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RNAi delivery by exosome-mimetic nanovesicles – Implications for targeting c-Myc in cancer

Taral R. Lunavat [a, 1], Su Chul Jang [a, b, 1], Lisa Nilsson [c], Hyun Taek Park [b], Gabriela Repiska [a], Cecilia Lässer [a], Jonas A. Nilsson [c], Yong Song Gho [a, b, **], Jan Lötvall [a, *]

[a] Krefting Research Center, Department of Internal Medicine and Clinical Nutrition, University of Gothenburg, Gothenburg 405 30, Sweden
[b] Department of Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea
[c] Department of Surgery, Sahlgrenska Cancer Center, Institute of Clinical Sciences, University of Gothenburg, Gothenburg 413 90, Sweden

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ABSTRACT

To develop RNA-based therapeutics, it is crucial to create delivery vectors that transport the RNA molecule into the cell cytoplasm. Naturally released exosomes vesicles (also called "Extracellular Vesicles") have been proposed as possible RNAi carriers, but their yield is relatively small in any cell culture system. We have previously generated exosome-mimetic nanovesicles (NV) by serial extrusions of cells through nano-sized filters, which results in 100-times higher yield of extracellular vesicles. We here test 1) whether NV can be loaded with siRNA exogenously and endogenously, 2) whether the siRNA-loaded NV are taken up by recipient cells, and 3) whether the siRNA can induce functional knock-down responses in recipient cells. A siRNA against GFP was first loaded into NV by electroporation, or a c-Myc shRNA was expressed inside of the cells. The NV were efficiently loaded with siRNA with both techniques, were taken up by recipient cells, which resulted in attenuation of target gene expression. In conclusion, our study suggests that exosome-mimetic nanovesicles can be a platform for RNAi delivery to cell cytoplasm.

1. Introduction

Over the last decade, many RNA-based therapeutic molecules have been developed for different diseases. However, RNA does not easily pass over biological membranes, which means that an efficient delivery vector is required for their successful transportation into the cytoplasm of a diseased cell. Thus, a major challenge is to achieve intracellular delivery of the different interference RNA species, and various delivery systems have been tested, including cationic lipids [1], cell-penetrating peptides [2], and cationic polymers [3]. In addition, exosomes and other extracellular vesicles have the intrinsic capacity to deliver multiple RNA species from one cell to another, and have therefore been proposed as delivery vehicles for therapeutic RNA molecules [4] [5], and [6]. Most types of extracellular vesicles have a lipid bi-layered membrane, and a diameter of approximately 30–300 nm [7], and contain various bioactive molecule including proteins, nucleic acids, and lipids [8] and [9]. Multiple studies have suggested that exosomes can deliver RNAs (specifically, mRNAs and miRNAs) to recipient cells, influencing their phenotype [10] [11], and [12]. Thus, extracellular vesicles are considered to be promising delivery vehicles, also because they are highly biocompatible [13] and [14]. However, there are big hurdles for therapeutic use of exosomes, primarily their low yield from cell cultures, and complicated current state-of-the-art purification processes [15].

RNA interference modulate the expression of RNAs, especially by inhibiting the function of specific mRNAs [16]. Since RNA interference selectively can attenuate specific genes, it has become a valuable research tool in cell biology, and is also considered as possible therapeutic approach for multiple diseases where specific gene expression is a causative mechanism [17] and [18]. Inhibition of specific mRNAs with siRNA (short interference RNA) or shRNA (short hairpin RNA) can be a powerful treatment strategy in cancer, because of their ability to target specific mutations and/or non-

* Corresponding author. Krefting Research Center, Department of Internal Medicine and Clinical Nutrition, University of Gothenburg, Gothenburg 405 30, Sweden.
** Corresponding author. Department of Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea.
E-mail addresses: ysgho@postech.ac.kr (Y.S. Gho), jan.lotvall@gu.se (J. Lötvall).
[1] These authors contributed equally.

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mutated overexpressed oncogenes [19] and [20].

It has previously been shown that exosome-mimetic nanovesicles (NV) with high production yield can deliver anti-cancer chemotherapeutics into cells [21]. Here, we hypothesize that exosome-mimetic nanovesicles, produced by multiple extrusion of cells through filters, can function as efficient siRNA delivery systems. We take two approaches to test this. Firstly, we produce a NV from the cell, and then load these vesicles with a specific siRNA by electroporation. We gave NV to TNF-α-treated endothelial cells to mimic the cell adhesion molecule interaction as described previously [21]. Secondly, we overexpress shRNA against human c-Myc, using a mouse fibroblast cell line that carries both human and mouse c-Myc, to generate a NV carrying the siRNA of interest, and gave these NV to mouse λ820 cells expressing human c-Myc, to examine the knock-down efficiency. We examined whether these siRNA-loaded NV can be taken up by recipient cells, and whether a targeted gene knock-down can be achieved.

2. Materials and methods

2.1. Cell culture

NIH3T3 cells were cultured in DMEM medium (HyClone, Logan, UT, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 1% L-glutamine (HyClone) and 100 U/ml penicillin (HyClone), 100 μg/ml streptomycin (HyClone). λ820 cells were cultured in RPMI-1640 with stable glutamine (Lonza, Basel, Switzerland) supplemented with 10% FBS (Sigma-Aldrich), 50 μM β-mercaptoethanol (Invitrogen) and antibiotics. The GFP expressing HMEC-1 and U937 cells were grown in RPMI 1640 with hygromycin (200 μg/ml) and RPMI 1640 medium which were supplemented with 10% FBS and 1% Antibiotic-Antimycotic, respectively. All cells were tested negative for mycoplasma and cultured at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. shRNA transduction

Lentiviral were produced by calcium phosphate–mediated transfection of 293T cells with packaging plasmids pCMV-dR8.2dvpr and pHCMV-Eco (Addgene) using X different MISSION short hairpin RNA (shRNA) constructs (Sigma) against the human MYC mRNA. Twenty-four hours post-transfection, media was changed and supernatants were harvested 4 times during 36 h and used to infect target cells. NIH 3T3 cells carrying overexpression of human MYC were infected with the different shRNAs by culturing the cells in the presence of viral particles and 4–8 μg/ml of polybrene. The cells were selected by culturing in the presence of 8 μg/ml puromycin.

2.3. siRNAs

Scramble siRNA, siRNA against GFP, and FITC labeled siRNA were purchased from Bioneer. Sequences of siRNAs used were: Scramble sense: CCUACGCCACCAAUUUCGU (dTdT), scramble antisense: ACGAAAUUGGUGGCGUAGG (dTdT), GFP sense GCAUCAAGGUGAACUUCAA (dTdT), GFP antisense UUGAAGUUCAC CUUGAUGC (dTdT). siRNA against GAPDH was obtained from Life Technologies.

2.4. Preparation of NV and siRNA-loaded NV

Cells (5×10^6 cells/ml) in phosphate-buffered saline (PBS) was serially extruded three times through 10 μm, 5 μm, and 1 μm polycarbonate membrane filters (Whatman) using a mini-extruder (Avanti Polar Lipids). To isolate the NV, two-step OptiPrep density gradient ultracentrifugation was performed. From bottom to top of an ultracentrifuge tube, 50% iodoxanol (Axis-Shield PoC AS), 10% iodoxanol, and the extruded samples was placed and then ultracentrifuged at 100 000 × g for 2 h at 4 °C. NV were obtained from the interface of the 50% and 10% iodoxanol layers. For the exogenous loading of siRNA, the amount of protein was adjusted to 1 mg/ml with PBS. Then, siRNA (20 μM) was added and electroporated at 200 V using Gene Pulser Xcell™ Electroporation Systems (Bio-rad). A two-step OptiPrep density gradient ultracentrifugation was performed once more as described above. The protein concentrations of NV were determined using the Bradford (Bio-Rad laboratories, Hercules, CA, USA).

2.5. Transmission electron microscopy

To view the NV with electron microscope, we used 10 μg of total NV protein suspended in HEPES buffer saline for the analysis. The detailed protocol was followed from Lunavat et al. under the methods section 'Transmission electron microscopy and cytospins' [22].

2.6. Western blot

NV were lysed using RIPA buffer (Thermo Scientific) and were sonicated for 5 min three times with intermittent vortexing in between to extract all the proteins from the vesicles. Proteins were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane (Bio-Rad), and blocked in 5% Non-Fat Dry Milk (Bio-Rad Laboratories). Membranes were then incubated with primary antibodies against PDGFR, Flotillin-1, CD9, Calnexin, c-Myc (1:1000; all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Cleaved PARP (Asp214) (Mouse Specific), Cleaved Caspase 3 (Asp175) and beta actin (1:1000; Cell Signaling Technology) at 4 °C overnight. Membranes were washed three times in TBST buffer and incubated with secondary antibodies for one hour at room temperature. The membranes were washed again three times for 5 min in TBST buffer and analyzed with ECL Prime Western Blotting Detection (GE HealthCare) and a VersaDoc 4000 MP (Bio-Rad Laboratories).

2.7. Quantification of siRNAs

To quantify the siRNA, siRNA-loaded NV were generated by using FITC-labeled siRNA. The siRNA-loaded NV (100 μl) were placed into the wells of 96-well plates and FITC fluorescence was detected using a Wallac 1420 VICTOR plate reader (Perkin-Elmer Life Sciences) with excitation/emission at 488 nm/530 nm.

2.8. Size and zeta potential measurement

NV (5 μg/ml total protein) were dispersed in PBS, and then the size and zeta potential of NV were assessed by Nanoparticle Tracking Analysis (NTA) and dynamic light scattering using Nano ZS (Malvern).

2.9. PKH67 labelling and uptake of NV

The NV (20 μg) from all the shRNA transduced NIH3T3 cells were used for labelling with PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labelling (Sigma-Aldrich) according to the manufacturer's protocol, with minor modifications in the washing process as described previously [23]. Appropriately, 20 μg of the PKH67 labeled NV or the same volume of the PKH67-PBS control was added and incubated for 12 h at 37 °C. The uptake of NV in λ820 cells was analyzed with a FACS and visualized with confocal microscope (LSM 700 Carl Zeiss microscope). For flow cytometry,