Biomaterials 35 (2014) 2383–2390

Contents lists available at ScienceDirect

Biomaterials

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

A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy

Yanhua Tian [a,b,1], Suping Li [a,1], Jian Song [c], Tianjiao Ji [a], Motao Zhu [a], Gregory J. Anderson [d], Jingyan Wei [b,*], Guangjun Nie [a,*]

[a] CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology of China, Beijing 100190, China
[b] College of Pharmaceutical Science, Jilin University, Changchun 130021, China
[c] College of Electronic Science and Engineering, Jilin University, 2699 Qianjin Street, Changchun 130012, China
[d] Iron Metabolism Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, Queensland 4029, Australia

ARTICLE INFO

Article history:
Received 15 October 2013
Accepted 27 November 2013
Available online 15 December 2013

Keywords:
Exosome
iRGD
Doxorubicin
αv Integrin-positive cancer cells
Tumor therapy

ABSTRACT

Targeted drug delivery vehicles with low immunogenicity and toxicity are needed for cancer therapy. Here we show that exosomes, endogenous nano-sized membrane vesicles secreted by most cell types, can deliver chemotherapeutics such as doxorubicin (Dox) to tumor tissue in BALB/c nude mice. To reduce immunogenicity and toxicity, mouse immature dendritic cells (imDCs) were used for exosome production. Tumor targeting was facilitated by engineering the imDCs to express a well-characterized exosomal membrane protein (Lamp2b) fused to αv integrin-specific iRGD peptide (CRGDKGPDC). Purified exosomes from imDCs were loaded with Dox via electroporation, with an encapsulation efficiency of up to 20%. iRGD exosomes showed highly efficient targeting and Dox delivery to αv integrin-positive breast cancer cells *in vitro* as demonstrated by confocal imaging and flow cytometry. Intravenously injected targeted exosomes delivered Dox specifically to tumor tissues, leading to inhibition of tumor growth without overt toxicity. Our results suggest that exosomes modified by targeting ligands can be used therapeutically for the delivery of Dox to tumors, thus having great potential value for clinical applications.

1. Introduction

Despite recent advances in packaging clinically approved drugs into nanoscale delivery vehicles (10–100 nm in diameter) for cancer therapy, achieving efficient drug tumor accumulation while avoiding immune activation and toxicity remains a challenge. Tissue-specific, non-toxic and non-immunogenic delivery technologies are critical to move these systems into clinical practice for cancer therapy. Exosomes are nano-sized membrane vesicles (30–100 nm in diameter) secreted by numerous cell types such as epithelial cells and hematopoietic cells [1]. They are released into the extracellular milieu upon fusion of multivesicular bodies, with the plasma membrane [2]. Their unique origin enables exosomes to contribute to intercellular communication through the transfer of mRNA, microRNA, receptors and enzymes between cells [3,4]. Besides transporting endogenous substances, two recent reports have shown that exosomes can be used as naturally derived nanovesicles to deliver exogenous RNAs (siRNAs and miRNAs) to target tissues/cells *in vivo*, leading to gene knockdown or inhibiting tumor growth in mouse models [5,6]. Indeed, a hydrophobic anti-inflammatory agent curcumin has been delivered with exosomes to targeted cells, leading to enhanced anti-inflammatory activity [7]. More importantly, exosome-mediated nucleic acid delivery *in vivo* did not induce short-term innate immune activation nor cause overt side effects [5].

In principle, exosomes used as drug delivery vehicles have multiple advantages over existing synthetic systems. First, as exosomes can be derived from a patient's own cells, they may be less immunogenic than artificial delivery vehicles. Second, exosomes have phospholipid bilayers, which may directly fuse with the target cell plasma membrane, thus improve the cellular internalization of the encapsulated drug [8]. Therefore, this fusion mode may bypass the endosomal–lysosomal pathway which is frequently utilized by synthetic materials and can lead to inflammasome activation [9]. Third, the naturally small size of exosomes allows them to avoid

* Corresponding authors.
E-mail addresses: jingyanweijlu@163.com (J. Wei), niegj@nanoctr.cn (G. Nie).
[1] These authors contributed equally to this work.

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.11.083

phagocytosis by the mononuclear phagocyte system and facilitates their extravasation through tumor vessels and their subsequent diffusion in tumor tissues [10]. These potential advantages of exosomes for therapeutic applications in cancer make them ideal vehicles for delivering encapsulated drugs and, in particular, chemotherapeutic agents. The clinical applications of such agents are significantly hindered by their low solubility, toxicity associated with non-specific targeting, and short half-life in the circulation.

Motivated by this rationale, we explored the feasibility of delivering the chemotherapeutic drug doxorubicin (Dox) to tumor tissue in a mouse model using engineered exosomes. In the work, immature dendritic cells (imDCs) were used to produce exosomes with low immunogenicity, probably due to the lack of immunostimulatory markers on their surface, such as CD40, CD86, MHC-I and MHC-II [11]. The tumor targeting capability of exosomes was conferred by engineering the imDCs to express lysosome-associated membrane glycoprotein 2b (Lamp2b), a well-characterized exosomal membrane protein [12], fused with iRGD (CRGDKGPDC) targeting peptide for αv integrin [13]. The iRGD exosomes (iRGD-Exos) were purified from cell culture supernatants and loaded with Dox by electroporation. The high antitumor activity of the iRGD-Exos loaded with Dox (iRGD-Exos-Dox) was then validated both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture

The human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from the American Type Culture Collection (ATCC) and propagated in DMEM (Wisent, Toronto, Canada) supplemented with 10% FBS and antibiotics. An immature mouse dendritic cell line (imDC) was kindly provided by Prof. Honglin Xu (National Vaccine and Serum Institute, China) and cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics. All cells were maintained in 5% CO_2 at 37 °C.

2.2. Construction of iRGD-Lamp2b plasmid and transfection

The pEGFP-C1-RVG-Lamp2b expressing vector was kindly provided by Dr. Yiqi Seow, University of Oxford [5]. We reengineered the vector by replacing the RVG fragment with iRGD (pEGFP-C1-iRGD-Lamp2b), which was obtained by insertion of the annealing synthesized single-stranded sequences for iRGD with restriction enzymatic sites (sense TCGATGTTAGACCTGGAAATAGTGGTGCTGTG, antisense CCGGCACAGCACCACTATTTCCAGGTCTAACA). Immature DCs were transfected with the vector expressing iRGD-Lamp2b fusion proteins using Lipofectamine 2000 transfection reagent (Invitrogen, USA).

2.3. Purification of exosomes

The exosomes were purified according to the literature with some modifications [14]. The cell culture supernatant containing exosomes were harvested 36 h after transfection by centrifugation at 200 × g for 5 min, to eliminate cells. The supernatant was then centrifuged at 12,000 × g for 45 min to remove dead cells and cell debris. The resulting supernatant was ultrafiltered through a 100 kDa membrane to concentrate the exosome-containing solution. The retained exosomes were further purified by layering them onto a 30% Tris/sucrose/D_2O solution (0.30 g sucrose and 0.024 g Tris base in 1 mL D_2O) and centrifuging at 100,000 × g for 90 min. The sucrose cushion (approximately 1 mL) containing purified exosomes was collected from the side of the tube with a 5 mL syringe fitted with an 18 G needle. The exosomes were washed with 9 mL cold PBS and centrifuged at 120,000 × g for 90 min. The purified exosomes were resuspended in PBS and stored at −80 °C prior to use. All procedures were carried out at 4 °C.

2.4. Exosome labeling

The fluorescent dyes 3, 3′-dioctadecyloxacarbocyanine perchlorate (DiO), 1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindotricarbocyanine iodide (DiR) and lipophilic styryl FM 4-64 (FM 4-64) were purchased from Invitrogen (USA) and used to label exosomes. Purified exosomes were incubated in the presence of each fluorescent dye (5 μM DiO, 5 μM DiR or 8 μM FM 4-64) for 15 min at 37 °C, then ultracentrifuged at 120,000 × g, 90 min to remove free dye. After being washed twice in PBS with 120,000 g centrifugation, the labeled exosomes were resuspended in PBS prior to use.

2.5. Identification of iRGD expression on exosomes

Purified $a_vβ_3$ integrins (Millipore, USA) were immobilized in the wells of microtiter plates (1–5 μg/mL, 50 μL/well) by adsorption overnight before blocking with casein blocker (Pierce). DiO-labeled exosomes were resuspended in binding buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM $MnCl_2$) and added to the microtiter wells [15]. After incubation for 2 h, the unbound exosomes were removed by washing the wells with the binding buffer twice. Bound exosomes were then quantitated by determining the fluorescence intensity at an excitation wavelength of 484 nm and an emission wavelength of 501 nm. To detect the binding specificity of iRGD-Exos to $a_vβ_3$, the $a_vβ_3$-coated wells were pre-incubated with synthetic iRGD peptide which shows competitive binding to $a_vβ_3$.

2.6. Electron microscopy

Purified exosomes from imDCs were resuspended in PBS and fixed with 2% paraformaldehyde for 30 min at room temperature. Eight microliters of mixture were then dropped onto EM grids that had been pretreated with UV light to reduce static electricity. After drying for 30 min, exosomes were stained twice (6 min each) with 1% uranyl acetate. The dried grids were examined using an HT7700 (Hitachi, Japan) transmission electron microscope (TEM) at 120 kV.

2.7. Characterization of exosomes by nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) measurements were performed with a NanoSight LM10-HSB instrument (A&P Instrument Co., UK) using purified exosomes (100 μL; 10 ng/μL). The mean size and size distribution data were captured and analyzed with the NTA 2.2 Analytical Software Suite. All procedures were performed at room temperature.

2.8. Loading therapeutic cargo

To load the exosomes with Dox, 100 μg of purified exosomes and 50 μg of Dox were gently mixed in 200 μL of electroporation buffer at 4°C. After electroporation at 350 V and 150 μF in 0.4 cm electroporation cuvetes using a Gene Pulser II Electroporator (Bio-Rad, USA), the mixture was incubated at 37 °C for 30 min to ensure the plasma membrane of the exosomes fully recovered. Recovery was assessed by TEM as described above. Exosomes were then washed with cold PBS twice by ultracentrifugation at 120,000 × g for 90 min to isolate exosomes and to remove unincorporated free Dox. The exosomes loaded with Dox were quantified for the encapsulated Dox by detecting the intrinsic fluorescence of Dox using Fluorescence Spectrophotometer F-4600 (HITACHI, Tokyo, Japan) at 594 nm with excitation at 480 nm.

2.9. Tumor-bearing nude mouse model

Six week old female BALB/c nude mice were purchased from Beijing Vital River Laboratories. Human breast cancer cells (MDA-MB-231, 2.0×10^6 cells in 50 μL PBS) mixed with 50 μL of matrigel were transplanted into the mammary fat pads of the mice, and allowed to grow to a tumor size ~0.1 cm^3 (volume = length × $width^2$/2, measured with a vernier caliper). The mice were then randomly divided into different experimental groups as described in Results. All procedures were approved by the Committee on the Ethics of Animal Experiments of the Health Science Center of Peking University (Beijing, China).

2.10. Statistics

Data analyses were performed with one-way ANOVA and the LSD multiple comparisons test. Tumor volumes were compared using a Kruskal–Wallis test followed by the Mann–Whitney test.

3. Results

3.1. Isolation and characterization of iRGD-Exos

To generate iRGD-positive exosomes, we fused the iRGD peptide to the extra-exosomal N terminus of murine Lamp2b protein by introducing the pEGFP-C1-RVG-Lamp2b plasmid into imDCs. To validate whether iRGD-Lamp2b was transfected successfully into the imDCs, we assessed the levels of iRGD-Lamp2b mRNA 36 h after transfection using RT-PCR. Relative to untransfected imDCs, the transfected imDCs expressed high levels of iRGD-Lamp2b message RNA (Fig. 1A).

We then purified exosomes from the culture supernatants of iRGD-Lamp2b transfected and untransfected imDCs by ultracentrifugation. In the purified materials, typical exosome structures were observed by TEM (Fig. 1B and C). Nanoparticle tracking analysis (NTA) showed that the exosomes had a narrow size distribution, with a mean particle diameter of 97 nm for exosomes from untransfected cells (blank-Exos). Exosomes from transfected imDCs (iRGD-Exos) were not significantly different in size from exosomes derived from untransfected cells (Fig. 1D and E). These