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Surface functionalized exosomes as targeted drug delivery vehicles for cerebral ischemia therapy

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ABSTRACT

The safe and effective delivery of drugs is a major obstacle in the treatment of ischemic stroke. Exosomes hold great promise as an endogenous drug delivery nanosystem for the treatment of cerebral ischemia given their unique properties, including low immunogenicity, innate stability, high delivery efficiency, and ability to cross the blood-brain barrier (BBB). However, exosome insufficient targeting capability limits their clinical applications. In this study, the c(RGDyK) peptide has been conjugated to the exosome surface by an easy, rapid, and bio-orthogonal chemistry. In the transient middle cerebral artery occlusion (MCAO) mice model, The engineered c(RGDyK)-conjugated exosomes (cRGD-Exo) target the lesion region of the ischemic brain after intravenous administration. Furthermore, curcumin has been loaded onto the cRGD-Exo, and administration of these exosomes has resulted in a strong suppression of the inflammatory response and cellular apoptosis in the lesion region. The results suggest a targeting delivery vehicle for ischemic brain based on exosomes and provide a strategy for the rapid and large-scale production of functionalized exosomes.

1. Introduction

Stroke is the leading cause of death and acquired disability worldwide, and ischemic stroke, induced by a thromboembolic occlusion of the cerebral artery, accounts for over 80% of all strokes [1,2]. To date, the only approved treatment for this type of stroke is recanalization *via* the application of recombinant tissue plasminogen activator (rt-PA) [1,3]. However, this approach is limited by a narrow therapeutic window (<4.5 h) and severe risk factors [4]. Many successful pharmacotherapy including various chemicals, peptides and genetic therapies that have been evaluated in experimental animal models are impeded by challenges in delivery to ischemic brain [5,6]. Hence, a safe and efficient delivery system is a critical need for therapy in the ischemic brain.

Exosomes are 40- to 150-nm extracellular vesicles (EVs) of endosomal origin and are secreted by all cell types [7,8]. As natural carriers that package bioactive molecules, various proteins, and coding and noncoding RNAs, they transfer information between cells and tissues [9,10]. In the past decade, exosomes have emerged as novel therapeutic effectors in immune therapy, regenerative medicine and drug delivery [11,12]. They are characterized by several favorable features, such as low immunogenicity, biodegradability, low toxicity, encapsulating endogenous bioactive molecules, strong protection for cargo and the ability to cross the blood-brain barrier (BBB) [13–16]. However, recent biodistribution studies of unmodified exosomes after intravenous injection revealed a rapid accumulation of exosomes in organs of the reticuloendothelial system (RES), such as the liver and spleen, and very few exosomes were delivered to the brain after systemic administration [17,18]. Thus, their targeting characteristics require improvement before exosomes can be used to deliver therapies against stroke.

The targeting ability of exosomes can be improved by surface modifications [16,19]. Recently, the popular method is the “cell

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engineering" technique, wherein donor cells are engineered to produce ligand-conjugated or drug-loaded exosomes [20–22]. These cell engineering processes are complex, high-cost, and most important cannot be readily applied to pre-isolated exosomes or exosomes from body fluids [16,19]. An exciting opportunity is translating nanoparticle techniques to exosome engineering, wherein exosomes are directly modified by biochemical conjugation or hydrophobic insertion. Several previous reports demonstrated chemical reactions can be applied to exosome conjugation [23–25]. However, they are proof-of-principle experiments and lack of *in vivo* conformation. Furthermore, few studies have modified exosomes with targeting ligands for ischemic stroke.

Here, we propose an easy, rapid and efficient method to conjugate functional ligands onto exosomal surfaces using bioorthogonal copper-free azide alkyne cyclo-addition (click chemistry). The cyclo(Arg-Gly-Asp-D-Tyr-Lys) peptide [c(RGDyK)], which exhibits high affinity to integrin $\alpha_v\beta_3$ in reactive cerebral vascular endothelial cells after ischemia specifically [26–29], was conjugated on mesenchymal stromal cell (MSC)-derived exosomes surface by our method. To generate an animal model of stroke, mice were subjected to middle cerebral artery occlusion (MCAO) and reperfusion (MCAO/R). Subsequently, c(RGDyK)-conjugated exosomes (cRGD-Exo) were intravenously administered through the tail vein. Near-infrared fluorescence (NIRF) imaging and immunofluorescence showed that cRGD-Exo targeted the lesion region of the ischemic brain and entered microglia, neurons and astrocytes. Furthermore, curcumin, a natural polyphenol from *Curcuma longa*, was loaded onto the cRGD-Exo. After the administration of cRGD-Exo containing curcumin (cRGD-Exo-cur), pro-inflammatory cytokines and activated microglia were detected. The resulting data showed that cRGD-Exo-cur suppressed the inflammatory response and cellular apoptosis in the lesion region more effectively than did curcumin or exosomes treatment alone. Also, cRGD-Exo was indicated to be a targeting delivery vehicle for ischemic brain.

2. Materials and methods

2.1. Mice and cerebral ischemia model

Eight-week-old male C57BL/6 mice were supplied by the Animal Core Facility of Nanjing Medical University (Nanjing, China). All animal experiments were carried out in compliance with institutional guidelines and were approved by the Animal Care and Use Committee of Nanjing Medical University (No. IACUC-1601160). Transient focal cerebral ischemia was induced by MCAO. After the induction of anesthesia, the right MCA was occluded by inserting a 6–0 nylon monofilament suture into the right internal carotid artery. The body temperature was sustained at 37 °C with a heated blanket throughout the procedure. Successful occlusion was assured by laser Doppler flowmetry (75–90% blood flow decrease in the MCA territory). One hour after occlusion, reperfusion was allowed by suture removal. In the sham group, an identical surgical procedure was performed without disturbing the arteries.

2.2. Cell culture

Bone marrow-derived MSCs were isolated from the tibias and femurs of mice and cultured in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS). The human glioblastoma cell line U87 and cervical carcinoma cell line HeLa were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). U87 cells expressing green fluorescent protein (U87-GFP) was purchased from Obio Technology Corp. (Shanghai, China). The three cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. All media and reagents for cell culture were purchased from Gibco (Carlsbad, CA, USA), and all cells were incubated at 37 °C in 5% CO_2.

2.3. Exosome isolation

α-MEM containing 20% FBS was centrifuged at 200,000 g for 18 h to deplete exosomes. MSCs from passages 4–6 were cultured with 10% exosome-depleted FBS for exosome production. Every 1.5–2 × 10^6 MSCs was cultured in a 100-mm dish for 48 h and 10 mL supernatant were collected. Then exosomes were isolated from the harvested supernatant according to a previous study [30]. Briefly, the supernatant was centrifuged at 300 g for 10 min, 1200 g for 20 min, and 10,000 g for 30 min at 4 °C to remove cells and debris and then filtered using a 0.22-μm filter (Millipore, Billerica, MA, USA). The filtrate was centrifuged at 140,000 g for 90 min at 4 °C in a Type Ti70 rotor using an L-80XP ultracentrifuge (Beckman Coulter, Brea, CA, USA). The exosome pellet was resuspended in PBS and ultracentrifuged again at 140,000 g for 90 min. Subsequently, the pelleted exosomes were resuspended in PBS and analyzed using a Micro BCA Protein Assay kit (Pierce, Rockford, IL, USA). To detect exosome markers and negative marker, Western blotting was carried out with anti-Alix, anti-TSG101, and anti-Calnexin antibodies (Abcam, Cambridge, UK).

2.4. Conjugation of ligands to exosomes

Reactive dibenzylcyclootyne (DBCO) groups were incorporated in amine-containing molecules on exosomes using a heterobifunctional crosslinker. Specifically, 3 μM dibenzocyclooctyne-sulfo-*N*-hydroxysuccinimidyl ester (DBCO-sulfo-NHS) (Sigma, St. Louis, MO, USA) was added to 0.5 mg/mL exosomes in PBS and allowed to react on a rotating mixer at room temperature (RT) for 4 h. Unconjugated DBCO-sulfo-NHS was removed by four washing steps on 100-kDa ultrafiltration tubes (Millipore). The DBCO-conjugated exosomes (DBCO-Exo) were then ready for linkage to azide-containing molecules *via* copper-free click chemistry. c(RGDyK) and scrambled c(RDGyK) peptide with an azide group on the lysine were synthesized by SciLight Biotechnology Co. (Beijing, China). According to the manufacturer, azide groups were introduced by conjugating 5-azidopentanoic acid to the side chain of lysine. Cy5.5 azide was purchased from Lumiprobe Co. (Hallandale Beach, FL, USA). Specifically, 0.3 μM c(RGDyK) or scrambled c(RDGyK) peptide with azide was added to DBCO-Exo in PBS, and 0.3 μM Cy5.5 azide was subsequently added if needed. The reaction was conducted on a rotating mixer at 4 °C for 12 h. All reactions were performed at pH 7.4. Then, the exosomes were floated on a 30% sucrose/D_2O cushion and centrifuged at 164,000 g for 90 min using an SW41Ti rotor (Beckman Coulter) to remove unincorporated ligands. After washing with PBS, the modified exosomes were resuspended and stored at −80 °C prior to use. To assess the successful conjugation of exosomes and azide ligands, Cy5.5-conjugated exosomes were stained by DiI, applied to coverslips and imaged by fluorescence microscopy (Nikon, Tokyo, Japan) with a 60 × objective (NA. = 1.49). As control, 0.5% BSA was subjected to the same reaction and the product was imaged.

2.5. Transmission electron microscopy (TEM), atomic force microscopy (AFM), nanoparticle tracking analysis (NTA) and zeta potential measurement

The exosomes were diluted in PBS, fixed with 1% glutaraldehyde, applied onto a carbon-coated copper grid, and stained with 1% phosphotungstic acid. A JEM-2100 transmission electron microscope (JEOL, Tokyo, Japan) was employed to observe the specimens. AFM images were recorded using a BioScope Resolve system