Biomaterials 35 (2014) 3060–3070

Contents lists available at ScienceDirect

Biomaterials

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

Selective eradication of tumor vascular pericytes by peptide-conjugated nanoparticles for antiangiogenic therapy of melanoma lung metastasis

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ARTICLE INFO

Article history:
Received 19 November 2013
Accepted 13 December 2013
Available online 3 January 2014

Keywords:
Pericyte
Antiangiogenic therapy
Nanoparticles
Melanoma
Lung metastasis

ABSTRACT

Antiangiogenic cancer therapy based on nanoparticulate drug delivery systems (nano-DDS) is emerging as a promising new approach besides the proved molecular-targeted antiangiogenic agents. The current nano-DDS are restricted to the targeting to tumor vascular endothelial cells, but seldom efforts have been made to target the tumor vascular pericytes which are also actively involved in tumor angiogenesis. In this study, we developed a new nano-DDS, TH10 peptide (TAASGVRSMH) conjugated nanoparticles loading docetaxel (TH10-DTX-NP) that can target the NG2 proteoglycan highly expressed in tumor vascular pericytes, for the investigation of therapeutic efficacy in the mice bearing B16F10-luc-G5 melanoma experimental lung metastasis. The results demonstrated that TH10-DTX-NP achieved controlled drug release in PBS and the mixture of rat plasma and PBS (1:1, v/v), and exhibited favorable *in vivo* long-circulating feature. TH10 peptide conjugation facilitated the nanoparticle internalization in pericytes via the interaction between TH10 and NG2 receptor, leading to more inhibition of pericyte viability and migration. TH10-conjugated nanoparticles could accurately target the vascular pericytes of B16F10-luc-G5 lung metastasis, where DTX-induced pronounceable pericyte apoptosis. TH10-DTX-NP significantly prolonged the mice survival with no obvious toxicity, and this enhanced antitumor effect was closely related with the decreased pericyte density and microvessel density in the lung metastases. The present research reveals the potency and significance of targeting tumor vascular pericytes using nano-DDS in antiangiogenic cancer therapy.

1. Introduction

Antiangiogenesis aiming at interfering with the supply of oxygen and nutrients to tumor cells by affecting the tumor vasculature is a cutting-edge treatment in anticancer therapy. FDA has approved several antiangiogenic inhibitors (Avastin, Nexavar, Votrient, Sutent, Caprelsa, Inlyta, Zaltrap, etc.) for the treatment of multiple types of cancer [1–3]. However, these molecular-targeted agents also lead to off-target effect on physiological functions and homeostasis, which in some circumstances seriously compromise their clinical performance [4].

Antiangiogenic strategy based on nanoparticulate drug delivery systems (DDS) is emerging as a new promising approach in this research field [5–7]. The capability to specifically deliver antiangiogenic therapeutics to tumor neovasculature and meanwhile minimize systemic toxicity is the major advantage for nano-DDS over the proved antiangiogenic agents. It is noted that most of the current nano-DDS are restricted to the targeting to tumor vascular endothelial cells (EC) [8], but seldom efforts have been made to target tumor vascular pericytes, a subtype of mural cells that are embedded within the basement membrane of tumor vessel and actively involved in tumor angiogenesis [9]. The activated pericytes may, at the initiation of angiogenesis, degrade the basement membrane and liberate matrix-bound growth factors [10]. They can regulate vascular sprouting by secreting factors such as VEGF to recruit EC from the parental vessel [10]. They also contribute to vascular stabilization and maturation and confer EC survival advantage through direct cell contact [10] and inducing autocrine VEGF-A signaling and Bcl-w expression in EC [11]. The contribution of pericytes in tumor angiogenesis has been proved to be responsible for the resistance in some anti-VEGF therapeutic

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http://dx.doi.org/10.1016/j.biomaterials.2013.12.027

settings [12,13]. Several studies have demonstrated that inhibition of the pericytes using kinase inhibitors or antibodies can add more anticancer efficacy to the treatment of targeting tumor EC alone [12–14]. Recently, doxorubicin-loaded liposomes modified with CPRECES peptides for APA (an angiogenic pericyte marker) targeting were generated for the combined therapy of targeting both tumor pericytes and EC to acquire synergistic enhanced anticancer efficacy [15].

In view of the critical role of pericytes in the maintenance of functional tumor vasculatures, which are necessary for nascent cancer metastasis, we hypothesize that it may be feasible to combat metastatic cancer by selective pericyte eradication through pericyte-targeting treatment. Here, for the pericyte-targeting drug delivery, a new nanomedicine, peptide (TAASGVRSMH, named TH10) conjugated biodegradable nanoparticles loaded with docetaxel (TH10-DTX-NP) was developed. TH10 was isolated from a phage display peptide library and has high affinity and specificity to NG2 proteoglycan [16], another highly expressed marker on the pericytes of tumor neovasculature [16–18]. The model drug docetaxel is a clinically well established anti-mitotic agent and was found in our lab to possess potent pericyte-killing activity. The targeting delivery ability of TH10-DTX-NP to pericytes *in vitro* and *in vivo*, and its potential therapeutic efficacy in experimental NG2-negative B16F10-luc-G5 lung metastases were investigated.

2. Materials and methods

2.1. Materials, cell culture, and animals

Aldehyde poly (ethylene glycol)-poly(lactide) (aldehyde-PEG-PLA, MW 64 kDa) and MPEG-PLA (MW 61 kDa) block copolymers were synthesized by the ring opening polymerization in our lab as previously described [19]. Docetaxel (DTX) was purchased from Knowshine Parma chemicals (Shanghai, China). TH10 peptide (TAASGVRSMH) was synthesized by GL Biochem (Shanghai, China). Coumarin 6, filipin, phenylarsine oxide, cytochalasin D, nystatin, chlorpromazine, and nocodazole were from Sigma–Aldrich (St. Louis, MO). Double distilled water was purified using a millipore simplicity system (Millipore, Bedford, MA). All other chemicals were of analytical grade and used without further purification.

Primary human brain vascular pericyte (HBVP) and pericyte medium (PM), were obtained from Sciencell (Carlsbad, CA). The cells at 3 to 5 passages were used in the experiments. B16F10-luc-G5, a luciferase expressing cell line derived from B16F10 mouse melanoma cells, was obtained from Caliper Life Sciences (Hopkinton, MA) and cultured in MEM/EBSS medium supplemented with 10% fetal bovine serum, 1× non-essential amino acids, 2 mM L-glutamin, 1 mM sodium pyruvate, 1× MEM vitamin solution and antibiotics (100 μg/ml of streptomycin and 100 U/ml of penicillin) (Life Technologies, Carlsbad, CA) at 37 °C in a humidified incubator with 5% CO_2.

Male and female Sprague–Dawley (SD) rats (180–200 g) and female BALB/c mice (~20 g) were provided by the Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). The animal experiment designed in this study was approved by the ethical committee of Shanghai Jiao Tong University School of Medicine.

2.2. Preparation and characterization of TH10-DTX-NP

TH10-DTX-NP were fabricated by emulsion and solvent evaporation method with a following surface functionalization. Briefly, 3 mg DTX was dissolved in 1 ml solution of 30 mg blend of aldehyde-PEG-PLA and MPEG-PLA (1:9, w/w) in dichloromethane. Next, 3 ml of 1% (w/v) sodium cholate was slowly poured into the solution and then the mixture was sonicated at 280 w for 25 s (Scientz Biotechnology, Ningbo, China). The O/W emulsion was further diluted in 40 ml of 0.5% (w/v) sodium cholate solution and the organic solvent was removed by rotary evaporation under reduced pressure. The resulting DTX-loaded nanoparticles (DTX-NP) were collected by centrifugation (11,000 × g, 30 min, 4 °C; Eppendorf AG 5810R, Germany) and washed twice to remove the excessive emulsifier. Then, DTX-NP was incubated with TH10 at 1:3 molar ratio of aldehyde to the N-terminal amine of TH10. The conjugation reaction was processed in 0.01 M PBS (pH 7.4) at room temperature for 10 h in the presence of $NaCNBH_3$ as a reducing reagent. The unconjugated TH10 was removed by centrifugation (11,000 × g, 30 min, 4 °C) and TH10-DTX-NP were collected. The coumarin 6-labeled nanoparticles were prepared in the same way except that in the oil phase DTX was mixed with 0.05% (w/v) coumarin 6.

The particle size and zeta potential were determined using a Zetasizer Nano ZS instrument (Malvern, Worcestershire, UK). The nanoparticles were negatively stained with 2% (w/v) sodium phosphotungstate and visualized using H-600 transmission electron microscopy (TEM) (Hitachi, Japan). Encapsulation efficiency (EE%) was expressed as the percentage of the drug amount found in the nanoparticles to the total amount used to prepare the nanoparticles, and drug loading (DL %) was expressed as the percentage of the drug amount found in the nanoparticles. The content of docetaxel and coumarin 6 were determined by high performance liquid chromatography (HPLC) methods. The peptide conjugation efficiency (CE%) was determined by estimating the amount of nanoparticle-associated peptide using CBQCA Protein Quantitation Kit (Life Technologies, Carlsbad, CA) [19].

2.3. Docetaxel release from TH10-DTX-NP

In vitro release experiments of DTX from the nanoparticles were performed under different pH conditions at 37 °C in 0.01 M PBS in order to evaluate if the release rate is associated with the pH, and also in the mixture of rat plasma and 0.01 M PBS (1:1, v/v). Briefly, 15 mg of TH10-DTX-NP or DTX-NP were put in a centrifuge tube and suspended in 5 ml phosphate buffer solution (PBS, pH 7.4 or 4.0) or mixture of PBS and rat plasma (1:1, v/v) containing 0.1% Tween-80 to maintain the sink condition. The tubes were placed in the gas bath at 37 °C shaking at 100 rpm. At specific intervals, the nanoparticles were centrifuged (11,000 × g, 30 min, 4 °C) and 1 ml release medium containing the free drug was transferred out and another 1 ml fresh medium was added to the test tubes to resuspend the particles for continuous release tests. The supernatant containing the drug was cryo-desiccated, then redissolved in 1 ml acetonitrile and analyzed by HPLC. Each measurement was performed in triplicate.

2.4. Pharmacokinetic study of TH10-DTX-NP in SD rats

Sprague–Dawley (SD) rats (180–200 g) were randomly divided into three groups (3 male and 3 female rats in each group) and fasted overnight with free access to water before drug administration. Rats in control group were intravenously administered through the caudal vein with 3 mg/kg Taxotere. The other two groups were injected with DTX-NP and TH10-DTX-NP formulations in saline solution (0.9%, w/v) at the same dose as the control group. After administration, 200 μl blood samples was collected from the retro-orbital plexus at 0.083, 0.5, 1, 2, 4, 8, 12, 24 and 36 h, respectively, and were placed into heparinized micro-centrifuge tubes. The blood sample was centrifuged at 5000 × g for 5 min and the separated plasma (100 μl) was stored at −80 °C until HPLC analysis. Briefly, 400 μl tert-Butyl methyl ether was added to the plasma sample to extract the DTX, the supernatant was collected, and the residue was extracted again in the same way. The supernatant containing DTX was volatilized by SpeedVac Concentrator (Thermo Scientific, Rockford, IL) and the residue was reconstituted in 100 μl mobile phase containing 20 μg/ml paclitaxel as internal standard. The chromatography system was composed of a Shimadzu LC-20AT chromatographic system (Shimadzu, Kyoto, Japan) with an LC-20AT binary pump and an SPD-20A UV-Vis spectrophotometry at 229 nm. Analysis was carried out on a Dikma Diamonsil C18 column (4.6 mm × 200 mm, 5 μm, Dikma Technologies, Beijing, China). The mobile phase was composed of H_2O-acetonitrile (48:52, v/v), and the flow rate was 1 ml/min. The column temperature was maintained at 25 °C, and the injection volume was 20 μl. The pharmacokinetic parameters were calculated with the WinNonlin software (Version 6.1 Pharsight, Mountain View, CA) according to non-compartmental model.

2.5. Targeted internalization of TH10-conjugated NP in HBVP

For cell uptake examination, HBVP (a cell model mimicking tumor vascular pericyte) [11,18] were cultured at a density of 5×10^3 cells/well in 96-well plates. When the cells reached about 80% confluence, the medium was replaced by 200 μl of coumarin 6-labeled nanoparticles (coumarin 6-labeled NP) or coumarin 6-labeled TH10-conjugated nanoparticles (coumarin 6-labeled TH10-NP) in medium at coumarin 6 dose of 0.2 μg/ml for 2 h. To investigate the internalization mechanism, the uptake studies were performed under low temperature (4 °C) or in the presence of soluble TH10 (50-fold excess), 10 μM filipin (to inhibit caveolae-mediated endocytosis), 3 μM cytochalasin D (to inhibit macropinocytosis), 10 μM nocodazole (to inhibit microtubule-mediated endocytosis), 30 μM chlorpromazine (to inhibit clathrin-mediated endocytosis), 30 μM phenylarsine oxide (to deplete ATP), or 10 μM nystatin (an inhibitor of lipid-raft mediated endocytosis) at 37 °C, respectively. HBVP were incubated with the inhibitors for 30 min before the incubation with nanoparticles for following 2 h. After removing the nanoparticles and washing the wells twice with PBS, the cells were fixed by 1% glutaraldehyde for 15 min, stained by 100 ng/ml DAPI (Invitrogen, Eugene, OR) for 8 min. Then, cellular uptake was assayed by quantifying the intracellular fluorescence intensity on the Thermo Scientific ArrayScan XTI High Content Analysis (HCA) Reader using a FITC filter (Ex: 490 nm, Em: 520 nm).

For the identification of NG2 expression in HBVP, we performed both western blot and flow cytometry assay with B16F10-luc-G5 cells as control. For western blot assay, the membrane proteins of HBVP and B16F10-luc-G5 cells were extracted by Novagen ProteoExtract Transmembrane Protein Extraction Kit (Merck, Darmstadt, Germany). Protein concentration was determined using BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and equalized before loading. Forty micrograms of membrane protein from the two samples were applied to 8% SDS-polyacrylamide gel and probed with rabbit anti-mouse NG2 antibody (Millipore, Temecula, CA) followed by exposure to a horseradish peroxidase-conjugated goat anti-rabbit