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Original Articles

Bovine milk-derived exosomes for drug delivery

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ABSTRACT

Exosomes are biological nanovesicles that are involved in cell–cell communication via the functionally-active cargo (such as miRNA, mRNA, DNA and proteins). Because of their nanosize, exosomes are explored as nanodevices for the development of new therapeutic applications. However, bulk, safe and cost-effective production of exosomes is not available. Here, we show that bovine milk can serve as a scalable source of exosomes that can act as a carrier for chemotherapeutic/chemopreventive agents. Drug-loaded exosomes showed significantly higher efficacy compared to free drug in cell culture studies and against lung tumor xenografts *in vivo*. Moreover, tumor targeting ligands such as folate increased cancer-cell targeting of the exosomes resulting in enhanced tumor reduction. Milk exosomes exhibited cross-species tolerance with no adverse immune and inflammatory response. Thus, we show the versatility of milk exosomes with respect to the cargo it can carry and ability to achieve tumor targetability. This is the first report to identify a biocompatible and cost-effective means of exosomes to enhance oral bioavailability, improve efficacy and safety of drugs.

Introduction

Over the last three decades, a number of nanoparticle-delivery systems have been developed for cancer therapy, including natural and synthetic polymer-based, lipid-based, and organic and inorganic materials [1,2]. However, due to inherent limitations, only a handful of them have been studied in clinics. The encapsulation of doxorubicin in liposomes (Doxil[R]) and paclitaxel in protein-based nanoparticles (Abraxane[R]) represents two of the successful applications [3–5]. Factors that have stalled the clinical introduction of other nanoparticles include high cost, difficulty in reproducibly synthesizing them in sufficient quantities, and/or toxicity issues [2,6]. The development of ideal nanoparticles with attributes such as long circulation time, evasion of the host immune system, and ability to target specific cells, minimum off site toxicity, and ability to carry versatile therapeutics remains elusive [7,8].

Nature-derived nanoparticles could potentially overcome some of the limitations of synthetic liposomes. Among the different secreted membrane vesicles, exosomes intrinsically possess many attributes of a drug delivery vehicle [9,10], such as they: (i) are well tolerated in the body, as evidenced by their wide distribution in various biological fluids (including milk) [11–13], (ii) have longer circulating half-life, (iii) are internalized by other cells, (iv) carry a cargo of macromolecules such as miRNA, mRNA, DNA and proteins that make these vesicles as delivery vehicles of therapeutics [14–16], and (v) are amenable to ligand attachment for tumor targetability.

Although the field of exosome-based therapeutics is in its infancy, the ability to engineer exosomes to display proteins, incorporate specific nucleic acid and protein cargos, load therapeutic agents, its targeted uptake and tolerance *in vivo* has been demonstrated to some extent [14,15]. However, before exosomes are accepted as a delivery vehicle in clinics, the development of biocompatible, economically-viable source and methods for harvesting exosomes, which are effective and well-tolerated *in vivo*, must be demonstrated.

We report here the suitability of bovine milk as a potentially scalable source of exosomes that could serve as a drug delivery vehicle. Bovine milk consumption is generally considered to be safe and to provide important nutritional benefits [17]. Thus, availability, cost and toxicity considerations make bovine milk a suitable natural source for large-scale production of exosomes. We demonstrate that milk-derived exosomes can serve as a vehicle to deliver both hydrophilic and lipophilic small molecules, including chemotherapeutic

Abbreviations: miRNA, microRNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, Phosphate-buffered saline; qRT-PCR, Quantitative reverse transcription-polymerase chain reaction; cDNA, Complementary deoxynucleic acid; SEM, Scanning electron microscopy; AFM, Atomic force microscopy; CUR, Curcumin; WFA, Withaferin A; Anthos, Anthocyanidins; FA, folic acid; PAC, Paclitaxel; DOC, Docetaxel; EMSA, Electrophoretic mobility shift assay (EMSA); UPLC, Ultra-performance liquid chromatography; TNF-α, Tumor necrosis factor-α; LPS, Lipopolysaccharide; MVB, Multivesicular bodies; VEGF, Vascular endothelial growth factor; EGFR, Epidermal growth factor receptor.

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(chemo) drugs. Using *in vitro* and *in vivo* models, we show enhanced biological efficacy of the exosomal formulations. This effect was further increased by the addition of tumor-targeting ligand, folic acid (FA). Therefore, milk exosomes represent a scalable, biocompatible and cost-effective means to potentially enhance oral bioavailability, improve efficacy and safety of drugs.

Materials and methods

Isolation of exosomes

Milk from pasture-fed Holstein and Jersey cows during the mid-lactation period was obtained from a local dairy; colostrum was from 1–2 days postpartum. Exosomes were isolated by differential centrifugation. Briefly, milk was centrifuged at 13,000 × *g* in 250 mL centrifuge bottles (Nalgene, Thermofisher Scientific, Holtsville, NY) using TA-10.250 rotor and Allegra 25R centrifuge (Beckman Coulter Inc, Fullerton, CA) at 4 °C for 30 min to remove fat globules, casein aggregates and other debris. The whey was collected by passing through layers of cheese cloth and subsequently transferred into 70 mL polycarbonate tubes and centrifuged at 100,000 × *g* in Type 45 Ti fixed angle rotor using Optima LE-80K Ultracentrifuge (Beckman Coulter Inc, Fullerton, CA) at 4 °C for 60 min to remove large particles and microvesicles. Forty-five mL of the supernatant was carefully removed from the top and the lower slush portion along with pellet was discarded. This supernatant (70 mL/tube) was finally centrifuged at 135,000 × *g* for 90 min at 4 °C in a Type 45 Ti fixed angle rotor using Optima LE-80K Ultracentrifuge. The supernatant was discarded and the exosome pellet thus obtained was washed thrice with PBS. The exosome pellets were pooled and resuspended in PBS to give homogenous suspension and filtered through 0.22 μm for sterilization. The total protein content of exosomes was determined and adjusted to get 6 mg/mL and stored in aliquots at –80 °C until use.

Protein determination

An aliquot of milk exosome preparation was used for protein estimation using the BCA kit (Thermo Scientific, Rockford, IL). Exosome preparations, usually diluted by 10-fold, were compared in triplicate against serially diluted BSA as standard according to manufacturer's instructions. Values were extrapolated from the standard curve, using a third-order polynomial equation, with $r^2 > 0.98$ for each assay.

NanoSight and zetasizer

The size distribution of the isolated exosomes was measured by NanoSight and Zetasizer (Malvern Instruments Ltd, Malvern, Worcestershire, UK). A monochromatic laser beam at 405 nm was applied to the diluted suspension of vesicles. Filtered PBS was used as a negative control. A video of 30 s was taken with a frame rate of 30 frames/s and particle movement was analyzed by NTA software (version 2.2, NanoSight). The NTA software is optimized first to identify and then track each particle on a frame-by-frame basis, and its Brownian movement is tracked and measured. The velocity of particle movement was used to calculate particle size by applying the two-dimensional Stokes–Einstein equation [18]. All samples were evaluated in 4 replicates. Size determination of isolated exosomes was also performed using a Zetasizer Nano ZS (Malvern Instruments). Exosomes were diluted in 1 mL PBS, and parameters such as zeta potential (electronegativity) and size distribution were analyzed at 37 °C according to the manufacturer's instructions.

Scanning electron microscopy (SEM)

Exosomes (6 mg/mL) were filtered through 0.22 μm syringe filter (Corning Inc, Manassas, VA) and diluted to 1000-fold using deionized water. Diluted exosomes (5 μL) were added onto clean silica (~300 nm SiO_2) wafers and air-dried for 30 min. A conductive layer of platinum metal was coated for 30 s at a current of 20 mA and grounded with copper tape. Exosomes were imaged in Zeiss Supra 35 VP SEM (Thornwood, NY) under low accelerated voltage (5 KV) using secondary electron detectors.

Atomic force microscopy (AFM)

Exosomes (6 mg/mL) were filtered through 0.22 μm syringe filter and diluted to 600-fold using deionized water. Then 5 μL of the diluted exosomes was added on to clean silica (~300 nm SiO_2) wafers and air-dried for 30 min. Asylum MF-3D (Asylum Research, Oxford Instruments, Goleta, CA) atomic force microscope in tapping mode, and silicon probes coated with aluminum (Force Constant = 40 Nm^{-1}; Resonant Frequency = 300 kHz, BudgetSensors.com) were used for imaging. Topographic height, amplitude and phase retraces were imaged concurrently with a fixed force (<1 nN) with a scanning rate of 1 Hz. The images were recorded at 256 × 256 pixels and processed using IGOR software.

Opti-prep density gradient

Buoyant density of the milk exosomes and the drug-loaded exosomes was determined by layering on top of an Opti-prep density gradient (10–60%; w/v) medium (Sigma-Aldrich, St. Louis, MO) at 150,000 × *g* and 4 °C for 16 h in a swing bucket rotor (SW 41Ti, Beckman Coulter Inc, Fullerton, CA). Distinct bands were collected from the tube, 10 mL of PBS was added to each sample, and exosomes were collected by centrifugation for 2 h at 135,000 × *g*.

Isolation of total RNA

mirVana miRNA Isolation kit (Applied Biosystems, Foster City, CA) was used to isolate total RNA for mRNA expression studies. Small RNA was further enriched from total RNA for qPCR analysis of miRNAs according to the manufacturer's protocol. Trace genomic DNA in the crude total RNA samples was removed by incubation with 10 units of DNase I per 100 μg RNA (Ambion, Austin, TX) at 37 °C for 30 min. The concentration of the total and small RNA was determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA integrity was verified with a Bioanalyzer 2100 (Agilent, Palo Alto, CA).

qRT-PCR for miRNA and mRNA expression

For miRNA analysis of the 5 selected miRNAs (miR-21, -181a, -155, -223 and -146a), the individual TaqMan human MicroRNA Assays were used. Briefly, 25 ng of total RNA was reverse-transcribed in a final volume of 20 μL with 12.5 nM of each RT primer using the TaqMan MicroRNA Reverse Transcription Kit. TaqMan miRNA PCR kit was used to perform PCR reactions on the ABI 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA). The reactions were initiated in a 96-well optical plate at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Relative miRNA expression was assessed using the differences in normalized Ct ($^{\Delta\Delta}$Ct) method after normalization to 5S rRNA.

To determine the exosome-associated mRNA expression levels, one-Step SYBR green qRT-PCR Kit (Quanta Biosciences, Gaithersburg, MD) was used to perform cDNA synthesis and PCR amplification simultaneously from 100 ng of total RNA according to the manufacturer's instructions. Bovine specific primers for CD36, CD63, E1α, FAS, MFG-E8, MHC-II, PIGR and XDH were designed using primer 3 express software and synthesized (IDT DNA Technologies, Coralville, IA). Reactions were run under the following conditions: hold at 50 °C for 10 min, 95 °C for 5 min, then 40 cycles at 95 °C for 10 s and 60 °C for 30 s. Relative gene expression was assessed using the differences in normalized Ct ($^{\Delta\Delta}$Ct) method after normalization to 5S rRNA.

Western blot analysis

Milk exosomes were analyzed for exosomal surface proteins by western blot as described [19] and blots were probed for CD63, CD81, Tsg101 and Alix (Cell-Signaling, Danvers, MA). To rule out the presence of contaminating multivesicular bodies (MVBs) and endoplasmic reticulum (ER) exosomes preparations were also probed with MVB markers such as integrin-β1, p-selectin and CD40 and ER marker calnexin (Cell-Signaling, Danvers, MA). Appropriate secondary antibodies were used and detection carried out using enhanced chemiluminescence reagent (Thermo Scientific, Waltham, MA). Equal loading of the proteins was confirmed based on coomassie brilliant blue staining of the gel.

In vivo *biodistribution of exosomes*

Athymic nude mice (n = 4 per group) were employed to study biodistribution of exosomes administered via oral and intravenous routes (*i.v.*). Animals were fed with purified AIN-93M diet and water *ad libitum*. Milk exosomes were labeled with near-infrared fluorescent dye DiR (20 μM) by incubation at 37 °C for 30 min, followed by centrifugation at 10,000 × *g* for 30 min to remove unbound dye. Labeled exosomes were concentrated with vivaspin 500 centrifugal filter devices (10,000 MWCO, Sartorius Stedim, Bohemia, New York) and washed thrice with PBS. Exosome pellets were suspended in PBS and sterilized by passing through 0.22 μm filter. Animals were administered with a single dose of DiR-labeled exosomes (60 mg/kg Exo protein; 100 μL). Animals were euthanized after 4 days of treatment; different organs were collected and imaged *ex vivo* using Photon Imager Optima (Biospace Lab, Paris, France). The relative intensities were measured and compared with untreated control. For *in vivo* stability study, after administration of DiR-labeled exosomes by oral gavage as described above, blood was collected at different time points (1, 4, 24, 48, 72 and 144 h) and imaged for fluorescent intensity.

Drug encapsulation and in vitro *release*

The drug loading of chemopreventive agents [withaferin A (WFA), bilberry-derived anthocyanidins (Anthos) and curcumin (CUR)] and chemotherapeutic drugs [paclitaxel (PAC) and docetaxel (DOC)] was achieved by mixing the test agent (dissolved in ethanol or 1:1 mixture of ethanol and acetonitrile) with exosome suspension in the proportion of 1:9 at room temperature (22 °C). In a separate experiment, we determined that these solvents had no effect on the particle size, coagulation, etc. Unbound drug was removed by a low-speed centrifugation (10,000 × *g*) for 10 min,