Veterinary Parasitology 236 (2017) 22–33

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Isolation and characterization of exosomes derived from fertile sheep hydatid cysts

Mar Siles-Lucas[a,*], Carlos Sánchez-Ovejero[a], María González-Sánchez[a], Esperanza González[b], Juan Manuel Falcón-Pérez[b,c], Belgees Boufana[d,e], Federica Fratini[d], Adriano Casulli[d,e], Raúl Manzano-Román[a]

[a] Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), Cordel de Merinas 40-52, 37008 Salamanca, Spain
[b] Laboratory of Exosomes, Metabolomics Unit. CIC bioGUNE, CIBERehd, Derio, Spain
[c] IKERBASQUE, Basque Foundation for Science, Bilbao, Spain
[d] Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
[e] WHO Collaborating Centre for the Epidemiology, Detection and Control of Cystic and Alveolar Echinococcosis (in Animals and Humans), Italy

ARTICLE INFO

Article history:
Received 19 September 2016
Received in revised form 16 January 2017
Accepted 21 January 2017

Keywords:
Echinococcus granulosus
Cystic echinococcosis
Extracellular vesicles
Exosomes
Proteomics
Virulence factors

ABSTRACT

Cystic echinococcosis (CE) is a chronic and complex zoonotic disease. Information on the mechanisms involved in parasite establishment, growth and persistence remain limited. These may be modulated by a crosstalk between extracellular vesicles (EVs). EVs including exosomes and microvesicles are able to carry developmental signaling proteins which coordinate growth and establishment of several parasites. Here, an exosome enriched EV fraction was isolated from hydatid fluid (HF) of fertile sheep cysts. A proteomic analysis of this fraction identified a number of parasite-derived vesicle-membrane associated proteins as well as cytosolic proteins. Additionally, the exosomal enriched fraction contained proteins of host origin. Specific proteins –antigen B2 and TSPAN14- in the exosomal fraction were further assayed by immunoblot and transmission electron microscopy. To the best of our knowledge, this is the first report on the presence of parasite exosomes in fertile hydatid cyst fluid. Further characterization of the exosome cargo will allow the discovery of new markers for the detection of CE in humans and animals, and the treatment of CE patients, and provide new insights regarding the role of these EVs in the establishment and persistence of hydatid cysts.

1. Introduction

Cystic echinococcosis (CE) is a chronic zoonotic disease caused by the metacestode stage of *Echinococcus granulosus* sensu lato (s.l.) species complex and is typically transmitted in a pastoral life cycle involving sheep and dogs. CE has a worldwide social and economic impact on veterinary and public health (Cardona and Carmena (2013), being the cause of million USD annual economic burdens in many countries (Budke et al., 2006; Benner et al., 2010; Venegas et al., 2014). In Europe, high human CE incidence rates are found in pastoral areas e.g., in the Mediterranean basin (Rojo-Vazquez et al., 2011; Brundu et al., 2015; Tamarozzi et al., 2015; Rossi et al., 2016; Van Cauteren et al., 2016; Herrador et al., 2016).

The metacestode stage, or hydatid cyst consists of a fluid-filled bladder-like structure with an inner germinative layer (GL) surrounded by the acellular, parasite-secreted laminated layer (LL), and an adventitial layer generated as a consequence of the host inflammatory response. The LL is a microfibrillar matrix composed mainly of high-molecular-weight carbohydrates and represents the first line of defense for survival of the cyst both mechanically and immunologically (Díaz et al., 2011a, 2011b). The GL reproduces asexually into the cyst lumen, resulting in the formation of brood capsules containing protoscoleces. The GL actively secretes the LL components to the outer surface of the cyst and, together with the protoscoleces, produces different molecules found in the hydatid fluid (HF) contained within the cyst. HF also contains host plasma proteins (e.g., albumin and immunoglobulins), components that cross the adventitial and LL barriers by unknown mechanisms (Silva-Álvarez et al., 2016). Some of the HF components, including antigens B and antigen 5, reach the host and elicit a strong specific antibody response, but the mechanism involved in their transport across the cyst membranes remains unknown.

Understanding parasite biology is needed in order to elucidate and interfere with the mechanisms by which the parasite pro-

* Corresponding author.
E-mail address: mmar.siles@irnasa.csic.es (M. Siles-Lucas).

http://dx.doi.org/10.1016/j.vetpar.2017.01.022
0304-4017/

gresses to the chronic stage of the disease. In this sense, potential virulence factors are crucial for parasite survival and could provide new markers with potential application in the treatment, diagnosis and prognosis of CE patients. Novel specific and specialized mechanisms based on cell-to-cell communication through secreted extracellular vesicles (EVs) have recently been proposed to be highly relevant in this context (Coakley et al., 2015). Additionally, EVs from some parasites are known to transport parasite molecules to different host cells where their effect is exerted, e.g. for immune evasion (Schorey et al., 2015). These EVs include proteins, carbohydrates, lipids, microRNAs and other small RNAs, and are mainly found in specific vesicles known as exosomes.

Exosomes have been identified in a number of parasite species, including several nematodes, trematodes and protozoa, and their influence on the host cells of some of these groups has recently been reported (Schorey et al., 2015). To the best of our knowledge, no studies to date have been conducted on EVs derived from *E. granulosus* hydatid cysts. This is the first report on the isolation of EVs and the purification of exosomes from *E. granulosus* sheep fertile hydatid cysts and their proteomic characterization.

2. Material and methods

2.1. Parasite material

Hydatid cyst fluid (HF) was aseptically obtained from fertile sheep hydatid cysts at the Coreses slaughterhouse (Zamora, Spain) using sterile syringes. The HF from individual cysts was placed in 50 mL falcon tubes and kept on ice during transportation. HF was analyzed under a microscope to determine the presence and viability of protoscoleces by checking flame cell activity. Protoscoleces and other solid material were removed from the HF by centrifugation at 2000 × *g* for 3 min. Protoscoleces-free HF (200 mL) from 15 different fertile cysts exhibiting at least 90% of viable protoscoleces was pooled and stored at −80 °C.

2.2. Extracellular vesicles isolation, exosome purification and characterization

EVs were isolated from the pooled HF as follows: 200 mL of clear HF was centrifuged for 15 min at 5000 × *g*. The supernatant was filtered using low-protein binding 0.22 μm pore filters (Whatman Klari-Flex). The filtered HF was subjected to ultracentrifugation at 110,000 × *g* for 2 h at 4 °C using a Beckman Coulter Optima-XL ultracentrifuge (Beckman Coulter, Spain) with a 70Ti low-angle small volume fixed-angle rotor. The pelleted EVs were then washed with PBS and further ultracentrifuged at 110,000 × *g* for 2 h at 4 °C. The obtained EVs were resuspended in 0.5 mL PBS and stored at −80 °C.

For exosome purification, a discontinuous iodixanol gradient was used as described by Greening et al. (2015) with minor changes. Briefly, solutions of 5, 10, 20 and 40% iodixanol were prepared by mixing appropriate amounts of homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCL, pH 7.4) with an iodixanol working solution (0.25 M sucrose, 6 mM EDTA, 60 mM Tris-HCl, pH 7.4) and a stock solution of OptiPrepTM (60% w/v) aqueous iodixanol solution (Axis-Shield). The gradient was obtained by layering 4 mL of 40%, 4 mL of 20%, 4 mL of 10% and 3.5 mL of 5% solutions on top of each other in a 26.3 mL polyallomer centrifuge tubes (Beckman Coulter). Subsequently, 0.5 mL of PBS containing EVs was overlaid on top of the gradient which was then centrifuged for 18 h at 110,000 × *g* at 4 °C.

Gradient fractions of 1 mL were collected from the top of the gradient and their density directly measured by weighing. Each fraction was then diluted with 10 mL PBS and centrifuged for 3 h at 110,000 × *g* at 4 °C. The resulting pellets were resuspended in 200 μL nuclease-free water and stored at −80 °C for exosome visualization or lysis with RIPA buffer for protein extraction.

Size distribution within exosome-enriched EVs preparations were analyzed by measuring the rate of Brownian motion using a NanoSight LM10 system equipped with a fast video capture and particle-tracking software (NanoSight, Amesbury, U.K.). This nanoparticle tracking analysis (NTA) enables the determination of individual particle size distribution using samples in liquid suspension (Gercel-Taylor et al., 2012). The analysis settings were as follows: detection threshold, 10 Multi; frames processed, 767 of 768; frames per second, 25.62; calibration, 166 nm/pixel; blur, auto; min track length, auto; min expected size: 80 nm; temperature: 24 °C; viscosity: 0.90 cP. The size information of the Optiprep enriched exosomes, including the mean, mode, median and distribution was obtained after analyzing a recorded video.

Cryo-electron microscopy was performed on the same preparations applying a 4 μL droplet of the exosome enriched suspension to a 200 mesh R 2/2 Quantifoil® holey-carbon grid (Quantifoil, Germany). The excess solution was removed using Whatman filter paper and the grid was rapidly plunged into liquid ethane and transferred under liquid nitrogen into the microscope using a side entry nitrogen-cooled Gatan 914 cryoholder (Gatan, USA). Sample analysis was carried out using a JEOL JEM 2200F (Cs = 1.4 mm) transmission cryoelectron microscope (JEOL, USA), with an acceleration voltage of 200 kV and defocus ranging from −1.2 to −2.5 μm, accurately determined by using enhanced power spectra. Images were recorded under low dose conditions (10 electrons per A2) with a 2k × 2k Gatan Utrascan™ 1000 CCD camera (Gatan, USA).

2.3. Proteomic analysis

For the quantification and visualization of proteins in the obtained exosomal fraction, pelleted exosomes were lysed with 200 μL RIPA buffer. The proteins were then reduced and concentrated in a 7.5% polyacrylamide gel. The gel was stained with SYPRO Ruby (ThermoFisher, Spain) following the manufacturerís instructions, for the accurate relative quantification of the protein bands intensity. This was done by image analysis with a ChemiDoc MP Imaging system (Bio-Rad, Spain) in comparison with standards of known concentration. After that, a 20 μg sample was loaded and resolved onto 12% stacking polyacrylamide gels following staining with silver nitrate (mass spectrometry compatible). The stained gel was sliced into twelve portions including all the proteins at different molecular weights. Each slice was digested with trypsin (Kreimer et al., 2015), and the supernatant was purified with C18 stop-and-go two-dimensional chromatography (Rappsilber et al., 2003). The resulting peptides were analyzed in three technical replicates (three injections) by firstly loading in 1% formic acid on a C18 RP-pre-column Symmetry (5 μm particle size, 20 mm × 180 mm; Waters, USA), then on a BEH RP-C18 column (1.7 μm, 7.5 cm × 25 cm; Waters, USA) and ran in LC–MS/MS CID Top20 mode using a 120 min linear gradient to 40% ACN. Mascot software v2.3.2 (Matrix Science, UK) was used to generate peak lists from raw files and to perform database searches against *Echinococcus granulosus* and *Ovis aries* entries in protein databases (Swiss-Prot and TrEMBL). Only proteins with at least one unique peptide with 1% FDR confidence and mascot ion score of 20 were selected for further analyses. Each gel portion was treated as described above using three replicates. The “in silico” studies of the subcellular location and Gene Ontology (GO) assignments for the identified proteins were performed using the UniProt software.