Biomaterials 35 (2014) 4390–4400

Contents lists available at ScienceDirect

Biomaterials

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

Microvesicle-mediated delivery of transforming growth factor β1 siRNA for the suppression of tumor growth in mice

Yaqin Zhang [a], Limin Li [b], Jianxiong Yu [b], Dihan Zhu [b], Yujing Zhang [b], Xihan Li [b], Hongwei Gu [b,*], Chen-Yu Zhang [b,*], Ke Zen [b,*]

[a] Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Nanjing Medical University, Nanjing, Jiangsu 210029, China
[b] Jiangsu Engineering Research Center for microRNA Biology and Biotechnology, State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, School of Life Sciences, Nanjing, Jiangsu 210093, China

ARTICLE INFO

Article history:
Received 18 January 2014
Accepted 4 February 2014
Available online 22 February 2014

Keywords:
Tumor therapy
Systemic delivery
Microvesicle
TGF-β1
siRNA

ABSTRACT

Cell-derived microvesicles (MVs) have been recently shown as an efficient carrier to deliver small RNAs into the target cells. In the present study, we characterized the inhibitory effect of TGF-β1 siRNA delivered by mouse fibroblast L929 cell-derived MVs (L929 MVs) on the growth and metastasis of murine sarcomas 180 cells both *in vitro* and *in vivo*. We found that, comparing to the same concentration of free TGF-β1 siRNA, TGF-β1 siRNA delivered by L929 MVs much more efficiently decreased the level of TGF-β1 in the recipient tumor cells. Functionally, MVs containing TGF-β1 siRNA significantly decreased the viability and migration of sarcomas 180 cells and promoted the apoptosis of tumor cells. Co-immunoprecipitation with Argonaute 2 (AGO2) via anti-AGO2 antibody indicated that the majority of TGF-β1 siRNA in the MVs were associated with AGO2 complex. A tumor implantation mouse model further showed that intravenous injection of TGF-β1 siRNA-containing MVs strongly suppressed TGF-β1 expression and TGF-β1 signaling downstream in the implanted tumor cells, and thus inhibited the growth and lung metastases of tumor cells. In conclusion, our results collectively demonstrate that the delivery of therapeutic TGF-β1 siRNA by cell-derived MVs provides an effective strategy to control tumor cell growth and metastasis.

1. Introduction

RNA interference (RNAi) is a remarkable endogenous regulatory pathway that guide sequence-dependent endonucleolytic cleavage of the mRNAs to bring about sequence-specific gene silencing, which become rapidly a promising tool for therapeutic applications in cancer and other diseases [1]. However, *in vivo* systemic delivery of short interfering RNA (siRNA) to tumor tissues/cells remains a challenge. The major limitations against the use of siRNA as a therapeutic tool are its degradation by extracellular nucleases, poor cellular uptake and rapid renal clearance following systemic administration [2]. Thus, it's necessary to develop the new tools for carrying siRNA into target cells. Recently, a number of biological delivery vehicles, such as cationic liposomes, viral vectors and nanoparticles, have been used to deliver siRNA to the desired target tissue [3–5]. Nevertheless, there are advantages and disadvantages with each method. Cationic liposomes can effectively package genetic materials, however, their clearance ratio and immunogenicity impose restrictions on clinical applications [6]. Viral vectors of gene delivery, such as adenovirus or adeno-associated virus, and retrovirus vehicles can efficiently carry genetic information into cancer cells, nevertheless, the usage of these viral vehicles is seriously limited by small packaging capacity, immune response to viral particles, and insertional mutagenesis [7]. Nanoparticles, containing polymeric siRNA nanoparticles, are recently developed as carriers for delivering siRNA, however, they can be prone to rapid clearance mediated by opsonin and complement in the blood [8]. Furthermore, viral vehicles and polyethylenimine (PEI) complexes are subjected to accumulate in liver, which makes it difficult for targeting other tissues [9]. Thus, further development of siRNAs for anti-cancer therapy replies on the development of safe and efficient carriers for systemic administration.

Microvesicles (MVs) are small vesicles and secreted by amount of cell types under both normal physiological and pathological conditions [10,11], which include some different vesicle types,

* Corresponding authors. Tel./fax: +86 25 84530231.
E-mail addresses: hongweigu@nju.edu.cn (H. Gu), cyzhang@nju.edu.cn (C.-Y. Zhang), kzen@nju.edu.cn (K. Zen).

http://dx.doi.org/10.1016/j.biomaterials.2014.02.003
0142-9612/

termed exosomes and shedding vesicles respectively, ranging from ~30 nm to 1000 nm in size [11]. Many cells can release the microvesicles, including macrophage [12], dendritic cells [13] and tumor cells [14]. Recently, studies by our laboratory [15] and others [16–18] have demonstrated that these vesicles can transfer protein, DNA, coding and non-coding RNA to neighboring or distant cells. It has been proven that exosomes contain components of the RNA-induced silencing complex, such as argonaute 2 (AGO2) [19,20], indicating that they are intrinsically implicated in miRNA induced gene silencing. Nevertheless, the protective effect of AGO2 complexes on the siRNA in the MVs has not been clearly defined. In addition, exosomes derived from many cell types can deliver exogenous siRNA to the brain in mice [16], the monocytes and lymphocytes [21] and the human cancer cell line [22] have also been reported. However, whether cell-secreted MVs can be explored as carriers to systemically deliver siRNA into cancer cells and execute gene silencing in transplanted tumor, remains largely unknown.

In the present study, we aimed to examine that MVs can be as an ideal exogenous siRNA delivery vehicle transporting therapeutic TGF-β siRNA for tumor therapy. Here, we selected mouse fibroblast L929 cell line and transfected with TGF-β1 siRNA using liposomes. Cells supernatant were harvested 24 or 48 h after transfection. After removing cells and other debris by centrifugation at 500, 1500, and 10,000 × *g*, the supernatant was centrifuged at 110,000 × *g* for 70 min (all steps were performed at 4 °C). MVs containing more TGF-β1 siRNA were collected from the pellet and resuspended in PBS or FBS-free medium, and used to treat murine sarcomas 180 tumor cells *in vitro* and *in vivo*. Moreover, we analyzed the TGF-β expression, cell functions, and TGF-β signaling suppression of the S180 tumor cells treated with MVs-delivered TGF-β1 siRNA. Our study serves to provide an effective strategy for controlling tumor cell growth and metastasis.

2. Materials and methods

2.1. Reagents

The mouse fibroblast L929 cell line, human embryonic kidney 293T cell line and the mouse sarcomas 180 (S180) cell line were purchased from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All the cell lines were cultured in DMEM supplemented with 10% FBS (GIBCO) and penicillin (100 U/ml)/streptomycin (100 mg/ml) at 37 °C in a 5% CO_2, water-saturated atmosphere. Anti-TGF-β1 (BS-1361) antibody was purchased from Bioworld Technology (Louis Park, MN, USA), anti-GAPDH (6C5) antibodies was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Ago2 (ab57113) antibody was purchased from Abcam (Cambridge, MA, USA), and anti-phosphorylated Smad2 (#3108) and anti-Smad2/3 (#3102) antibodies were purchased from Cell Signaling Technology (Cell Signaling, Beverly, MA). Quantikine™ TGF-β1 ELISA kit was purchased from R & D System (Minneapolis, MN, USA).

2.2. Transfection of S180 cells with TGF-β1 siRNA

Four siRNA oligonucleotides were synthesized from GenePharma Biotechnology (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). The sequences of these siRNAs duplex are: TGF-β1 siRNA-1 sense, 5′-GCUCUUGUGACAGCAAAGAUATT-3′; antisense, 5′-UAUCUUUGCUGUCACAAGAGCTT-3′; TGF-β1 siRNA-2 sense: 5′-GAGCCCUGGAUACCAACUAUUCTT-3′, antisense: 5′-AAUAGUUGGUAUCCAGGGCUCTT-3′; TGF-β1 siRNA-3 sense: 5′-AUGUGGAACUCUACCAGAAAUTT-3′, antisense: 5′-AUUUCUGGUAGAGUUCCACAUTT-3′; TGF-β1 siRNA-4 sense: 5′-GUACAGCAAGGUCCUUGCCCUTT-3′, antisense: 5′-AGGGCAAGGACCUUGCUGUACTT-3′; scrambled negative control sense: 5′-GUCUCCACGCGCAGUACAUUUTT-3′, antisense: 5′-AAAUGUACUGCGCGUGGAGACTT-3′. S180 cells were seeded on 6-well plates or 10-mm dishes prior to transfection. When the cells reached approximately 70–80% confluence, they were transfected with TGF-β1 siRNA-1, TGF-β1 siRNA-2, TGF-β1 siRNA-3, TGF-β1 siRNA-4 or scrambled negative control (NC siRNA), using Lipofectamine 2000 as suggested by the manufacturer (Invitrogen). Twenty-four hours after transfection, total RNA and protein were harvested and analyzed for TGF-β1 expression. In a separate experiment, S180 cells were plated on 10-mm dishes and when the cells reached approximately 70–80% confluence, they were cultured in serum-free medium containing lipofectamine-mediated TGF-β1 siRNA-1 or TGF-β1 MVs (L929 MVs-delivered TGF-β1 siRNA-1) along with the 8 μM TPF [23] for 24 h. Then, total RNA and protein were harvested and analyzed for TGF-β1 expression. Data are representative of three independent experiments.

2.3. Preparation of bio-nanoparticles-MVs

MVs were isolated from the L929 cell culture medium by differential centrifugation according to previous publications [15]. Briefly, L929 cells were seeded on 10-cm dishes overnight and transfected the following day using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. For the overexpression of TGF-β1 siRNA-1, 60 pmol per 1 × 10^5 cells of TGF-β1 siRNA-1 was used. Scrambled negative control siRNA (NC siRNA) were used as control for TGF-β1 siRNA-1. Cells supernatant were harvested and centrifuged at 500 × *g*, 1500 × *g*, 10,000 × *g* and 110,000 × *g* (all steps were performed at 4 °C). MVs were collected from the pellet and resuspended in PBS or FBS-free medium.

2.4. Fluorescence labeling of MV and confocal microscopy analysis

L929 cells were labeled with DiI-C_{16} for 1 h and then washed three times with PBS. The cells were re-suspended and cultured for 24 h in DMEM medium supplemented with 10% FBS. The supernatants were then collected and centrifuged to harvest MVs. L929 MVs were re-suspended in FBS-free DMEM medium or PBS and incubated with cultured S180 cells. After a fixed time interval (0 h, 2 h or 4 h), S180 cells were washed, fixed, and observed under confocal microscopy (FV1000; Olympus, Tokyo). The pictures were taken under these conditions: Objective Lens: PLAPON 60× O NA: 1.42; Scan Mode: XY; Excitation Wavelength: 405 nm for DAPI and 543 nm for DiI-C_{16}; Image Size: 1024 × 1024 Pixel.

2.5. Transmission electron microscopy assay

For conventional TEM, the MV pellet was placed in a droplet of 2.5% glutaraldehyde in PBS buffer at pH 7.2 and fixed overnight at 4 °C. Samples were rinsed in PBS buffer (3 times, 10 min each) and postfixed in 1% osmium tetroxide for 60 min at room temperature. The samples were then embedded in 10% gelatin and fixed in glutaraldehyde at 4 °C and cut into several blocks (less than 1 mm^3). The samples were dehydrated for 10 min each step in increasing concentrations of alcohol (30%, 50%, 70%, 90%, 95%, and 100% × 3). Pure alcohol was then exchanged by propylene oxide, and specimens were infiltrated with increasing concentrations (25%, 50%, 75%, and 100%) of Quetol-812 epoxy resin mixed with propylene oxide for a minimum of 3 h per step. Samples were embedded in pure, fresh Quetol-812 epoxy resin and polymerized at 35 °C for 12 h, 45 °C for 12 h, and 60 °C for 24 h. Ultrathin sections (100 nm) were cut using a Leica UC6 ultra-microtome and poststained with uranyl acetate for 10 min and with lead citrate for 5 min at room temperature before observation in a FEI Tecnai T20 transmission electron microscope, operated at 120 kV.

2.6. Quantitative real-time PCR

The total RNA from MVs, cells or tissues were extracted using TRIzol Reagent (Invitrogen). Quantitative RT-PCR was carried out using TaqMan miRNA probes (Applied Biosystems; Foster City, CA) according to the manufacturer's instructions. Briefly, 5 μl of total RNA was reverse transcribed to cDNA using AMV reverse transcriptase (TaKaRa; Dalian, China) and a stem-loop RT primer (Applied Biosystems). Real-time PCR was performed using a TaqMan PCR kit on an Applied Biosystems 7300 Sequence Detection System. All reactions, including no-template controls, were run in triplicate. After the reaction, the CT values were determined using fixed threshold settings. To calculate the absolute expression levels of TGF-β1 siRNA-1, a series of synthetic TGF-β1 siRNA-1 oligonucleotides at known concentrations were also reverse transcribed and amplified. The absolute amount of TGF-β1 siRNA-1 was then calculated by referring to the standard curve. In the experiments presented here, because there is no current consensus on the use of housekeeping genes for quantitative RT-PCR analysis in MVs, the expression levels of TGF-β1 siRNA-1 in MVs were directly normalized to the total protein content of MVs in our study.

To detect the expression level of TGF-β1, TGF-β2 or TGF-β3, primers specific for TGF-β1, TGF-β2 or TGF-β3 messenger RNAs (mRNAs) were designed using Primer Express v.2.0 software (PE Applied Biosystems, Foster, CA). The following primer sequences were used: TGF-β1 forward, 5′-ACCGCAACAACGCCATCTAT-3′, TGF-β1 reverse, 5′-GTAACGCCAGGAATTGTTGC-3′; TGF-β2 forward: 5′-CGTCCGCTTTGATGTCTC-3′, TGF-β2 reverse, 5′-AGTTCAATCCGCTGCTCG-3′; TGF-β3 forward, 5′-AGGGTGGAAGCCATTAGG-3′, TGF-β3 reverse, 5′-AAGACAGCCATTCAGCGG-3′; β-actin forward, 5′-GAGACCTTCAACACCCCAGC-3′, β-actin reverse, 5′-ATGTCACGCACGATTTCCC-3′. The level of TGF-β1, TGF-β2, and TGF-β3 genes was evaluated on the basis of the copy number of mRNA related to 1 μg of total RNA by the use of SYBR Green Reagent (Invitrogen) on an Applied Biosystems 7300 Sequence Detection system according to the manufacturer's instructions. Briefly, RNAs are polyadenylated by poly(A) polymerase and subsequently converted into cDNA by reverse transcriptase with oligo-dT. The cDNA is then used for real-time PCR quantification of mRNAs. β-actin was used as internal controls in each single qRT-PCR for all samples.

2.7. Western blot analysis

Samples of tissues and cultured cells were prepared and used for immunoblotting as described previously [24]. Briefly, Samples of tumor and cultured cells