Available online at www.sciencedirect.com

ScienceDirect

Biochimie Open 4 (2017) 61–72

http://www.journals.elsevier.com/biochimie-open

Research paper

Purified horse milk exosomes contain an unpredictable small number of major proteins

Sergey E. Sedykh [a,b], Lada V. Purvinish [a,b], Artem S. Monogarov [a], Evgeniya E. Burkova [a,b], Alina E. Grigor'eva [a], Dmitrii V. Bulgakov [c], Pavel S. Dmitrenok [d], Valentin V. Vlassov [a,b], Elena I. Ryabchikova [a,b], Georgy A. Nevinsky [a,b,*]

[a] SB RAS Institute of Chemical Biology and Fundamental Medicine, 8 Lavrentiev Ave., Novosibirsk 630090, Russia
[b] Novosibirsk State University, Pirogova 2, Novosibirsk 630090, Russia
[c] Institute of Biology and Soil, Far East Division, Russian Academy of Sciences, Vladivostok 690022, Russia
[d] Pacific Institute of Bioorganic Chemistry, Far East Division, Russian Academy of Sciences, Vladivostok 690022, Russia

Received 16 January 2017; accepted 23 February 2017
Available online 1 March 2017

Abstract

Exosomes are 40–100 nm nanovesicles containing RNA and different proteins. Exosomes containing proteins, lipids, mRNAs, and microRNAs are important in intracellular communication and immune function. Exosomes from different sources are usually obtained by combination of centrifugation and ultracentrifugation and according to published data can contain from a few dozens to thousands of different proteins. Crude exosome preparations from milk of eighteen horses were obtained for the first time using several standard centrifugations. Exosome preparations were additionally purified by FPLC gel filtration. Individual preparations demonstrated different profiles of gel filtration showing well or bad separation of exosome peaks and one or two peaks of co-isolating proteins and their complexes. According to the electron microscopy, well purified exosomes displayed a typical exosome-like size (30–100 nm) and morphology. It was shown that exosomes may have several different biological functions, but detection of their biological functions may vary significantly depending on the presence of exosome contaminating proteins and proteins directly into exosomes. Exosome proteins were identified before and after gel filtration by MALDI MS and MS/MS spectrometry of protein tryptic hydrolyzates derived by SDS PAGE and 2D electrophoresis. The results of protein identification were unexpected: one or two peaks co-isolating proteins after gel-filtration mainly contained kappa-, beta-, alpha-S1-caseins and its precursors, but these proteins were not found in well-purified exosomes. Well-purified exosomes contained from five to eight different major proteins: CD81, CD63 receptors, beta-lactoglobulin and lactadherin were common to all preparations, while actin, butyrophilin, lactoferrin, and xanthine dehydrogenase were found only in some of them.

The article describes the morphology and the protein content of major horse milk exosomes for the first time. Our results on the decrease of major protein number identified in exosomal preparations after gel filtration may be important to the studies of biological functions of pure exosomes.

Keywords: Horse milk; Exosomes; Proteins identification; MALDI mass spectrometry

1. Introduction

Mammalian breast milk is known as functionally active nutrient system. However, breast milk contains significant amounts of various factors: many bioactive proteins, peptides and antibodies. These compounds of breast milk promote neonatal

Abbreviations: EVs, extracellular vesicles; FPLC, fast protein liquid chromatography; MM, molecular mass; 2D-electrophoresis, two-dimensional electrophoresis (isoelectric focusing and SDS-PAGE); SDS-PAGE, SDS polyacrylamide gel electrophoresis.

* Corresponding author. SB RAS Institute of Chemical Biology and Fundamental Medicine, 8 Lavrentiev Ave., Novosibirsk 630090, Russia. Fax: +7 (383) 3635153.
E-mail address: nevinsky@niboch.nsc.ru (G.A. Nevinsky).

http://dx.doi.org/10.1016/j.biopen.2017.02.004

growth protecting newborns from viral and bacterial infections and they are usually considered as integral parts of the infant's intestinal physiology [1,2]. Milk is an essential source of a wide variety of proteins; during the last 16 years more than 200 publications examining the milk proteome were appeared in PubMed [2].

Secreted membrane-enclosed vesicles include microvesicles, microparticles, ectosomes, exosomes, apoptotic bodies as well as other extracellular subsets. All of them were collectively called extracellular vesicles (EVs) and currently belong to the rapidly growing field of biology and medicine [3].

Exosomes are 30–100 nm membrane vesicles. They are secreted into the extracellular liquids by different types of cells in various biological fluids: saliva, serum, amniotic fluid, breast milk, and urine [3–6]. Exosomes contain several major mRNAs, microRNAs, proteins, and lipids, and are considered as alternative pathway of secretion. It is shown that exosomes are involved in the development of pathological processes such as growth and spread of cancer, the transfer of infectious agents including the whole virus particles or viral RNA, and prion proteins (for review see Ref. [6]). In addition, recent findings suggest that the exosomes are specialized vesicles involved in intracellular communication in multicellular organisms, carrying on its surface various lipid and protein ligands, and passing the recipient cells are novel proteins and nucleic acid [7–11]. Exosomal components are usually transferred to the recipient cells and can change biological functions of the target cells [3–6]. It was suggested that exosomes may be involved in several neurodegenerative diseases [10,11].

Exosomes containing proteins and/or RNA were obtained from human [12–17], bovine [2,18,19] and porcine milk [20].

It has been suggested that preparations of exosomes may be used for clinical purposes as effective carriers of various drugs including proteins, lipids, and RNA and other compounds to mammalian cells [21]. It was reported that exosomes can deliver curcumin anti-inflammatory agent in vivo to activated myeloid cells and that this drug directing to the cells is associated with therapeutic, but not toxic, effects [22]. It was shown that catalase included into exosomes by special method is stable against degradation by proteases. In vitro exosomes effectively interacted with neuronal cells and after intranasal administration a significant amount of exosomes was detected in the brain of mice with Parkinson's disease [11]. Since catalase of exosomes leads in vivo to significant neuroprotective effects it was proposed that such approach may be useful for treat inflammatory and neurodegenerative disorders. One can assume that to deliver of different drugs to the human cells may be used exosomes of mammals. One of the main available sources of preparative amounts of exosomes is the milk of cows and horses.

To date, no data concerning horse milk exosomes have been published. At the same time, the only article devoted to horse exosomes evaluates these vesicles as markers of erythrocyte regeneration, as horses do not release reticulocytes into the peripheral blood [23]. The authors have proposed that transferrin receptor 1 (TfR1) expressed in exosomes of serum can provide a regeneration of new marker for anemic horses.

Many very different methods were used for isolation of exosomes from milk and other mammalian biological liquids including precipitation by centrifugation in special conditions, ultracentrifugation, ultracentrifugation in density gradients etc. [12–25]. The combination of sequential centrifugations with the increasing number of rpm (from 300 *g* up to 16,500 *g*) is universal and frequently used approach: the final step of ultracentrifugation is usually performed at 100,000 *g* [19,24–26]. Some protocols also contain ultrafiltration through 0.10–0.22 μm filters [25]. A protocol of exosome purification suitable for clinical use was proposed, in which ultracentrifugation into a 30% sucrose/deuterium oxide cushion was added [27]. A method of biological fluids incubation in the ice at acidic pH has also been proposed [28]: neutrally charged exosomes are precipitated and then may be separated by centrifugation. The authors believe that exosomes obtained by this method do not differ from those purified by ultracentrifugation. It should be mentioned that these methods basically allow obtaining samples enriched with exosomes, but not pure ones.

In some publications the analysis of some vesicular and exosomal proteins was carried out. Small membranous vesicles (25–75 nm in diameters) were purified from the ram cauda epididymal fluid by only high-speed centrifugation [29]. These vesicles according to SDS-PAGE protein pattern are specific and significantly differ from those of the seminal plasma, sperm extract and the caudal fluid. After 2D-electrophoresis more than 40 proteins were revealed. Using mass MALDI mass spectrometry analysis approximately 30 proteins were identified. These 30 proteins were grouped into a) vesicle-associated proteins including lactadherin (MFEG8-PAS6/7) and vacuolar ATPase; b) membrane-linked enzymes including neprilysin (NEP), dipeptidyl peptidase IV (DPP-IV), protein G-beta and phosphodiesterase-I (E-NPP3), c) several cytoskeleton-associated proteins including annexin and ezrin; and d) metabolic enzymes.

After 2D-electrophoresis using MALDI MS/MS sequencing there were identified 188 proteins spots of cell-derived exosomes obtained from mouse fibroblast NIH3T3 cells and Ras-transformed NIH3T3 cells (isolated using several centrifugations) many of which were previously revealed in exosomes from cell of other type [25]. However, some proteins, for example, Serpin B6, have been identified as novel for fibroblast exosomes. It was shown that more than 34 proteins including collagen alpha-1 (VI), guanine nucleotide-binding proteins (G proteins), milk fat globule EGF factor 8 (lactadherin), collagen alpha-1 (VI), 14-3-3 species, the eukaryotic translation initiation factors elF-3 gamma as well as elF-5A are accumulated in exosomes (>2-fold) upon Ras-induced oncogenic transformation.

Dendritic cell-derived exosomes were purified by three successive centrifugations at 300 *g*, then at 1200 *g*, additionally at 10,000 *g*, and finally at 110,000 *g* [30]. These preparations of exosomes according to SDS-PAGE analysis contained ~30 protein bands, 11 of which were major proteins corresponding to several cytosolic proteins such as heat shock cognate protein hsc73, annexin II, and heteromeric G protein