactome between liver cells, however, their specific role in the paracrine communication hepatocytes-macrophages related to inflammation-associated insulin resistance during MAFLD remains to be studied.

Objective

To study the impact of hepatocyte- or circulating-derived EVs secreted under MAFLD conditions in liver inflammation and hepatocyte insulin signaling.

Methods

Primary hepatocyte-derived EVs (PH-EV) were characterized and analyzed by lipidomics. PH-EV were added to mouse Kupffer cells (KCs) to monitor internalization, pro-inflammatory effects and modulation by a TLR4 inhibitor. Conditioned media (CM) from PH-EV-loaded macrophages were added to hepatocytes to analyze insulin signaling. Intravenous PH-EV injections in mice were conducted to study biodistribution, liver inflammation and hepatocyte insulin signaling. The crosstalk macrophages-hepatocytes was also evaluated using circulating EVs from mice and humans with MAFLD.

Results

Quantity of EVs released by hepatocytes was increased under MAFLD conditions. Hepatocyte-derived lipotoxic EVs were internalized by macrophages through the endosomal pathway and induced pro-inflammatory responses that were ameliorated by TLR4 inhibition. CM from macrophages/KC loaded with hepatocyte-derived lipotoxic EVs impaired insulin signaling in hepatocytes. Both lipotoxic EVs and recipient macrophages were enriched in palmitic (C16:0) and estearic (C18:0) SFAs, well-known TLR4 activators. Upon intravenous injection, hepatocyte-derived lipotoxic EVs reached KCs and triggered a pro-inflammatory response in the liver monitored by JNK phosphorylation, NF-kB nuclear translocation and pro-inflammatory cytokine expression. Again, this effect was attenuated by TLR4 inhibition. Macrophage inflammation and following hepatocyte insulin resistance was also induced by circulating EVs from mice and humans with MAFLD.

Conclusions

SFAs are essential components in the cargo of lipotoxic hepatocyte-derived EVs, that are transferred to macrophages and, via TLR4, trigger liver inflammation and insulin resistance in hepatocytes. Evidences of this macrophage-hepatocyte interactome have been also found in MAFLD patients pointing the relevance of EVs as additional SFAs transporters and mediators of lipotoxicity in MAFLD.

OC40. Functional role of extracellular vesicles in the communication of obese adipose tissue with the liver, and their involvement in the establishment and maintenance of human hepatic steatosis

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Keywords: Extracellular vesicles, adipose tissue, NAFDL, obesity, insulin resistance.

Introduction
The rediscovery of extracellular vesicles (EVs) and their role in normal and pathological physiology is changing the paradigm in many aspects of biomedical research. Specifically, in the study of metabolic diseases, EVs, and, in particular, exosomes, emerge as a new cellular communication pathway independent of the classical endocrine pathway still very little explored; additionally, these vesicles are being postulated as good candidate biomarkers of malignancy. We postulate that obese adipose tissue sheds pathological-EVs that may participate on obesity and its comorbidities such as diabetes or NAFLD (Non-Alcoholic Fatty Liver Disease).

Objective
To determine the functional role of the EVs released by obese adipose tissue in the context of obesity and the associated hepatic diseases.

Methods
We isolated pathological vesicles from murine adipocyte cell models of insulin resistance (IR) and hypertrophy (HGHI, palmitate/oleic acid) by ultracentrifugation. Functional in vitro analysis of isolated vesicles was assayed by the study of the insulin signaling pathway and gluconeogenic and lipogenic pathways on primary culture of murine hepatocytes through immunodetection and PCR.

Results
Functional assays have shown that EVs from lipid-hypertrophied, insulin-resistant adipocytes induce pathology in healthy murine primary hepatocytes. Interestingly, pathological vesicles, especially EVs from lipid-hypertrophied adipocytes, are able to induce insulin resistance due to alteration of the insulin signaling pathway in healthy hepatocytes. Furthermore, these vesicles secreted by hypertrophied adipocytes promote inflammation and activation of gluconeogenic pathways in healthy primary murine hepatocytes.

Conclusions
Hypertrophied adipocytes release Trojan horse extracellular vesicles capable of inducing metabolic alterations in healthy hepatocytes and probably exacerbating liver disease once established.

Acknowledgements
This work was funded by the Instituto de Salud Carlos III-FEDER (PI16/01212). Camino T is funded by a FPU17 contract (Ministerio de Educación, Cultura y deporte) and by the FPU2021 stay. Lago-Baameiro L is funded by Ayudas a la etapa Predocotoral GAIN-XUNTA de GALICIA.

FLASH POSTER

P1. Extracellular vesicles from the third stage larvae of Anisakis pegreffii: investigating possible proteins implicated in the host-parasite adaptation and pathogenesis

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Keywords: *Anisakis pegreffii*; extracellular vesicles (EVs); third stage larvae (L3); proteomics

Anisakis pegreffii is a nematode parasite species of the *A. simplex* (s. l.) complex, requiring marine homeothermic (cetaceans) and heterothermic (crustaceans, fish and squids) organisms to complete its life cycle. It is also a zoonotic species, being humans accidental hosts.

To investigate the biological mechanisms and molecular signals involved in this host-parasite interaction and pathogenesis, the proteomic composition of the extracellular vesicles (EVs) released by the third stage larvae (L3) of *A. pegreffii,* was characterized. L3 were cultured at 37°C to simulate the homeothermic host environment. Isolated EVs showed rounded-shaped structures (size 65-295 nm). Proteomic analysis was performed by shotgun analysis and results were blasted against the *A. pegreffii* specific transcriptomic database, and 153 unique proteins were identified. Several proteins belonging to distinct metabolic pathways were predicted by Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis. The similarity search employing selected databases revealed that the *A. pegreffii* EVs cargo contains proteins essential for the parasite survival and adaptation, potential pathogenicity-related proteins and allergens. Further, analysis of predicted host-parasite interactions between the *A. pegreffii* EVs and both human and cetaceans' proteins, were identified.

This study represents the first proteomic characterization of *A. pegreffii* EVs and provides useful data that expands our knowledge on the host-parasite interaction and pathogenesis.

This study was supported by the Italian Ministry of Health (RF) 2018 – 12367986, title "Innovative approaches and parameters in the diagnosis and epidemiological surveillance of the Anisakis-related human diseases in Italy"

P2. Proteomics of porcine seminal plasma: inside and outside of extracellular vesicles

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Keywords: Extracellular vesicles; Porcine; Proteomics; Seminal plasma.

Seminal plasma (SP) is rich in proteins, which are involved in several physiological reproductive events, including sperm functionality and embryo development. Little is known about whether these SP-proteins would circulate free or inside of extracellular vesicles (EVs). This study aimed to quantitively compare the protein cargo in porcine SP-EVs with those freely circulating in SP. Three SP pools (three ejaculates per pool) were centrifuged sequentially (3,200xg/15min and 20,000xg/30 min at 4ºC), and the ultrafiltrated supernatants were subjected to size exclusion chromatography (SEC). SEC-eluted fractions 7–10 (EV-enriched) and 18–20 (EV-free) were selected and mixed separately to generate two pools (EV-enriched and EV-free). SP-EVs were characterized by morphology (Transmission electron microscopy), size distribution (Nanoparticle tracking analysis), purity (albumin assessment by flow cytometry, FC) and EV-specific protein markers (CD63, HSP90β and CD44, by FC). Quantitative proteomics analysis was carried out by SWATH-MS strategy and proteins with a $p<0.05$ and a Log2 fold-change > ±2 were considered quantitatively different. Isolated EVs showed a high degree of purity (albumin < 3.5%), were heterogeneous in shape, electron density and size (30 to 350 nm); and were positive to CD63 (mean ± SD: 77.62 ± 6.89%), HSP90β (90.74 ± 4.02%) and CD44 (99.28 ± 0.07%). A total of 737 proteins were quantified, and 35 of them were underexpressed and two were overexpressed in EV-enriched samples compared to EV-free ones. GO enrichment for biological processes revealed that the proteins up-regulated in SP-EVs were involved in developmental processes, whereas those down-regulated were related to growth and localization. In summary, most of the SP proteins were in similar amounts in the EV-enriched and EV-free samples, and the few quantitative different proteins do not show defined reproductive functions at this time. Fundings: MCIN/AEI/10.13039/501100011033 (PID2020-113493RB-I00 and PID2019-105713GB-I00); European Comission (H2020-MSCA-IF-2019-891382); and Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Valencia, Spain (Grant PROMETEO/2020/071).

P3. Ferroptosis transmission by small extracellular vesicles in epithelial ovarian cancer cells

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Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy as its early diagnose is complex owing to unspecific symptoms on the initial stages of the disease. Currently available pharmacological treatments seem to be effective at the beggining and remmission states can be reached. However, eventually, many patients relapse as cancer cells become resistant leading to a poor prognosis. Hence, the study of novel approaches to fight resistant cancer cells is needed. Ferroptosis is a type of non-apoptotic-regulated cell death triggered by an excessive ionic iron accumulation. Since it has been demonstrated that ovarian cancer cells exhibit an alteration in iron metabolism, specifically an increase in its intracellular concentration, ferroptosis has emerged as a potential tool to fight cancer cells resistant to available treatments such as inmunotherapy or angiogenesis inhibitors, among others. It has been reported the removal of intracellular iron by sEV as a way to induce ferroptosis in cancer cells. That is why intercellular communication through sEV is an attractive field to fight OVCA (epithelial ovarian cancer cells).

Aim

The objective of this study is to unravel the molecular mechanisms that regulate ferroptosis and, pecularly, determine whether it is capable of paracrine transmission.

Methodology

To achive this we analyze the capacity of ferroptotic inducers treated OVCA to generate sEV, assesing its size and number. The potential of sEVs in paracrine ferroptosis was also studied by analyzing different parameters: cell viability, MDA levels, Fe^{2+} levels, GSH:GSSG ratio and ROS levels.

Results

OVCA cells treated with ferroptotic inducers are capable of modifying intercellular communication by means of sEV, inducing cell death in recipient cells.

Futhermore, these receptor cells are able to generate a greater amount of sEV, contribuing to a much higher ferroptosis paracrine transmission.

Conclusions

This study has, for the first time, provided evidence of the capacity of ovarian cancer cells treated with ferroptotic inducers to modify their intercelular enviroment and comunicate surrounding cells. Thus, it opens up other avenues of cancer treatment, being ferroptotic modulators a feasible one.

P4. Identification of exosome modulators in 3D breast cancer models using exoscreen and cell painting technologies

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Keywords: ExoScreen, Cell Painting, Exosomes, Breast cancer, Polypeptide-based combination conjugates

Introduction

3D spheroid cell cultures recapitulate the tumor micro-environment[1] better than their adherent 2D counterparts[2]. Spheroid models represent a valuable cancer research tool, allowing optimized drug selection and improved tumor distribution. 3D models of breast cancer will reduce the number of animals employed and drug screening costs[3-5]. Of note, the molecular complexity of breast cancer, especially when targeting metastasis, will require combinatorial drug treatments[6,7]. Exosomes - extracellular vesicles (EVs) that play essential roles in intercellular communicators[8] - help form the pre-metastatic niche[9] and support drug resistance[10]. EV markers include tetraspanins[11]; however, current methodologies to purify exosomes remain time-consuming and challenging to translate to clinical practice. We recently optimized a quick and reliable HTS methodology[12] to identify exosome modulators that combines external signals measured by ExoScreen technology (a sensitive assay that measures protein-protein interactions) and internal exosomal markers in 2D models[13]. Our study employed MCF7 cells (Luminal A subtype).

Objectives

1. Study the effect of polymer-drug conjugates (single/combinations) on exosomes via the ExoScreen assay
2. Combine ExoScreen and Cell Painting[14] to discover exosome modulators (and other effects)

Methodology

Using low adherence plates, we cultured MCF7 mammospheres with EGF2/B27. We added free or polymer-conjugated drugs for 72 h and performed the ExoScreen assay using anti-CD9 acceptor beads and a biotinylated-anti-CD63 antibody. For Cell Painting, we employed markers of the mitochondria, endoplasmic reticulum, cell membrane, intraluminal/extracellular vesicles, and nucleus. Cell viability evaluations employed the MTS assay.

Results

We identified "Drug 1" as an exosome biogenesis inhibitor (reducing ExoScreen and CD63 signals, extra- and intracellularly, respectively). "Drug 3" inhibited exosome release, manifested as a lower ExoScreen signal and accumulated intracellular CD63 signal, and modulated the endoplasmic reticulum. Both drugs mimic the behavior previously observed in a 2D model[13].

Conclusion

Employing this screening technique in 3D spheroids ensured the homogeneous distribution of labeling in a preclinically relevant model. We combined analysis of exosomal intracellular markers (CD63) with morphological features to create a multiplexed approach. We employed our approach to validate the antitumor/antimetastatic properties of a polypeptide-based-drug combination conjugates[7,15], with findings correlating with in vivo data. The following steps include applying suitable image analysis software and artificial intelligence tools to enhance intracellular signal quantification of different markers and establishing correlations with therapeutic outputs[7].

Acknowledgments
We thank Dr. Zoraida Andreu for 3D model optimization, David Charbonnier for ExoSreen assay development, Dr. Alicia García for cell painting, Alberto Hernández for confocal imaging, and Dr. Stuart P. Atkinson for English editing. This work is supported by the Tentacles excellence Network, RED2018-102411-T, and the Spanish Ministry of Science and Innovation PID2019-108806RB-I00. Equipment funded by the Generalitat Valenciana and co-financed with European Regional Development Fund (FEDER) funds (PO FEDER of Comunitat Valenciana 2014-2020).
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P5. Divergent contributions of extracellular vesicle subpopulations to huntington's disease neuropathology

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Keywords: Exosomes, microvesicles, Alix, Annexin-2, neurodegeneration

Introduction

Huntington's disease (HD) is an inherited autosomal dominant disease characterized by progressive degeneration of the striatum and the cerebral cortex caused by a mutation in the Huntingtin gene comprising an expansion of a CAG repeat. Mutant huntingtin accumulates in endosomal compartments and triggers the activation of the endo-lysosomal system, which may contribute to huntingtin proteolysis and to an autophagic process of cell death. The altered trafficking through the endosomal pathway can affect the production, secretion and content of exosomes, extracellular vesicles (EVs) with an endosomal origin.

Objective

In this work, we investigated whether the levels and content of small EVs, including exosomes, are altered in the brains of Huntington's disease patients.

Methods

We isolated and characterized microvesicle- and exosome-enriched EVs from the striatum and dorsolateral frontal cortex of Huntington's disease brains presenting signs of either early or advanced degeneration, and control brains, by differential ultracentrifugation followed by a high-resolution iodixanol density gradient. EV secretion by human fibroblasts from Huntington's disease patients and healthy individuals was also explored.

Results

Our data indicate that the level of Annexin-2-positive EVs is enhanced in HD cortex, whereas the EV population enriched in Alix is reduced in HD striatum and cortex compared to controls. The Annexin-2 and Alix loading per EV were also increased and reduced in the disease, respectively. The altered EV cargo loading of Alix was consistent with a drastic decrease in Alix protein levels in HD striatum and cortex, and lower levels of the protein in the cerebrospinal fluid. In HD fibroblasts with Alix levels similar to controls, there was less also secretion of Alix-positive EVs.

Conclusions

We propose a model in which lower level of brain exosomes in HD impacts the endosomal pathway, and enhanced secretion of microvesicles is an attempt to relieve brain cells from the accumulation of mutant huntingtin and other pathological proteins. Taken together our data provide new insights into the mechanisms of HD pathogenesis that could contribute to the development of novel therapeutic strategies targeting EVs.

P6. Extracellular vesicles released by adipose or hair follicle mesenchymal stem cells induce neuroprotection and modulate neuroinflammation in primary cell cultures

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Keywords: Neurodegenerative diseases • neuroinflammation • neuro-protective • extracellular vesicles • mesenchymal stem cells

Introduction

During the last decade, cell replacement therapies, mainly focused on mesenchymal stem cells (MSC), have been long an attractive prospect for treating the complexity of neurodegenerative diseases (NDs). They exhibit immunomodulatory effect, migratory capability and regenerative potential, which result in a neurological recovery and neo-angiogenesis through the secretion of neurotrophins and angiogenesis regulatory factors. Lately, numerous research articles suggest that the therapeutic effect appears to be a consequence of the paracrine action of different biomolecules, including the extracellular vesicles (EVs). EVs are nanometer-sized lipid membrane-enclosed vesicles secreted by most cells and contain lipids, proteins and various nucleic acid species of the source cell with great potential in NDs treatment.

Objective

Study and compare the properties of EVs derived from two different sources of MSC as a "holistic" approach for NDs treatment using neuroprotective and anti-inflammatory assays in primary cell cultures.

Methods

We isolated, characterized and compared medium-large EVs (m-lEVs) obtained from hair follicle-derived MSCs (HF-MSCs) against, EVs isolated from adipose tissue-derived MSCs (hAT-MSCs) We performed neuroprotective and inflammatory assays to probe the therapeutic potential of EVs modulating the hallmarks of NDs.

Results

The obtained EVs were around 220-240 nm in both cases. Moreover, HFEVs, as well as hATEVs, expressed typical MSCEVs markers (CD9, CD44, CD63, CD81 and CD105) among other different functional markers. We observed that both cell types were able to increase the percentage of dopaminergic neuron living cells after its exposure to 6-OHDA toxin. Finally, both MSCEVs were able to decrease the release of pro-inflammatory cytokines, such as TNF-α and IL-1β, after LPS *stimuli* in microglia cell cultures.

Conclusion

HF-EVs demonstrated, for the first time, to exhibit comparable potential to AT-EVs. The positive outcome obtained after the incubation with both m-lEVs in neuroprotective and anti-inflammatory assays confirm the suitability of EVs as multifaceted biopharmaceuticals for NDs treatment.

Acknowledgements

S Hernando thanks UPV/EHU for her postdoctoral fellowship grant (MARSA 21/36) funded by the European Union-Next Generation EU. This project was partially supported by the Basque Government (Consolidated Groups, IT1448-22).

P7. Gamma catenin containing extracellular vesicles are detected in multiple pathologies

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Keywords: Anaphylaxis, Diabetic Retinopathy, Gamma Catenin, Neutrophils, Proteomics

Introduction

Multiple proteomic approaches around extracellular vesicles (EVs) research render thousands of identified proteins, some of which can be detected in multiple datasets. The function of many of these proteins associated with their detection in vesicles preparations are unknown. Interestingly, some of them are detected in multiple disease vesicles datasets which could share common mechanisms not fully understood yet.

Objective

Our data presented certain similarities among apparently different pathologies, like anaphylactic reaction and diabetic retinopathy, so we aim to evaluate selected targets as crossover translational markers.

Methods

We performed proteomic analysis on human EVs from different sources and pathologies. We purified and characterized EVs from 86 human plasma samples, and we performed proteomic analysis on the vesicles content. We analyzed human retina tissue explants derived EVs and urinary EVs from diabetic retinopathy patients.

Results

First, we hypothesized that anaphylaxis derived EVs could provide potential markers of anaphylaxis due to their participation in the underlying molecular mechanisms. We identified 526 proteins exclusively detected in anaphylactic reaction EVs of which 83 were increased in the acute phase, thus suggesting their potential as candidate biomarkers. Similar results were obtained in independent analysis of diabetic retinopathy derived EVs, in which we identified 57 proteins detected both in retina tissue explants from diabetic retinopathy patients and in their urinary EVs.

In silico Ingenuity pathway analysis showed around 25% of the protein signature participating in leukocyte trans-endothelial migration and cell degranulation. In particular, the gene JUP, which codifies for Junction plakoglobin, or Gamma catenin captured our attention due to its role as a component of desmosomes and adherent junction structures, and its predicted role in neutrophils degranulation.

Conclusion

Gamma-Catenin is specifically detected in EVs associated with pathological states in different diseases with common phenomena. Its influence on disease progression needs further clarification to understand general effects of EVs on pathological scenarios.

P8. "Monitorization of response in multiple myeloma patients treated with the anti-BCMA drug conjugate Belantamab mafodotin through circulating extracellular vesicles"

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Keywords: Multiple myeloma, Belantamab mafodotin, extracellular vesicles, plasma

Introduction

Multiple myeloma (MM) is a hematological malignancy of clonal plasma cells that proliferate and accumulate in the bone marrow and produce high monoclonal Ig or M-protein. Recently, immunotherapeutic treatments directed against the B-cell maturation antigen (BCMA) have become increasingly relevant in MM, due to the specificity of this target. The first anti-BCMA treatment approved by the FDA/EMA was Belantamab mafodotin or Belamaf, which is an anti-BCMA monoclonal antibody conjugated to the microtubule toxin monomethyl auristatin F. However, the efficacy of anti-BCMA treatments may be hampered by the release by MM cells of extracellular vesicles (EVs) containing BCMA. **Objectives**
To determine several EV-related parameters in plasma from healthy donors and MM patients included in the GEM Bela-VRd clinical trial (NCT04802356). To compare circulating EVs from healthy donors and patients at three stages of treatment. EV-related analyses will then be associated/correlated to MM standard measures of response as assessed in the GEM Bela-VRd clinical trial.

Materials and methods

EVs were isolated from plasma from healthy donors (n=3), or MM patients at three time points [screening -before treatment- (SC) n=22; week 10 (wk 10) n=20, and C6D28 -end of induction therapy- n=4]. EVs were isolated using a precipitating agent (SBI, ExoQuick).

Mean EV size (nm) and EV plasma concentration (particles/mL) were determined by Nanoparticle Tracking Analyses (Nanosight NS300, Soft-ICMAB, Barcelona). After EV protein isolation, EV protein concentration in plasma (μg/mL plasma) and EV cargo (μg/10^9 particles) were calculated.

BCMA content in circulating EVs was determined by capillary-based electrophoresis nanoimmunoassay (Protein Simple).

Results

EV protein concentration in plasma at SC was high and was significatively reduced to levels similar to that of healthy donors after Bela-VRd treatment. Both EV protein concentration and EV cargo were significatively diminished in pairwise comparisons from patients at SC and wk10.

BCMA was detectable in EVs from MM samples by capillary-based nanoimmunoassay. Likewise, a significative reduction in BCMA-EV content was observed between plasma at SC and after treatment.

With data and measured responses at time of this report, a multivariable EV approach showed promise as predictor of Belantamab-VRd response.

Conclusions

1. EV protein concentration and BCMA-content in circulating EVs from patients in the GEM Bela-VRd trial were significatively diminished after treatment with Bela-VRd
2. Combined EV-variables may have value as predictors of response to Belantamab mafodotin

Funding: Pfizer RFP Research Grant (ID 70094931), GlaxoSmithKline Research Grant (ref 12380), Instituto de Salud Carlos III (PI19/01384 and PI18/01600) co-funded by the European Union; and AECC (PROYE20047GUTI).

P9. Combined exosomal and plasma non-coding RNA signature associated with urinary albumin excretion in hypertension

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Keywords: Exosome, non coding RNA, hypertension, urinary albumin excretion

Introduction

Non-coding RNA (ncRNA), released into circulation or packaged into exosomes, play important roles in many biological processes in the kidney.

Objectives

The purpose of the present study is to identify a common ncRNA signature from plasma and urine exosomes and plasma, associated with early renal damage and its related molecular pathways by constructing a RNA-based transcriptional network.

Methods

This is an observational case-control study which included twenty-one patients with essential hypertension (n=21) and twenty-two without persistent elevated urinary albuminuria (UAE) (≥30 mg/g urinary creatinine). Three individual libraries (plasma and urinary exosomes and total plasma) were prepared from each hypertensive patient for ncRNA sequencing analysis. Next, a RNA-based transcriptional regulatory network was constructed.

Results

The three RNA biotypes with the greatest number of differentially expressed transcripts were long ncRNA (lncRNA), microRNA (miRNA) and piwi-interacting RNA (piRNA). We identified a common 24 ncRNA molecular signature related to hypertension-associated albuminuria, of which lncRNA was the most representative. In addition, the transcriptional regulatory network analysis showed five lncRNA (LINC02614, BAALC-AS1, FAM230B, LOC100505824 and LINC01484), and the miR-301a-3p to play a significant role in network organization and to target critical pathways regulating filtration barrier integrity, tubule reabsorption and systemic endothelial dysfunction.

Conclusion

Our study found a combined ncRNA signature associated with albuminuria, independently of biofluid origin identifies a handful of potential targets involved in filtration barrier, tubule reabsorption and endothelial function that may be utilized to treating hypertension-associated albuminuria and cardiovascular damage progression.

Grants

Health Institute Carlos III" [PI12/02615; PI19/01796; CD18/00166 (Sara Borrell for Ana Ortega) and FI20/00096 (PFIS for Olga Martínez-Arroyo)]. European Social Funding and European Regional Development Fund (ERDF).

P10. Implementation of an interdisciplinary core facility for the study of extracellular vesicles at the national hospital of paraplegics

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Keywords: MIFlowCyt-EVs, MISEV, Flow Cytometry, Proteomics

In the last years, preclinical research at hospitals has revealed as an important factor to facilitate translational research focused on biomarker discovery. The analysis of Extracellular Vesicles (EVs) as biomarkers is gaining importance, although their characterization needs for a highly qualified staff specialized in diverse state-of-the-art techniques. MISEV 2018 and MIFlowCyt guidelines are recommendations for the standardization and reporting of EVs studies which are not always easy to be followed. In this sense, an "Interdisciplinary Core Facility" (ICF) provides the access to specialized technologies, and instrumentation, with the support of a highly trained staff which will ensure the generation of reproducible quality data. OBJECTIVE: Creation of an ICF specialized in the study of EVs following MISEV 2018 and MIFlowCyt-EVs guidelines.

Methods and Results

Flow Cytometry (FC) and Proteomics Core Facilities were associated in a new ICF specialized in the study of EVs to provide researchers the necessary technologies from the first step of EVs enrichment to the last one for protein cargo characterization.

EVs isolation from cell culture supernatant samples was performed using ultracentrifugation, Size Exclusion Chromatography (SEC) or a combination. Characterization was performed by FC and Western-blot. Exosome standards were used for titration and standardization of staining conditions with antibodies against tetraspanins, as well as for optimizing western blot analysis of TSG101, Hsp70 and tetraspanins. Calibration of VSSC with polystyrene (PS) NIST beads, and fluorescence calibration with Rainbow particles were carried out in a CytoFLEX S, following MIFlowCyt-EVs guidelines, resulting in detection of 80 nm PS beads and organosilica core beads with an estimated size of 187 nm (Verity Shells A, Exometry).

Samples from all the steps of the EVs enrichment protocol were analyzed, and the presence of EVs were easily detected with a cell type-specific marker by FC. Serial dilution controls for assuring no swarming effect and detergent controls were performed following MIFlowCyt-EV guidelines.

Proteomics analysis were carried out using different lysis buffers and digestion protocols. The cargo was determined by LCMS using a TripleTOF 6600 plus.

Conclusions

It is possible to have a dedicated EV ICF with high quality standards for researchers who have difficulty or do not have the possibility to access the technologies needed for their highly specialized study. Its existence in a clinical environment will help to promote the necessary translation of basic research into the real clinical practice.

Acknowledgements

This work was supported by Bristol Myers Squibb research agreement to DC. AMR holds a Technical Staff fellowship from the Spanish *Ministerio de Ciencia, e Innovación (*PTA2020-018340-I). GBG, AGA, MCO, DC and VV-dS are hired by SESCAM.

P11. Intelectin-1, epithelial and protector marker on cardiovascular disease, is identified in extracellular microvesicles of neutrophils.

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Keywords: Intelectin-1 (ITNL-1), Orosomucoid (ORM1), Acute *de novo* Heart Failure (ADHF), plasma, neutrophils.

Introduction

Intelectin-1 (ITNL-1) is mainly expressed in epithelial cells and reduces inflammation. Orosomucoid (ORM1), which is known to be an acute phase reactant, is mainly expressed in hepatocytes. Patients with acute *de novo* heart failure (ADHF) with low ITLN-1 and high ORM1 at discharge have a higher probability to rehospitalize and/or dying during the following 90 days. Neutrophils have an important function during cardiovascular inflammation and repair through microvesicles (MVs).

Objective

Study ITNL-1 and ORM-1 levels in neutrophils and their MV on HF patients.

Methods

Circulating plasma at discharge from patients with ADHF and controls were used. Neutrophils from subjects or patients were centrifuged and isolated with Polymorphprep. Then, the isolated neutrophils were stimulated with fMLP for 1h30', and the MVs excreted to the supernatants were precipitated using ExoGAG, according to the manufacturer's protocol, and finally identified by Nanoparticles Tracking Analysis (NTA). Same protocol was used for MVs precipitation from plasma (250 uL). Then, mRNA ITLN-1, DEFA3 (a specific protein found on neutrophils) and ORM1 were analyzed in plasma, neutrophils and neutrophils´ MVs by real time PCR and the amplified products were confirmed by sequencing according to the Sanger method. Proteins were tested by Western Blot.

Results

Plasma´s MVs from patients with ADHF and subjects exhibited ORM1, ITNL-1 and DEFA3 mRNA levels. While mRNA ORM1 in MVs was increased in those patients with events after discharge, ITNL-1 and DEFA3 were similar among subjects and patients with and without events. At this point, we analyzed their levels on neutrophils and their MVs. We found a higher ORM1 mRNA expression profile on neutrophils and their MVs. Although ITNL-1 levels were lower than ORM1, we described for the first time its expression in these cells and their MVs by three different methods: sequencing, real time PCR and Western Blot.

Conclusions

ITNL-1 and ORM1 mRNA levels were identified in plasma MV, neutrophils and their MV. However, they were not significantly modified in ADHF patients with or without events. For the first time, ITNL-1 mRNA was detected in neutrophils and their MV. Since ITNL-1 is known to play an anti- inflammatory role, these results might suggest a protective effect of these cells.

Acknowledgements
We wanted to thank the *Fondo de Investigaciones Sanitarias 19* (PI19/01330) from the *Plan Estatal de I+D+I 2019–2023* for supporting it.

P12. Isolation and characterization of extracellular vesicles from fluidic samples in the context of *Mycobacterium tuberculosis* infection

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Keywords: *Mycobacterium tuberculosis*, Extracellular vesicles, Urine samples, Size exclusion chromatography

Tuberculosis (TB) is the leading cause of death due to a single bacterial infectious agent worldwide, though it is a preventable, treatable and curable disease. The control of the disease is difficult for several reasons, including the long-time treatment of antibiotics, the rapidly spreading of the multidrug-resistant *Mycobacterium tuberculosis* (Mtb) strains that are resistant to the conventional treatment, and the currently available diagnostic methods that have inherent limitations, such as long incubation times (sputum-based culture) or high financial cost (rapid molecular tests). These reasons highlight the need for new simpler, cheaper and faster diagnostic methods, to reduce the gap between the beginning of symptoms and diagnosis.

Recently, extracellular vesicles (EVs) have emerged as a powerful tool for biomarker characterization in several diseases. Mycobacterial extracellular vesicles (MEVs) are membrane walled spheres of 60-300 nm in diameter released by Mtb that contain iron scavenging molecules, virulence factors, and immunologically active complex lipids and lipoproteins, and that play a role in immunomodulation, modifying the response of host cells to infection. All these features have created the bases to investigate MEVs as a potential source of biomarkers to know about the status of both the bacterium and the host during an ongoing disease.

Towards this end, we first generated hybridoma cell lines by mice immunization with Mtb vesicles, obtaining highly specific antibodies against MEVs. Followed by the design of a protocol to isolate host- and Mtb-EVs from fluidic samples by size exclusion chromatography (SEC) and to carry out a comparative analysis of the EVs population by DotBlot and by nanoparticle tracking analysis (NTA). These methodologies were validated isolating and analysing EVs from THP-1 macrophages cultures infected with Mtb. The results showed that the designed procedures are valid to use with different types of fluidic biological samples. Specifically, we started collecting clinical samples of urine from a cohort of ten TB patients, five male and five female, and at different times of treatment: baseline, two and six months. Their analysis will provide us information about heterogeneity and compositional changes of EVs in different stages of the disease and treatment.

P13. Secondary Focal Segmental Glomerulosclerosis (FSGS) biomarker discovery from urinary Extracellular Vesicles (uEV)

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Keywords: Urinary extracellular vesicles, Nephrology, FSGS, Biomarkers, Exosomes

Introduction

Chronic kidney disease (CKD) affects about 10% of the population world-wide. The incidence of CKD is projected to rise due to the increasing prevalence of obesity, diabetes and hypertension. These pathologies cause glomerular hyperfiltration, secondary focal segmental glomerulosclerosis (FSGS) and CKD progression. FSGS is a glomerular disease defined by the findings in kidney biopsy, thus, novel non-invasive biomarkers are needed to improve early diagnosis and/or substitute biopsy when is contraindicated or not recommended.

Objective

Our aim was to perform proteomic analysis of urinary extracellular vesicles (uEVs) isolated by size exclusion chromatography (SEC) from FSGS patients with CKD stages 3-4 to discover novel biomarkers for FSGS monitoring.

Methods

Nine patients with biopsy proven FSGS and glomerular filtration rate (GFR) of 60-15 ml/min/1.73 m^2 and ten age/sex matched healthy controls (HC) were recruited. All FSGS patients were overweight (n=5) or obese (n=4), so 10 HC were further subdivided (5+5) into body mass index (BMI) >25 Kg/m^2 (overweight-OW) and BMI <25 Kg/m^2 (lean control-LC) to asses BMI influence. uEVs were isolated from urine by SEC, and characterized according to MISEV18 guidelines. Proteomic analysis of uEV was performed by LC-MS/MS (Orbitrap Fusion Lumos Tribrid). Significance threshold for the identifications was set to $p<0.05$, minimum ions score of 20 and expression of a minimum of 2 unique peptides.

Results

Comparison of FSGS and Healthy controls (OW and LC) showed 126 and 70 proteins differentially expressed (DE) respectively (p-value <0.05 and log2FC >2). FSGS uEV proteome was enriched in biological processes related to humoral immune responses, while HC uEV proteins were enriched in regulation of vesicle organization and exocytosis. Principal component analysis (PCA) showed clustering of OW donors closer to FSGS patients. FSGS vs LC showed 126 and 79 DE proteins, while FSGS vs OW only showed 36 and 51 DE proteins respectively.

Conclusions

Analysis of uEVs proteins highlighted an enrichment of markers involved in immune responses that constitute potential FSGS biomarkers. OW controls showed fewer DE markers that cluster closely to FSGS patients, while LC showed the most significant differences when compared to FSGS patients.

Aknowledgements

This work was supported by Instituto Carlos III project PI20/00097, integrated in the National R + D + I and funded by the ISCIII and the European Regional Development Fund. SGG is supported by the Catalan Health department ("Departament de Salut") in receipt of a grant from PERIS-PIF-Salut (SLT017/20/000158), and MF is supported by ISCIII (MS19/00018), co-funded by ERDF/ESF, "Investing in your future".

P14. Co-isolation of highly abundant soluble proteins in exosome samples trips biomarker discovery up

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Keywords: biomarkers; exosomes; methodology; proteomics

Introduction
Exosomes are extracellular vesicles whose cargo depends on their cell origin. Exosomes are a potential source of disease biomarkers, but potential contamination with highly abundant proteins could occur during their isolation, which must be considered when using proteomic approaches.

Objective
To investigate the influence of the exosomal source in the purity of exosomes isolated by ultracentrifugation (UC).

Methods
We have analysed the contamination of exosome samples isolated by UC from different sources (cell culture supernatants, sera, and urine) by western blot, Dynamic Light Scattering (DLS) and LC-MS/MS (data-dependent acquisition (DDA); Triple TOF).

Results
We found higher levels of exosomal markers (CD9, CD63) in those derived from cell culture supernatants compared to serum and urine. Using DLS, we observed an abnormal peak at <50 nm (protein aggregates) in serum and urine exosomes. Salts treatment (KBr) or density gradients during UC did not decrease such contamination. In agreement, our proteomic analysis revealed a higher number of exosomal proteins in vesicles isolated from cell culture supernatants vs. serum. Moreover, gene ontology (GO) analysis for cellular component showed a higher enrichment in proteins from exosomes (GO:0070062) in samples from cell culture, but not in blood microparticles (GO:0072562), proteins from the extracellular region (GO:0005576) or the extracellular space (GO:0005615). Altogether, our results suggest that exosomes from culture supernatants are less contaminated with non-exosomal proteins, making UC a suitable method in those samples.

Conclusion
Although UC is a suitable method for miRNA biomarker discovery, its use in proteomic analyses of exosomes from serum or urine (but not cell culture supernatants) is questionable due to the presence of non-exosomal protein contamination.

P15. Comparison of techniques for the isolation of VP40 virus-like particles and extracellular vesicles

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Keywords: Virus-like particles, exosomes, isolation, VP40 matrix protein

Introduction

VP40 matrix protein plays an essential role in the assembly and budding of Ebola virions. In fact, the expression of VP40 protein in cell culture induces the formation and release of virus-like particles (VLPs) to the cell culture medium [1]. However, VP40 protein is also secreted inside extracellular vesicles (EVs) [2].

Objective

The similarities in the physicochemical properties of VLPs and EVs [3] result in a difficult separation of both populations for their study. Our objective is to compare different techniques and to select the most appropriate for the enrichment of our sample either in VLPs or in EVs.

Methods

We transfected HEK293T cells with a plasmid encoding VP40 matrix protein and submitted the cell culture medium to different protocols (based on ultracentrifugation and size-exclusion chromatography). Then, we characterized the obtained products in terms of physicochemical properties, molecular composition, and morphology.

Results

Different levels of HA-VP40 and CD63 tetraspanin were detected after each isolation protocol.

Isolated particles had an average hydrodynamic diameter around 200 nm. Filamentous particles with similar shape to Ebola virions were observed, mostly accompanied of extracellular vesicles.

Conclusion

Our results show that complete purification is not achieved with any of the studied methods. However, we can selectively enrich the sample in VLPs or in exosomes.

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Acknowledgements

This work has been funded by Ministerio de Economía, Industria y Competitividad (MINECO-RETOS, Grant MAT2017-84361-R, Feder Funds).

P16. High-throughput EV purification from biological fluids for diagnostic application

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Keywords: EV, liquid biopsy, high- throughput EV purification, proteomics

Extracellular vesicles (EV) enriched from liquid biopsies such as blood have great potential as a source for biomarkers. Lipid encapsulation protects the cargo from degradation and allows the EV to be transported throughout the body and across physiological barriers. EV purification is a critical step for the application of high-throughput omics technologies in a non-invasive manner for predictive and prognostic applications.

Here we focused on optimization of size exclusion chromatography (SEC) approach compatible with high-throughput purification from low amounts of serum samples.

SEC and differential ultracentrifugation (UC) were applied as EV enrichment methods followed by quality control (QC) assessment with NanoFCM, Western blotting, Transmission electron microscopy (TEM) and high-performance mass spectrometry based proteomics analysis.

SEC first elution fraction (fraction 1) and EV purified by UC were showing highest enrichment in EV and EV markers compared to other elution fractions or direct measurement of undepleted and depleted serum samples. Principal component analysis shows separation of samples enriched by UC and SEC fraction 1 from other SEC fractions and serum.

Early implementation of proteomic analysis as a part of EV QC influenced the selection of SEC as method of choice for EV purification from low volume serum samples.

We acknowledge Mario Soriano for the great support with TEM analysis.

P17. Optimization of an RNA isolation method for EV-Based Non-invasive Testing of *ALK* fusions in lung cancer patients.

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Keywords: *ALK* fusion, digital PCR, lung cancer, RNA, SEC

Introduction

Detection of *ALK* fusions in lung cancer (LC) identifies patients who can benefit from *ALK* inhibitors, improving the patient's quality of life and survival. Due to compromised tumor tissue availability in LC patients, *ALK* rearrangements testing using liquid biopsies, although challenging, is a promising approach. The main purpose of this study is the development of an adapted EV-associated RNA isolation methodology for *ALK* fusions detection as a novel diagnostic approach in LC patients that could be transferred to automated methods.

Methods

Five protocols were tested for EV-associated RNA isolation using a pool of plasma obtained from *ALK*-positive LC patients. Briefly, we analyzed by Qubit RNA-HS Assay kit and Agilent-2100 Bioanalyzer the yield and quality of RNA obtained from plasma processed using exoRNeasy kit (Qiagen) [with/without proteinase-K pre-treatment (exoRNeasy-PK/exoRNeasy)], plasma/serum exosome and free-circulating RNA isolation kit (Norgen) or ultracentrifugation (with/without proteinase-K pre-treatment) followed by RNA isolation with exoRNeasy kit. RNA fusion transcripts were analyzed by nCounter-NanoString and digital PCR (dPCR). *ALK* detection was also evaluated in RNA from different Size-Exclusion Chromatography (SEC) fractions from the supernatant of *ALK*-positive LC cell lines (H2228, H3122) in order to identify which type of EVs are enriched in *ALK* fusions transcripts. Finally, *ALK* detection was tested in RNA from plasma samples (healthy donors) spiked-in with H3122–EVs concentrated with bio-inert filters Nanosep (Pall).

Results

Among all the RNA isolation methods tested, we obtained the highest EV-associated RNA yield using the exoRNeasy kit. However, nCounter analysis showed that *ALK* fusion transcripts were more clearly detected in samples isolated using the Norgen kit. These results were confirmed by dPCR which detected the *ALK* fusion variant *EML4(6)-ALK(20)* at a variant allele frequency (VAF) of 2.61% in Norgen samples and at a VAF of 0.79% in exoRNeasy samples. Regarding SEC analysis, dPCR results revealed that *ALK* fusions were more abundant in CD9, CD81, and CD63-enriched SEC fractions in both LC cell lines (VAF-H2228=12.88% and VAF-H3122=24.76%). In addition, a combination of 0.45 µm and 300 KDa pore size filters allowed the concentration EVs containing *ALK* fusion transcripts.

Conclusion

Our results show that EV-associated RNA is a promising approach for the detection of *ALK* fusions in plasma from LC patients.

Acknowledgments

DTS, TENTACLES, ERDF/ESF ("A way to make Europe"/ "Investing in your future"), BECAS MB-Erasmus fellowship.

P18. Proof of concept of using a membrane sensing peptide for sEVs affinity-based isolation

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Keywords: sEVs, purification, affinity chromatography, bradykinin-derived peptide

Differential ultracentrifugation and size exclusion chromatography are the mostly used EVs isolating methods, but none of them can efficiently purify EVs from all contaminants present in a complex biofluid sample. Until now, affinity-based methods may not be a good option as there is no a known universal marker of EVs, leading to the selection of a given subpopulation depending on the capture antibody and membrane protein composition of EVs. Recently, a membrane-sensing peptide, derived from bradykinin and that selectively binds to highly curved membranes was reported and applied in a microchip-based method to analyse small EVs (sEVs) (Gori et al., 2020).

Our objective is to study the capacity of this peptide to work in selectively isolating sEVs from conditioned media through an affinity chromatographic method.

To address it, the membrane-sensing peptide was modified with a poli-His tag to allow binding to divalent cations-carrying agaroses. After peptide-agarose binding under rotation, concentrated conditioned medium from melanoma SKMEL-147 cell line was incubated ON at 4ºC with the agarose under rotation. We used different antibodies to estimate peptide (anti poly-His) and sEVs (anti-CD81) binding. We used anti-ApoB antibody to analyse lipoprotein contamination.

Firstly, we observed that cobalt agarose was the most efficient cation in peptide binding. We optimized the amount of peptide and incubation time necessary for a proper EV attachment. We did not observe unspecific binding of lipoproteins. A modified agarose with a longer spacer arm resulted in better peptide and EVs binding. A branched peptide was also tested. Finally, we did some EV recovery test, and binding and elution conditions were set up.

In conclusion, bradykinin-derived peptide is a promising method for sEVs isolation, which allows proper

purification without any bias for a given protein marker.

P19. Standardization of an ELISA test for direct CD73 functional evaluation of MSC-derived extracellular vesicles

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Keywords: Adenosine, inflammation, CD73, quantitation, potency assays

Introduction

Extracellular purines (ATP, ADP and Adenosine) are key regulators of inflammatory responses. Specifically, ATP is released to the extracellular space upon tissue injury due to cell necrosis, inducing inflammation. After sequential hydrolysis of ATP, Adenosine is produced, which participates in potent anti-inflammatory signaling. Mesenchymal stromal/stem cells (MSCs) and their derived EVs (MSC- EVs) express CD73 on their surface. CD73 is a 5'-nucleotidase enzyme that hydrolyses 5'AMP to Adenosine producing inorganic phosphate (Pi) as a by-product, which can be measured to assess CD73 activity. Adenosine production through CD73 has been pointed out as one of the anti-inflammatory mechanisms of MSC and MSC-EVs, and the direct quantification of this function can be a valuable potency assay to identify active batches.

Objective

In this study, we aimed to standardize an ELISA test to determine both the presence of CD73 in MSC-EVs and to measure their enzymatic activity, as surrogate markers of MSC-EV quantification and potency.

Methods

SEC-enriched MSC-EVs were either directly adsorbed to 96-well plates or immune-captured by CD63 or CD90 antibodies (EV and MSC markers, respectively). CD73 enzymatic activity was determined by measuring Pi production after addition of 5'AMP at different concentrations. Specificity was ensured by addition of the CD73 specific inhibitor APCP. Finally, in the same wells, CD73 expression was measured on adhered or immune-captured EVs using a biotinylated CD73 monoclonal antibody.

Results

CD73 functional activity was observed through Pi production in both EV directly adsorbed or immune-captured with CD90. Of note, specific immune-capture rendered less functional activity, thus indicating that not all EV carrying CD73 may be expressing CD90. In a similar ratio, not all CD9-positive EV could be detected with CD90 capture. Analogous results were obtained when CD63 was used as capture antibody. Thereafter, CD73 expression was measured in the same wells, and the results were in line with those observed in the functional activity. Then, protein fraction of SEC was analyzed following the same workflow, and no enzymatic activity nor CD73 presence could be detected, indicating CD73 specific presence on EV surface.

Conclusions

Our results suggest that combining a functional test with an ELISA determination in the same wells could be a feasible assay for quantitatively measure the activity of MSC-EV using a low sample volume. This data is of most relevance to establish quantification and potency assays.

P20. Tangential flow filtration as a proper alternative for extracellular vesicles isolation

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CIC bioGUNE

Keywords: Extracellular Vesicles, Isolation, Tangential Flow Filtration, Makers

The extracellular vesicles (EV) isolation process presents as a challenge for scientific and clinical applications. Current techniques, such as ultracentrifugation (UC), size exclusion chromatography (SEC) or density gradient (DG), have limitations: low recovery rates, time consuming, can damage EV structure or poor yield. Tangential Flow Filtration (TFF) seems to be a good alternative; it uses a flow that moves tangential to a porous membrane, avoiding clogging and EV extortion through the pores. Moreover, it is able to process rapidly high volumes of fluid in sterile conditions.

Our goal is to develop an isolation method based on TFF. The work here describes an EV isolation method using TFF that serially combines two different pore sizes. This approach is able to separate and concentrate small EVs (sEVs) secreted by different cell lines. Checking different EV markers (CD63, CD10, LAMP1) and others (caveoline), we could compare the isolation output between TFF and ultracentrifugation (UC), the gold standard.

Using TFF appears to be a proper and better isolation methodology than UC since it is faster and capable of separating and concentrating EVs with a higher yield.

P21. The Oxylipin profile of Human Milk Extracellular Vesicles

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Keywords: Extracellular vesicles (EVs), oxylipins, human milk, Ultra Performance Liquid Chromatography – tandem Mass Spectrometry (UPLC-MS/MS)

Introduction

Extracellular Vesicles (EVs) are nano-size vesicles that are present in all biofluids with especially high abundances in Human Milk (HM). They play a crucial role in cell-to-cell communication and stimulate the neonatal immune system. Furthermore, oxylipins, which are circulating bioactive lipids generated from polyunsaturated fatty acids (PUFAs) by cyclooxygenase, lipoxygenase, and cytochrome P450 enzymes, are involved in inflammatory response.

Objective

This study aims at the characterization of a panel of oxylipins presents in HM and HMEVs to boost our understanding of the anti-inflammatory and pro-healing capacities of HM and potentially, HMEVs.

Methods

EVs were isolated from HM samples by multi-stage ultracentrifugation. EVs were assessed in HM and in isolations using the ExoView R100 platform (NanoView Biosciences). A method for the simultaneous detection of a panel of 56 oxylipins (i.e., 12,13-DiHOME; 17,18-DiHETE; 14,15-DiHETE; 8(S),15(S)-DiHETE; 19,20-DiHDPA; 14,15-DiHETrE; 9,10-DiHOME; Resolvine-D5; Maresin 2; 17-HDHA; 14-HDHA; PGF2α; PGE2 and the semi-quantitative analysis of 43 additional oxylipins) in 200 µL of HM and 200 µL of HMEVs was developed based on reversed-phase UPLC-MS/MS operating in positive electrospray ionization mode and multiple reaction monitoring. For sample clean-up and preconcentration, solid phase extraction was performed using Oasis® MAX plates. The recovered extracts were evaporated and dissolved in 60 µl CH3OH/CH3CN (50/50 v/v).

Results

The method was applied to the analysis of HM and their EVs from a cohort of HM donors (N=15), with pre-term (N=8) and term (N=7) infants. The average particle size and concentrations were 61 nm and $5x10^{13}$ particles/mL in HM and 59 nm and $4x10^{15}$ particles/mL in isolated EVs. Eight and ten of 13 biomarkers were detected with ranges between 0.03 nM – 4.2 nM and 0.04 nM – 124 nM for HMEVs and HM, respectively. The most abundant biomarkers found in HM samples were 12,13-DiHOME and 9-10-DiHOME (linoleic acid derivatives) and 14-HDHA and 17-HDHA (docosahexaenoic acid derivatives) in HMEVs. No significant differences in the concentration of these compounds were found according to the gestational age of the study groups (p-value > 0.05, Wilcoxon ranksum).

Conclusions

Oxylipins are present at detectable concentrations in HM and HMEVs. Future studies will focus on the determination of the oxylipin profiles of HMEVs and the study of their anti-inflammatory and pro-healing capacity, with potential applications in preterm infant care, specifically the prevention of severe intestinal complications such as necrotizing enterocolitis.

Acknowledgments

Instituto de Salud Carlos III, Spain [grant numbers CD19/00176, FI21/00193, CP16/00034, CPII21/00003, PI20/]; Fact-based personalised nutrition for the young (NUTRISHIELD) [grant agreement nr. 818110]

P22. Bone marrow-on-a-chip for the study of EVs in malaria cryptic infections

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Keywords: Vivax malaria, Extracellular vesicles, Organ-on-a-chip, Host-parasite interactions, Bone marrow

Introduction

Intercellular communication mediated by extracellular vesicles (EVs) plays a key role in the pathophysiology of *Plasmodium vivax*, the most widely distributed human malaria parasite. Our group has recently shown that *P. vivax* gametocytes (sexual forms transmitting the disease) are always present in the bone marrow (BM) of infected patients.

Objectives

We hypothesize that circulating EVs from natural vivax infections signals the BM to favour gametocyte differentiation, thus facilitating transmission from this cryptic niche.

Methods

To overcome the ethical and technical difficulty of working with the human bone marrow, we are constructing organs-on-a-chip (OOC), mimicking functional 3D units of this human tissue. The two-channel microfluidic OOC culture chips are fabricated using polydimethylsiloxane (PDMS) and soft-lithography techniques. The BM channel houses ex vivo generated erythroblasts and BM stromal cells embedded in a fibrin-collagen hydrogel mimicking the extracellular matrix of the organ. The vessel channel contains endothelial cells, emulating a blood vessel.

Results

As a first step, we have implemented 3D microcultures in a fibrin-collagen hydrogel to determine the capacity of BM-derived CD34+ cells to differentiate into reticulocytes. Moreover, we are using a transgenic *P. falciparum* line constitutively expressing GFP and luciferase to follow and quantify infections. Notably, after 3D microculture for several days, all stages of the parasite erythrocytic cycle were observed in polychromatic, orthochromatic erythroblasts and reticulocytes. In parallel, we are studying the diffusion properties of labelled EVs within this hydrogel inside the microfluidic compartment under flow conditions. Moreover, chips are being populated with erythroblasts, stromal and endothelial cells as well as with EVs from natural infections, thus recapitulating BM physiological properties. Results on these experiments will be presented.

Conclusions

Here, we present the development of a BM-on-a-chip to study the role of EVs as intercellular communicators during *Plasmodium vivax* infections. This bioengineering device will facilitate studies of the role of EVs at a space and velocity that will ensure interactions with all cells and will contribute to unveil molecular insights of parasite cryptic infections in this hemopoietic tissue. Moreover, it will reduce the use of animals in human experimentation. As parasites in this niche are largely sheltered from antimalarials, these studies will discover alternative control strategies ultimately contributing to malaria elimination.

Acknowledgements
We are grateful to Melanie Giorgi for managerial assistance. This project is funded by "la Caixa" Foundation (LCF/PR/HR21/52410021).

P23. Characterization of extracellular vesicles secreted by thymic regulatory T cells (thyTreg-EVs)

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Keywords: Extracellular vesicles, regulatory T cells

Introduction

Regulatory T cells (Treg) are crucial components of the immune system due to their suppressive function. When detecting an antigen identified as dangerous for the organism, the immune system attacks. Treg cells ensure this response ends after the antigen is neutralized and eliminated, therefore reaching the so-called immune homeostasis.

Because of these properties, Treg cells are currently used in therapy and are mostly obtained from peripheral blood. However, a different cellular profile is observed by using the human thymus as a novel Treg source. These cells (thyTreg cells) seem to manifest more suitable features for therapy than peripheral Treg cells, such as purity, suppressive capacity, stability, and cell numbers without extensive proliferation.

Extracellular vesicles (EVs) constitute an important cell-communicating mechanism that has been broadly studied during the last years. In immunology, regulatory EVs from mesenchymal or Treg cells are thought to be pivotal in maintaining the immune homeostasis. Therefore, EVs are thoroughly studied as a potential treatment for a great variety of diseases. For that, EVs show characteristics that make them an interesting alternative to cell therapies:

- A small size that allows a wider distribution.
- Little risk of differentiation, modification, and proliferation because of their lack of nucleus.
- More feasible storage and stability.

Objective

The aim is to understand thyTreg-derived EVs (thyTreg-EVs), their properties and characteristics. To reach this goal, the following secondary objectives were defined:

- Successfully isolate EVs from thyTreg cells.
- Characterize the obtained EVs by Western Blot and Nanoparticle Tracking Analysis in order to obtain information regarding EV protein enrichment, size distribution and particle concentration.

Materials and Methods

Firstly, thyTreg cells were purified from human thymuses and later cultured and activated. EVs were obtained from the conditioned media by different isolation methods such as Immunocapture, Ultrafiltration and Size Exclusion Chromatography and further characterized by Western Blot and Nanoparticle Tracking Analysis.

Results

EVs from thyTreg cells were characterized by the presence of EV-enriched proteins (CD9 and TSG101) by Western Blot. Size distribution was also studied, observing two main EV subpopulations, one of which proved to be small EV-like (30-150 nm).

Conclusion

These findings show that thyTreg-EVs can be isolated from the human thymus by isolation methods such as Immunocapture, Ultrafiltration and Size Exclusion Chromatography. This is a first step that opens new possibilities regarding EV therapy.

P24. Comparison of the proteomic profile of plasma-derived extracellular vesicles isolated by different methods from patients with leishmaniasis.

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Keywords: Extracellular vesicles; ultracentrifugation; size exclusion chromatography; proteomics; plasma

In the last decade, extracellular vesicles (EVs) have received special attention due to their role in physiological and pathological conditions. Their cargo, representing tissue-specific molecules, can serve as biomarkers for specific diseases, such as visceral leishmaniasis, which is re-emerging in the context of increasing numbers of immunocompromised individuals in the European Mediterranean region. Since plasma is one of the preferred samples for the rapid diagnosis of visceral leishmaniasis (VL), plasma-derived EVs provide a unique source of proteins that can fill up the lack of biomarkers for surveillance of these patients. Isolation of plasma-derived EVs presents some unique technical and analytical challenges that need to be further addressed.

In order to determine the most effective assay method for EV isolation from low volume of plasma samples stored at -80oC for 1-9 years, a comparison of plasma-derived EV proteomic profiles was conducted using three isolation methods using 500ul of human plasma samples.

EVs were isolated using three different isolation methods: ultracentrifugation (UC method), a commercial qEV size exclusion chromatography (SEC method), and a combination of these two methods using first purification with SEC and then further concentration with UC (SEC &UC method).

Size, concentration, and morphological integrity of the particles were analyzed by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM), showing that all methods isolated exosomes within the expected size and morphology (100-200 nm). In the EVs sample isolated by SEC, there were multiple vesicle subpopulations with different sizes and concentration peaks. However, UC had the highest particle number and protein content, which was confirmed by NTA quantification and BCA protein assay, respectively. In addition, Western blot analysis was performed to verify differential expression of both EV proteins CD63 and CD9. Exosome purity was determined by the ratio between particle concentration and protein content, which was significantly lower in exosomes isolated by UC. In a final step, we analyzed the protein content of EVs by liquid chromatography-tandem mass spectrometry (LC-MS /MS) and found differences in the identified proteins associated with EVs.

Our comparative study has helped us to select the most appropriate method for analyzing these type of clinical samples based on the proteomic profile and characterization of EVs. Future research will include the search for biomarkers of cure in patients with visceral leishmaniasis.

P25. Detecting and diagnosing the liver stage of *Plasmodium* infection in circulating extracellular vesicles

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Keywords: Malaria; *Plasmodium*; Liver stage infection; Extracellular vesicles; Biomarkers.

Introduction

Malaria remains a global health problem, with half of the world's population at risk of infection. In 2020, 241 million new cases were reported, leading to 627,000 deaths. This disease is caused by protozoans of the genus *Plasmodium* and is transmitted by infected female *Anopheles* mosquitoes. *Plasmodium* parasites exhibit a complex life cycle, with two stages of infection in humans: the liver stage and the blood stage. The liver stage of infection is the initial, obligatory, and asymptomatic phase of mammalian infection before the onset of malaria symptoms. Therefore, the early detection of this stage of infection is essential to hamper its progress into the blood and the ensuing pathology.

Objective:

We propose to identify *Plasmodium*-specific biomarkers of liver stage infection associated with extracellular vesicles (EVs). In this way, we expect to be able to obtain a *Plasmodium* infection-specific fingerprint to be used for its early detection and diagnosis.

Methods

As a proof-of-concept, the proteomics identification of *P. berghei* proteins associated with EVs in mice with an ongoing *P. berghei* liver infection was performed using size exclusion chromatography followed by mass spectrometry (MS). The acquired spectra from MS were analyzed using the Proteome Discoverer and the MaxQuant software.

Results

Notably, parasite proteins were identified albeit not in all biological replicates and with one unique peptide.

Conclusion

Thus, to increase the MS signal, we are implementing direct immune-affinity using a liver specific antibody. In the future, we will apply the same principles used on the rodent model system to investigate and characterize human plasma samples. Results of these experiments will also be presented.

P26. Proteomics analyses of Extracellular Vesicles from peritoneal exudates reveal the elicited inflammatory/autoimmune response in the chronic graft versus host disease lupus model

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Keywords: Extracellular vesicles, cGVHD lupus-like, CD38, proteomics, inflammation

Introduction

Systemic Lupus Erythematous (SLE) is a complex autoimmune disease, characterized by increased cellular death by apoptosis and defective clearance of apoptotic bodies and nuclear fragments, resulting in increased antinuclear antibody production. We have used the inducible bm12 lupus model, where an abnormal chronic graft versus host disease (cGvHD) is induced in non-autoimmune C57BL6 mice (WT) by the adoptive transfer of MHC Class II IA-incompatible bm12 spleen cells. In the absence of CD38 in the host (CD38KO mice), we have observed a significant decrease in the severity of the disease (doi: 10.3389/fimmu.2021.713697).

Objectives

Analyze the protein composition and function of EVs released in the peritoneal cavity of cGvHD mice, to identify predictive or diagnostic biomarkers of the disease

Methods

EVs were isolated by qEV size-exclusion-chromatography (SEC) from peritoneal exudates of cGVHD lupus-mice, two weeks after the adoptive transfer of bm12 cells. Protein extracts from isolated EVs were analyzed by LC-MS/MS. The protein identification was performed with ProteinScape, and MASCOT data searching using Swiss-Prot database. To quantify protein abundance, the emPAI-based method was used. We used ClueGO and CluePedia apps within the Cytoscape software for functional analysis.

Results and Conclusions

Within the common proteins significant for Fold Change, are multiple complement factors (C3, C1QB, C1QC, C4B, properdin and CFB) or fibrinogen. Among proteins with increased abundance in EVs-CD38KO, are TGM2, BIRC3 and CD5L, involved in the regulation of cell death processes. The proteins over-represented in EVs-WT show a completely different profile. Among them, Pentraxin 3 (PTX3) is involved in the regulation of phagocytosis, and the vitamin D binding protein, DBP is involved in the inactivation of macrophages and in the chemoattraction of neutrophils to inflamed tissues via C5α. The quantitative and functional proteomic analyses of the EVs allowed us to distinguish whether the auto-inmune response implicated increased cell death rate versus defective clearance of the apoptotic bodies and cellular remains. EVs- WT showed increased abundance of proteins with pro-apoptotic, chemoattractant, or proinflammatory capabilities. Likewise, proteins related with proliferation, differentiation and activation of immune cells, and with the immunoproteasome. Histones, characteristic of neutrophil extracellular traps, were also identified. In contrast, in EVs-CD38KO predominated terms and proteins involved in activating es, which may be crucial to eliminate cellular remains and keep the inflammatory and autoinmune processes under control.

P27. *Comparative study of pEVs isolated from different platelet sources in wound healing.*

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Keywords: Platelet-derived Extracellular Vesicles (pEVs), platelet lysates (PL), fresh platelet concentrates (fP), aged platelet concentrates (aP), wound healing.

Introduction

Platelet-derived Extracellular Vesicles (pEVs) have been proposed as an emerging therapeutic asset in regenerative therapies. However, there are still many studies that need to be addressed to reach the clinics, for instance, the method used for platelet concentrate generation or activation, or the age of the platelet source. In this work, we performed a comparative study of pEVs obtained from different platelet concentrate sources in terms of their characteristics and functionality in wound healing.

Material and Methods

pEVs were isolated by Size Exclusion Chromatography (SEC) using 0.9% NaCl as eluant from three different sources: (1) platelet lysates (PL), (2) fresh platelet concentrates (fP) and (3) aged platelet concentrates (aP). pEVs obtained were characterized by Transmission Electron Microscopy (TEM), Western Blot (WB), Nanoparticle Tracking Analysis (NTA), miRNA content using Tanscriptome Analysis Console (TAC) and their functionality was evaluated by an *in vitro* Wound Healing Assay, a cell viability assay (PrestoBlue™) and a cytotoxicity assay (LDH) on hTERT/hNOF fibroblasts.

Results

The PL-EVs, fP-EVs and aP-EVs presented significant differences in their characterization, for instance, the amount of pEVs and their size distribution. The miRNA analysis showed differential content on the pEVs evaluated (PL-EVs and aP-EVs). In addition, in the functional study PL-EVs treated cells showed significantly higher wound closure.

Conclusion

The obtained results show that a better functionality was obtained when isolating pEVs from PL, achieving also a higher pEVs enrichment yield. These functional differences could be explained, at least in part, by differences in their miRNA cargo. Thus, we propose PL as the most suitable pEVs platelet source for regenerative therapies.

P28. Exogenously loading monocytes extracellular vesicles with mRNA

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Keywords: mRNA therapeutics, exogenous loading, extracellular vesicles, therapeutics use

Introduction

mRNA has been recently recognized as a potent macromolecule for prophylaxis and therapeutics. However, it is highly labile and it cannot penetrate plasmatic membranes. Therefore, its protection using a nanosystem is required for its in vivo use.

Objective

Our work aims the set up of exogenous mRNA loading on extracellular vesicles (EVs) obtained from monocytes. EVs have advantages over synthetic carriers: they occur naturally; transfer their cargo to recipient cells; and are naturally loaded with biological molecules.

Methods

EVs were isolated from human and murine monocyte cell lines, by differential centrifugation, and characterized by transmission electron microscopy (TEM), Western blot (WB), and nanoparticle tracking analysis (NTA). EVs were loaded with two model mRNAs (GFP as a reporter or OVA as a model antigen) by passive diffusion. The mRNAs loading and stability, after EVs incubation with RNases, were assessed by fluorescence measurement of labelled mRNA. Finally, *in vitro* preliminary studies to evaluate the uptake of EVs and mRNA expression by recipient cells were performed, by flow cytometry and fluorescence microscopy.

Results

EVs were characterized using three techniques. NTA and TEM confirmed EVs sizes below 200 nm. TEM showed an EVs homogeneous population, with the expected morphology. WB confirmed the presence of EV-related proteins. Importantly, mRNA loading efficiencies into EVs higher than 75% were found, which enabled stable mRNA levels for longer than 6h, in the presence of nucleases. Finally, *in vitro* experiments confirmed that fluorescently-labelled mRNAs loaded into EVs can penetrate and be expressed in recipient cells.

Conclusions

Preliminary results support the idea of using EVs as mRNA delivery systems, given the ability of mRNA artificially-loaded EVs to penetrate recipient cells, which can then express the delivered mRNAs.

Funding

European COST Action- STRATAGEM CA17104 (short-term mission fellowship) and Grupo Español de Investigación en Vesículas Extracelulares (GEIVEX mobility fellowship) allowed CF's work at i3S, Portugal.The MHV group is supported by FEDER through COMPETE 2020 and by FCT (POCI-01-0145-FEDER-030457).

P29. Exosomal miRNAs changes following cancer therapy-induced cardiotoxicity

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Keywords: Cardiotoxicity, exosomes, microRNAs, biomarkers, H9c2

Introduction

Cardiotoxicity is a relatively frequent and potentially serious side effect of cancer treatment on heart function and structure. Cardiotoxicity is particularly common in breast cancer patients, in which anthracyclines and trastuzumab are commonly used. These antitumoral agents have been associated with heart failure. Current cardiac biomarkers lack of sensitivity, and cardiotoxicity is typically identified by a reduction in left ventricular ejection fraction, assessed by echocardiography. However, this detection occurs when there is already a damage on the myocardium. Thus, there is an urgent need of new sensitive biomarkers to be incorporated into diagnostic algorithms for early detection, adequate risk assessment, prognosis, and adoption of cardioprotective strategies. Exosomes are small extracellular vesicles of endocytic origin that mediate cell-to-cell communication, both locally and systematically, by transferring cellular cargoes including microRNAs (miRs). MiRs have been extensively studied in a wide range of regulatory functions of cellular pathophysiological processes, including cardiovascular diseases.

Objective

In this work, we aim to determine if chemotherapy-induced cardiotoxicity modulates exosome-derived miRs in a rat cardiomyoblast cell line (H9c2).

Methods

Minimal individual and combined cardiotoxic concentrations of doxorubicin and trastuzumab were determined by viability and dose-response MTT assays. Cardiotoxicity was assessed by oxidative stress (dihydroethidium) and apoptosis/necrosis (annexin-V/propidium iodide) studies through flow-cytometry analysis. MiRs were extracted from isolated exosomes from cell-culture media and RT-qPCR was performed to determine exosomal miR profile. In silico KEGG pathway targeting and enrichment analysis was performed for the differentially expressed miRs.

Results

Our results showed that doxorubicin reduces H9c2 viability, which is enhanced in combined therapy. Exosomal miR profile was altered under cardiotoxic conditions. In silico analysis showed enrichment in signal transduction pathways that could be involved in the damage mechanism.

Conclusion

These data suggest that exosomal-miR profile could be used as a subclinical biomarker of cardiotoxicity but also provide further insight into the pathways involved in cardiac injury.

Acknowledgements

This work was supported by grants from the Spanish Society of Cardiology (SEC 2019), Fundación Mutua Madrileña (AP153482014), Xunta de Galicia: Programa de Consolidación de Unidades de Investigación Competitivas do SUG (GPC 2016/022 and GRC 2019/02), Centro Singular de Investigación de Galicia 2019–2022 (ED431G 2019/02), European Union European Regional Development Fund (ERDF), and Centro de Investigación Biomédica en Red Enfermedades Cardiovasculares (CIBERCV).

P30. Extracellular vesicles from the conjunctiva: Isolation, characterization, and antioxidant effect.

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Keywords: Conjunctiva, Cytotoxicity, Extracellular Vesicles, Flow Cytometry, ROS.

Introduction

Extracellular vesicles (EVs) play an important role in intercellular communication and can participate in many physiological and pathological processes. Although there is increasing knowledge about ocular EVs, their presence and role in the conjunctival tissue of the ocular surface is limited.

Objective

To use the Immortalized Human Conjunctival Epithelial Cell line (IM-HConEpiC) and human conjunctival mesenchymal stromal cells (Conj-MSCs) as a source of conjunctival EVs, to characterize them and to analyze their antioxidant effect.

Methods

IM-HConEpiC and Conj-MSCs cells isolated from cadaveric donor tissues were cultured with DMEM/F12 and DMEM supplemented medium, respectively. To obtain EVs, cells were grown to 70% confluence and cultured with EVs-free FBS supplemented medium for 48h. Secretome was collected and EVs isolated by differential ultracentrifugation (UC). The ExoStep Kit was used for the detection and quantification of EVs by flow cytometry. A Beckman Coulter CytoFLEX LX flow cytometer was used to individually characterize the EVs and expression of EV markers was determined by EV bead conjugated flow cytometry. EVs were morphologically analyzed by atomic force microscopy (AFM) and their size distribution was determined by dynamic light scattering (DLS). To analyze the effect of conjunctival EVs on oxidative stress, IM-HConEpiC were exposed to 50 µg/ml Conj-MSCs-derived EVs, then loaded with 2',7'- Dichlorofluorescin diacetate and finally exposed to 200 µM H2O2 to induce oxidative stress. Fluorescence was measured to quantify reactive oxygen species (ROS) production. The effect of Conj-MSCs-EVs on IM- HConEpiCs viability was determined by alamarBlue assay. Data were shown as mean ± standard deviation.

Results

IM-HConEpiC-derived and Conj-MSC-derived EVs expressed the EV markers CD9, CD63, CD81, and CD147. Main EV subpopulations were <300nm, having a similar violet SSC to 80nm Apogee beads, but larger differential subpopulations were also observed. That was corroborated by DLS. Zeta-potential determined that the extracted EVs were stable. EVs were morphologically round as determined by AFM. Conj-MSC-derived EVs significantly reduced ROS levels to 0.42±0.46 ($p<0.0001$), compared to untreated cells whose ROS levels were set as 1, and equivalent to that of 250 µM ascorbic acid (0.50±0.59; $p<0.0001$), used as control. Also, EVs did not negatively affect cell viability.

Conclusion

It is possible to isolate EVs from conjunctival cells by UC. Conj-MSC-derived EVs showed no cytotoxicity and promising antioxidant activity on IM-HConEpiC, warranting further research to determine their potential therapeutic effect.

Acknowledgements
This work was supported by the Agencia Estatal de Investigación (AEI), Ministerio de Ciencia, Innovación y Universidades (MCIU) and Fondo Europeo de Desarrollo Regional (FEDER); Grant number RTI2018-094071-B-C21. RC-I was supported by the "Ministerio de Ciencia e Innovación" PRE2019-089985 predoctoral contract.

P31. Funcionalization of MSC-EVs with quercetin as a potential senolytic treatment for atherosclerosis

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Keywords: Atherosclerosis, senescence, extracellular vesicles, senolytics.

Cardiovascular disease is the main cause of death worldwide, atherosclerosis being the first underlying condition. Cellular senescence, defined as an irreversible and stable cell cycle arrest, has been associated with the establishment and progression of age-related diseases, such as atherosclerosis. Moreover, senescence is closely linked to inflammation, which is very present within the atherosclerotic plaque.

Senolytic drugs are compounds that induce the apoptosis of senescent cells. One of the most used is quercetin (Q), a natural flavonoid that targets PI3K/AKT, BCL-2/BCL-XL and p53/p21/serpine cellular pathways.

In the context of cardiovascular disease, several studies have demonstrated that mesenchymal stem cell-derived EVs (MSC-EVs) exert anti-inflammatory effects *in vitro* and *in vivo*. The use of EVs for drug delivery offers some advantages, such as the increase of the drug's bioavailability and the capability of passing through the blood-brain barrier. Furthermore, EVs can be engineered in order to improve their properties. Thus, the objective of our study is the combination of MSC-EVs and quercetin treatments by using EVs as a vehicle for quercetin delivery in a context of atherosclerosis.

In this part of the study, we present EVs' isolation from immortalized adipose tissue MSCs by ultrafiltration and size exclusion chromatography (SEC) and their characterization by nanoparticle tracking analysis (NTA), western blot and transmission electron microscopy (TEM). In addition, optimisation of the conditions for quercetin's internalisation has been performed. The functionalization of the EVs with quercetin has been evaluated by high performance liquid chromatography (HPLC) and fluorescence spectroscopy. Moreover, preliminary *in vitro* experiments suggest a senolytic effect of the functionalized EVs in human endothelial cells. Finally, ongoing experiments decorating MSC-EVs with aptamers are being conducted with the objective of specifically delivering the treatment to the vascular tissue.

In conclusion, we have been able to encapsulate the senolytic drug quercetin in MSC-EVs and the first *in vitro* experiments indicate a senolytic effect of the treatment in senescent human endothelial cells.

Acknowledments

This study has been financed by the Programa de Atracción de Talento Modalidad 1 of the Comunidad Autónoma de Madrid 2019-T1/IND-13794

P32. *In vivo* loading of recombinant proteins into extracellular vesicles for the treatment of lysosomal storage diseases

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Keywords: Extracellular Vesicles, Enzymatic Replacement Therapy, Lysosomal Storage Diseases, Recombinant Proteins, Cell Penetrating Peptides

Introduction
Extracellular vesicles (EVs) are increasingly being studied as drug delivery systems (DDSs) due to their high blood stability and low immunogenicity. Moreover, EVs can be naturally loaded with therapeutic proteins through the genetic modification of producing cells. Remarkably, vehiculization of lysosomal enzymes in EVs has proved to increase their stability and catalytic activity, making EVs a promising drug nanocarrier for the development of the enzyme replacement therapy in lysosomal storage diseases (LSDs).

Objective
Production, isolation, functionalization and testing of HEK293F and CHO DG44-derived EVs as DDSs for three model LSDs, namely Fabry, pycnodisostosis and Niemann Pick diseases.

Methodology
Cells were transfected using polyethylenimine (PEI) with plasmids coding for the enzymes alfa-galactosidase A (GLA) and cathepsin K (CTSK) and the chaperone Heat Shock Protein 70 (Hsp70) as therapeutic proteins for Fabry, pycnodisostosis and Niemann Pick diseases, respectively. EVs from the conditioned medium were diafiltered and concentrated using Tangential Flow Filtration (TFF) and subsequently purified by Size Exclusion Chromatography (SEC). Morphology and composition of EVs was characterized by NTA, DLS, WB and TEM and the functional activity of loaded proteins was verified by crude enzymatic activity assays and *in vitro* cellular assays. Finally, a cell-penetrating peptide developed in-house named F7 was chemically conjugated onto EVs surface and the changes in the EV internalization rate were monitored by flow cytometry.

Results
NTA and DLS data as well as TEM images showed an optimal purity-yield ratio of EVs isolated by SEC. Vesicles also displayed appropriate size and morphology. WB allowed to detect basic EV protein markers (e.g. CD63, CD81, Alix) and to confirm the presence of recombinant proteins in the EV cargo. Enzymatic activity of EV-GLA was 10-fold higher than clinical soluble GLA (alfa-agalsidase) and *in vitro* efficacy was also notably higher (87%). Conjugation with F7 peptide increased EVs internalization rates in ranges of 3 to 4.5 fold.

Conclusion
EVs loaded with GLA, CTSK and Hsp70 were optimally isolated from transiently transfected cells and accurately characterized. EV-GLA showed higher *in vitro* enzymatic activity and efficacy than free enzyme. In addition, EV internalization rates can easily be enhanced by chemical functionalization of the EV surface. These data contribute towards the implementation of EVs as an efficient DDS for lysosome-related therapeutic proteins.

Acknowledgements
This work has been funded by the P21/00936 grant of the Instituto de Salud Carlos III and co-funded by the European Regional Development Fund (ERDF), the European Mimic-key project (GA 964386) and the ADVERT- Proyectos Valorización CIBER-BBN. DBM-A was supported by a FPU grant (FPU20/04210) from the Ministerio de Educación y Ciencia and MMA received a PERIS-FI grant (SLT/017/20/000181) from the Catalan Government.

P33. Nanoengineering exosomes with nanoparticles

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Keywords: Exosome, Exosome engineering, Nanoparticles, Noble metals

Introduction

Exosomes are small membrane vesicles released from mostly all kinds of cells. They take part in cell-to- cell communication and have been shown to play a significant role in the diagnosis as well as in the treatment of many diseases. Regarding the latest, they can be used as drug delivery vehicles due to their tropism for the cells which they are secreted from. One of the cargoes they can be loaded with are nanoparticles, which are capable of modulating physiological or pathological processes and endow exosomes with unique properties. However, the methods used to introduce them usually damage exosomes and they cannot be further utilized. On the other hand, bio-orthogonal catalysis, often based on noble metal-catalysed chemistry, has emerged as a field where prodrugs are converted into bioactive drugs, being a promising solution to the drug side effects caused by off-target effect. In this work, exosomes engineered with metal nanoparticles will be considered as vectors to deliver biogenic nanoparticles and activate bio-orthogonal reactions without compromising exosomes' features.

Methods

Exosomes are isolated from cells that have been harvested for 48 hours and, once isolated, they are quantified, loaded with a noble metal precursor and subjected to a high-pressure CO reduction process. Afterwards, characterization through Western blot, TEM, MP-AES and Nanosight of the loaded exosomes is made to ensure that the desired nanoparticles have been produced and that exosomes have not been damaged. Additionally, cytotoxicity assays with Au and Pt salt precursors have been performed.

Results

In cytotoxicity assays with Au and Pt salt precursors, a concentration of 0,031 mM and 0,25 mM, respectively, was proved not to be toxic on human placenta mesenchymal stem cells (hpMSC).
A protocol to achieve a high load of catalytically active Pt and Au nanoparticles inside exosomes was provided and allowed the loading of these vesicles without disrupting their membranes, hence exosomes' shape and properties were maintained, which permitted their use for therapeutic purposes.

Conclusion

Bio-orthogonal catalysis is a method to introduce biogenic metallic nanoparticles inside exosomes without affecting their inherent functionality, as they do conventional methods such as electroporation, or sonication. The protocol provided here involves a multistage process that can be conducted by any researcher with basic biology and chemistry knowledge in three days of time and whose technology is proven to be scalable.

Acknowledgements

VS acknowledges Fundación Ramón Areces (XX concurso nacional-ciencias de la vida y la materia) and beca Leonardo a Investigadores y creadores culturales 2021 de la Fundación BBVA.

P34. Nanoparticles-like vesicles from food and their impact on S. Aureus and E. Coli growth

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Keywords: Nanoparticles; vesicles; Bacterial growth; food; prebiotics

Introduction

The increase of multiresistant bacteria has generated the necessity to search for new substances. Natural products like edible plants and other food have been traditionally used for their role as antibacterial agents. In this sense, recently, it has been discovered that Nanoparticles-Like Vesicles (NLVs) from different food could exert antimicrobial effects.

Methods

We selected three samples: pomegranate juice, pulp carob and honey. NLVs were isolated by differential followed by size-exclusion chromatography (SEC). The protein cargo was determined by BCA assay. Escherichia coli and Staphylococcus aureus were co-incubated with 25-50 µg/mL of NLVs in 96-well plates at 37°C, with agitation at 400rpm for 24h. The bacterial growth curve was measured at 595nm (OD595) every hour.

Results

Carob-NLVs (C-NLVs) and Pomegranate-NLVs (Pg-NLVs) at 50 µg/mL increased E. Coli growth whereas concentration at 25µg/mL did not show difference concerning PBS control. Respect to Honey NLVs (H-NLVs), low concentrations also showed an increase in E. Coli growth but it decreased as the concentration increased. However, NLVs from the three samples reduced the S. Aureus growth.

Summary/Conclusion

Our results demonstrate that the origin and concentrations of the NLVs exert different effects on bacterial growth. C-NLVs and Pg-NLVs at higher concentrations and lower concentrations of H-NLVs could use as a prebiotic for E Coli. However, for S Aureus, NLVs could be potential antimicrobials.

Acknowledgements

Supported by AEI-Ministerio de Ciencia e Innovación, Spain (Grant PID2019-105713GB- I00/AEI /10.13039/501100011033), and Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Spain (Grant PROMETEO/2020/071). Part of "Red Traslacional para la Aplicación Clínica de Vesículas Extracelulares, Tentacles, (RED2018-102411-T, Agencia Estatal de Investigación, Spain.

P35. Pasteurization influences on honey vesicle-like nanoparticles and its biological activity

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Keywords: Honey; nanoparticles; vesicles; pasteurization; inflammation

Introduction

The presence of vesicle-like nanoparticles it has been demonstrated in raw honey (H-VLN). However, before its commercialization, honey suffers different industrial operations such as pasteurization. Here, we compared H-VLN from raw and commercial pasteurized honey of two different botanical origins (eucalyptus and rosemary).

Methods

Honey samples were provided by a local industry (Valencia, Spain). H-VLNs were isolated by differential centrifugation and size-exclusion chromatography (SEC) and characterized using Nanoparticle Tracking Analysis (NTA) and Transmission Electron Microscope (TEM). The total protein amount was measured with the BCA assay. Bioactive properties and safety of H-VLNs were studied in-vitro in CACO-2, HEPG-2 and THP-1 cells. Concentrations of H-VLNs between 2.5 and 20 µg/mL protein were evaluated.

Results

H-VLNs from raw honey showed significant higher particle concentrations than pasteurized. In any case, the protein content of the rosemary H-VLNs was lower than eucalyptus. Treatment with higher concentrations (20 µg/mL) of H-VLNs resulted in reduced cell viability, being more significant in pasteurized honey. Finally, pasteurization had an effect on the anti- inflammatory and citoprotective activity of H-VLNs.

Summary/Conclusion

The industrial processing of honey could affect the protein content and integrity of H-LVNs. Moreover, the bioactive properties of H-LVNs could alter due to the botanical origin and processing of honey. Further research needs to be focused on honey processing and its impact on H-LVNs and health benefits.

Acknowledgements

Supported by AEI-Ministerio de Ciencia e Innovación, Spain (Grant PID2019-105713GB- I00/AEI /10.13039/501100011033), and Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Spain (Grant PROMETEO/2020/071). Part of "Red Traslacional para la Aplicación Clínica de Vesículas Extracelulares, Tentacles, (RED2018-102411-T, Agencia Estatal de Investigación, Spain.

P36. Pomegranate-derived extracellular vesicles as therapeutic nanocarriers of exogenous miRNAs

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Keywords: 'Extracellular vesicles', 'miRNA', 'pomegranate', 'cross-kingdom gene regulation'

Introduction

The wide-ranging biological activities of microRNAs (miRNAs) promoted research on disease mechanisms and is suggesting appealing therapeutic applications. When unprotected, miRNAs suffer from rapid degradation and appropriate strategies need to be developed to improve their therapeutic potential. As miRNAs can be naturally transported by extracellular vesicles (EVs), the latter have been proposed as specific transport means for drug delivery, conferring stability and increasing resistance against RNase degradation. However, a standard, reproducible and cost-effective protocol for EV isolation as well as a natural source of EV is lacking.

Objective

To study the potential of pomegranate-derived EVs as a therapeutic vehicle for extracellular RNA drug delivery.

Methods

EVs were isolated from pomegranate, combining ultracentrifugation and size exclusion chromatography methodology. The isolated EVs were then loaded with exogenous miRNAs. The miRNAs uptake was tested in intestinal Caco-2 cell line.

Results

Pomegranate-derived EVs were efficiently isolated, characterized and loaded with exogenous miRNAs. Loaded EVs efficiently ferried extracellular RNAs to cells.

Conclusion

An edible fruit (i.e., pomegranate) is proposed as a natural source of EVs, which are capable of transporting exogenous miRNAs with potential therapeutic effects.

P37. Therapeutical use of EVs derived from platelets and from mesenchymal stromal cells: a comparative miRNA study

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Keywords: Extracellular Vesicles, miRNA, osteoarthritis, collagen, platelets

Introduction

Extracellular Vesicles (EVs) have emerged as potential functional therapeutic effectors in regenerative medicine, for example in the field of osteochondral injuries such as osteoarthritis (OA). However, their cargoes remain yet to be thoroughly evaluated; being non-coding micro-RNAs (miRNA) among the most interesting cargoes in the vesicles, since they are important regulators of gene expression. In this study, our aim was to evaluate the therapeutic effect of platelet lysates (PL)-derived EVs (pEVs) and human umbilical cord mesenchymal stromal cells (hUC-MSC) derived EVs (cEVs) on an osteoarthritis in vitro model and to study their differential miRNA content to contribute elucidating their biological effect.

Material and Methods

Isolated EVs by Size Exclusion Chromatography (SEC) from PL and hUC-MSC conditioned medium were characterized by Transmission Electron Microscopy (TEM), Western Blot (WB) and Nanoparticle Tracking Analysis (NTA). EVs therapeutic functionality was evaluated by collagen quantification on an OA in vitro model using human cartilage explants. Then, EVs miRNA content was evaluated using the gene chip miRNA 4.0 array and the data was analysed with the Transcriptome Analysis Console (TAC).

Results

pEVs and cEVs presented significant differences in their morphological characterization in terms of concentration and size distribution. The biological effect was also different since cartilage explants treated with pEVs showed statistically higher collagen content compared to the explants treated with cEVs. In agreement, a differential profile in miRNA content is observed between both EVs groups.

Conclusion

pEVs and cEVs show different miRNA content that might influence their biological activity on the OA-induced cartilage explants. However, a more exhaustive analysis of the EVs molecular content is needed to elucidate the mechanisms underlying their biological effects.

ABSTRACTS