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Efficient production and enhanced tumor delivery of engineered extracellular vesicles

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ABSTRACT

Extracellular vesicles (EV), including exosomes and microvesicles, are nano-sized intercellular communication vehicles that participate in a multitude of physiological processes. Due to their biological properties, they are also promising candidates for the systemic delivery of therapeutic compounds, such as cytokines, chemotherapeutic drugs, siRNAs and viral vectors. However, low EV production yield and rapid clearance of administered EV by liver macrophages limit their potential use as therapeutic vehicles. We have used a hollow-fiber bioreactor for the efficient production of bioactive EV bearing the heterodimeric cytokine complex Interleukin-15:Interleukin-15 receptor alpha. Bioreactor culture yielded ~40-fold more EV per mL conditioned medium, as compared to conventional cell culture. Biophysical analysis and comparative proteomics suggested a more diverse population of EV in the bioreactor preparations, while serum protein contaminants were detectable only in conventional culture EV preparations. We also identified the Scavenger Receptor Class A family (SR-A) as a novel monocyte/macrophage uptake receptor for EV. In vivo blockade of SR-A with dextran sulfate dramatically decreased EV liver clearance in mice, while enhancing tumor accumulation. These findings facilitate development of EV therapeutic methods.

1. Introduction

Extracellular vesicles (EV), including exosomes and microvesicles, are nano-sized membrane vesicles secreted by most cell types. Given their intrinsic properties, e.g. immunomodulation [1,2] and their ability to distribute systemically, EV are being developed as biocompatible, targeted therapeutic particles [3,4]. Techniques to load bioactive cargo, such as protein [5,6], siRNA [7], viral vectors [8], and chemotherapeutics [9] are also currently investigated in several settings.

EV for therapeutic applications are typically purified from cell culture conditioned media of cell lines or primary cells. Purification methods are comprised of combinations of techniques, including ultracentrifugation, filtration, precipitation, and chromatography [10–12], each resulting in somewhat different mixtures of EV species and non-EV contaminants. Reported EV yields range from 1 to 10 μg/mL culture supernatant, even from "high-yield" systems, such as the Integra CELLine bioreactor [7,13,14]. Thus, obtaining sufficient material for *in vivo* studies currently comprises a

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technical bottleneck for therapeutic EV development, irrespective of the purification method.

With regard to systemic delivery of EV, modifying surface proteins with targeting ligands has enabled increased delivery to the CNS (via the RGD targeting peptide) [3] and to EGFR-expressing tumors (via the GE11 peptide) [15]. However, systemically delivered EV are rapidly cleared by the monocyte/macrophage or reticuloendothelial system (RES), resulting in minimal accumulation within the desired target sites [4,15–19], which is a major hurdle for EV-based systemic therapeutic approaches. Depletion of monocytes/macrophages was shown to significantly prolong EV systemic half-life in mice, suggesting uptake by these cells as an important mechanism of EV clearance *in vivo* [17]. Clearance of synthetic nanoparticles by the RES was shown to be largely mediated by the Scavenger Receptor Class A family (SR-A), which recognizes a variety of negatively charged ligands, including phosphatidylserine [20], a phospholipid shown to be enriched on EV. Indeed, masking phosphatidylserine with annexin-V decreased EV uptake *in vitro* [21].

Herein, we describe approaches to overcome obstacles of EV production and delivery, which will facilitate further development of this therapeutic platform. We compare EV production using an efficient hollow-fiber culture system to that of conventional culture methods. We show that this method produces bioactive EV retaining surface proteins and can be used for the production of bioactive, EV associated heterodimeric interleukin-15. We also identify SR-A as a major receptor for the clearance of EV by monocyte/macrophages, and assess the applicability of SR-A blockade to achieve tumor delivery of administered EV in mice.

2. Materials and methods

2.1. Cells

HEK293 and all mouse cell lines (RAW264.7, 4T1, B16, LLC1, MC38 and EG.7) were obtained from ATCC. HEK293 cells expressing high levels of hetIL-15 were previously described [22,23]. NK92 cells were kindly provided by Dr. Howard A. Young (Cancer and Inflammation Program, National Cancer Institute, USA). Blood samples from healthy blood donors were collected in acid-citrate-dextrose tubes, under approved protocols for human subjects' research by the National Cancer Institute Investigational Review Board. Peripheral blood mononuclear cells (PBMC) were purified by gradient centrifugation over Histopaque-1077 (Sigma-Aldrich), according to the manufacturer's protocol.

4T1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. RAW264.7 and E.G7 cells were cultured in RPMI 1640 medium, supplemented with 5% fetal calf serum. The remaining cell lines were cultured in DMEM medium, supplemented with 10% fetal calf serum.

Human PBMC were cultured overnight in RPMI medium supplemented with 10% fetal calf serum and 100 U/mL penicillin/streptomycin. The cells were used within 24 h of purification from whole blood.

2.2. Cell culture method for EV production

EV/protein aggregate depleted cell culture medium was obtained by ultrafiltration of complete medium through a 500 kDa commercial hollow fiber ultrafiltration module (mPES MidiKros 500 kDa filter module, Spectrum Laboratories; Rancho Dominquez, CA), as previously described [14,24]. Specifically, a peristaltic pump was used to slowly circulate culture medium through the filter module, and filtrate was collected to be used as EV/protein aggregate depleted medium. The entire procedure was carried out using sterile materials within a biosafety cabinet. Ultrafiltered supernatant was filtered a second time through a 0.22 μm filter device to ensure sterility.

To obtain conditioned medium from conventional cultures, 3 million cells were seeded in 175 cm^2 tissue culture flasks in DMEM supplemented with 10% fetal calf serum and 100 U/mL penicillin/streptomycin. After overnight incubation, cell monolayer was gently washed with PBS, and 15 mL of fresh EV/protein aggregate-depleted medium was added to each flask. 48 h later, conditioned medium was harvested and pooled for immediate EV purification. After conditioned medium was removed, cells were collected in PBS and pelleted by centrifugation at 300 × *g*. Cell lysates were prepared by addition of N1 lysis buffer to cell pellet, incubation on ice for 1 h, and two rounds of sonication for 6 s.

2.3. Fibercell hollow-fiber bioreactor culture

The HEK293 cell clone stably expressing hetIL-15 (clone 19.7) was expanded in conventional culture flasks and used to seed a medium-sized, hollow-fiber culture cartridge, with a 20 kDa molecular weight cut-off (Fibercell Systems; Frederick, MD). Cells were adapted over two weeks to bioreactor culture conditions by gradually increasing the proportion of protein-free medium (DMEM + 10% Fibercell Systems CDMHD protein-free supplement + 100 U/mL penicillin/streptomycin). Bioreactor conditioned medium (20 mL) was collected for each harvest three times per week. Harvests were cleared of cells by 300 × *g* centrifugation, and supernatants stored at −80 °C for further purification.

2.4. EV purification

After removing large cell debris by centrifugation at 3000 × *g* for 15 min, the supernatants were carefully moved to polycarbonate tubes, and spun for 45 min at 20,000 × *g* in a type 45Ti rotor (Beckman-Coulter; Brea, CA). Supernatants were then filtered through 0.22 μm Stericup device (EMD Millipore; Billerica, MA), moved to Snakeskin 10 kDa MWCO dialysis tubing (ThermoScientific; Grand Island, NY), and dialyzed overnight in >30 volumes of Tris-buffered saline (TBS). Dialyzed supernatants were centrifuged for 2 h at 110,000 × *g* in a type 70.1Ti rotor (Beckman-Coulter) to pellet EV. Pellets were resuspended to the original volume in TBS, by passing through a 27G needle approximately 5 times (until aggregates were no longer visible), and centrifuged again at 110,000 × *g* to wash away contaminating soluble proteins. EV pellets were resuspended in 1/50 original volume of TBS following the procedure described above. Finally, EV were additionally cleared of residual aggregation by 3 min centrifugation at 20,000 × *g* in a microfuge, and the supernatants containing the EV were transferred to a clean Lobind protein tube (Eppendorf; Hauppauge, NY), and stored at −80 °C for downstream applications. All purification steps were conducted at 4 °C.

For experiments directly comparing conventional culture to bioreactor EV, conditioned cell culture media were concentrated by ultrafiltration (Centricon-70, 100 kDa MWCO; EMD Millipore) prior to 110,000 × *g* centrifugation. This allowed for a larger volume of conventional flask-derived supernatants to be pooled prior to ultracentrifugation. Furthermore, initial EV pellet was not washed, but rather immediately resuspended in 100 μL TBS. These protocol modifications were implemented to allow for sufficient EV yields from conventional flask supernatants for downstream comparison analyses.

2.5. Biophysical characterization and imaging

Nanoparticle tracking analysis (NTA) was performed on fresh