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A tissue factor targeted nanomedical system for thrombi-specific drug delivery

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ABSTRACT

Tissue factor (TF) is a 47 kDa membrane-bound glycoprotein, which is present at high concentrations on damaged endothelium, atherosclerotic plaques or tumor vasculature, and is an important trigger of coagulation cascade. In this study, we have expressed and purified the TF targeting protein-EGFP-EGF1, which was thiolated and conjugated to the malemide of the PEG-PLGA nanoparticle to form a TF targeting nanomedical system: EGF1-EGFP-NP. The system was carefully characterized and the targeting efficiency was systematically evaluated. The EGF1-EGFP-NP could significantly facilitate specific uptake by TF overexpressed BCEC via EGF1/TF mediated endocytosis pathway. In addition, the pharmacokinetic study demonstrated that EGF1-EGFP-NP has the same blood circulation time as NP. Enhanced accumulation of EGF1-EGFP-NP in the cortex infarction region was also observed by real-time fluorescence image. Confocal microscopy and TEM further showed that EGF1-EGFP-NP combined with TF and further transfected through the damaged endothelium. Moreover, in vitro cell viability experiment and in vivo coagulation ability confirmed that the EGF1-EGFP-NP was safe.

1. Introduction

In the classic concept of coagulation, tissue factor (TF), the 47 kDa membrane-bound glycoprotein, is an important trigger. The TF is always present on subendothelial cells under normal physiological conditions, endothelial disruption leads to exposure of TF to the blood stream. Exposed TF can bind with its natural ligand factor Ⅶ (FⅦ), which then becomes activated FⅦ(FⅦa). The thus formed TF: FⅦa complex converts factor Ⅹ(FⅩ) to activated factor Ⅹ(FⅩa) and FⅩa in turn activates prothrombin leading to formation of thrombin. Thrombin subsequently activates platelets and converts fibrinogen into fibrin, two essential components of a stable thrombus [1,2].

The primary function of subendothelial TF is to serve as a hemostatic envelope surrounding the vasculature. However, under certain conditions the expression of TF is induced in monocytes and endothelial cells by various stimuli such as tumor necrosis factor-a (TNF-a) and bacterial endotoxin lipopoly saccharide (LPS) [3,4]. TF is also often expressed on cancer cells and the tumor vasculature, which is present at high concentrations in atherosclerotic plaques [5,6]. In our previous research we have identified the TF expression in the thrombi of photochemical cerebral thrombosis rats [7]. Therefore, as an upstream component of the cascade, the TF might be a potent target for thrombolytic agents or anti-tumor drugs.

Monoclonal antibody-mediated TF targeted drug delivery maybe a good strategy.

[8]. However, the potential immunogenicity and difficulty for large quantity production may restrict its application in clinical practice. As the natural ligand of TF, the light chain of FⅦ contains an epidermal growth factor-like domain-EGF1. Recent evidence showed the EGF1 domain plays an important role in TF binding and the TF: FⅦa complex formation [9,10]. The intriguing results prompted us to develop a TF targeted drug delivery system by taking advantage of the TF-targeting property of EGF1 peptide.

Poly (D,L-lactide-coglycolide) (PLGA), polylactide (PLA) and PEG have already been approved by FDA for producing non-cytotoxic, biodegradable and sustained-release drug carriers [11]. In our previous research, the EGF1 domain peptide was cloned from rat FⅦ and EGFP-EGF1fusion protein was obtained from *Escherichia coli* BL21cells. Furthermore, the EGFP-EGF1 protein was conjugated

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with PEG-PLA nanoparticles and the following EGFP-EGF1-NP have shown cerebral thrombus targeting property by taking advantage of the TF-targeting property of EGFP-EGF1 fusion protein. In vivo multispectral fluorescent imaging demonstrated that EGFP-EGF1-NP had high specificity and sensitivity in targeting thrombi compared with EGFP-NP [7]. However, both the distribution of the NPs in the cerebral infarctions and their relationship with the endothelium derived TF were still obscure.

It had been recently reported that NPs formulated using PLGA polymer demonstrated greater gene transfection than those formulated using PLA polymer [12,13]. In this study, we developed a new TF targeted drug delivery system: EGFP-EGF1 protein-conjugated PEG-PLGA nanoparticles (EGF1-EGFP-NP). The EGFP-EGF1 fusion proteins were further purified and the system was carefully characterized. The targeting efficiency was systematically evaluated in vitro at the cellular level by performing intracellular accumulation, vitro cytotoxicity in primary cultured rat brain capillary endothelial cell (BCEC). Besides the localization in rats was examined by using in vivo multispectral fluorescent imaging, the relationship between TF and EGF1-EGFP-NP was investigated by confocal microscopy and TEM. The system's pharmacokinetics and effect on the coagulogram of SD rats were also investigated.

2. Materials & methods

2.1Materials and animals

The *E. coli* strain BL21 (DE3) and plasmid pET-28a-EGF1-EGFP were maintained in our laboratory. Isopropyl-β-D-thiogalacto-pyranoside(IPTG), 2,3,5-Triphenyl tetrazolium chloride (TTC), rose bengal sodium salt (dye content ~90%),DNase I, Goat anti-rabbit IgG/Gold Colloid, coumarin6 and 4′,6-Diamidino-2-phenylindole (DAPI) were purchased from Sigma Co. (USA). 2-iminothiolane (Traut's reagent) and BCA Protein Assay Reagent were from Thermo fisher scientific inc. (USA).Ni^{2+}-NTA affinity chromatography and Sephacryl S-100 HR chromatography were from GE healthcare (USA). The medium 131/microvascular growth supplement (MVGS), DMEM-F12, collagenase II, DiI-Ac-LDL were from Invitrogen Co. (USA). The Percoll™ PLUS was from GE Healthcare Co. (Sweden).Collagenase/dispase and bovine serum albumin (BSA) were purchased from Roche Co. (USA). The Rabbit anti-histag polyclonal antibody, rabbit anti rat TF polyclonal antibody were obtained from Santa Cruz Co. (USA). Rabbit polyclonal to Von Willebrand Factor (VWF) and CY3 labeled goat anti-rabbit IgG were from Abcam Co. (USA). Recombinant rat tumor necrosis factor alpha (TNF-a) was from R&D Systems (USA). Dir (DiIC18 (7) or 1, 1′-dioctadecyl-3, 3, 3′, 3′- tetramethyl indotricarbocyanine Iodide, λex\λem (MeOH) = 748/780 nm) was from Biotium (USA). Poly (DL-lactic-co-glycolic acid) (50:50) (PLGA, inherent viscosity 0.89, Mw ~ 100 kDa) was purchased from Absorbable Polymers (USA). Methoxy-poly (ethylene glycol) (MePEG, MW 3000 Da) was supplied by NOF Corporation (Japan) and Maleimide-PEG (MW 3400 Da) was purchased from Nektar (Huntsville, AL). Sodium cholate was from Shanghai Chemical Reagent Co. (China). Ellman's reagent was from Acros Co. (Belgium). Cell counting kit-8 (CCK-8) was from Dojindo Inc. (Japan). All the other chemicals were analytical reagent grades and used without further purification.

Sprague–Dawley rats (50–60g, 180–220g, ♂) were provided by the Center of Experimental Animals of Tongji Medical College (Wuhan, China). The protocols for treating the animals in the experiment were evaluated and approved by the ethical committee of Tongji Medical College.

2.2. Preparation of nanoparticles

MePEG-PLGA and maleimide-PEG-PLGA block copolymers were synthesized and characterized as described previously [14,15]. Nanoparticles were made of a blend of MePEG-PLGA and maleimide-PEG-PLGA (weight ratio 10:1) by using a double-emulsion solvent-evaporation technique as described elsewhere with minor modification [13,15–18].The preparation of nanoparticles loaded with coumarin6, Superparamagnetic Ferumoxides (Fe_3O_4) or Dir were the same as that of blank nanoparticles, except that 100 μl coumarin6 (3 mg/ml stock solution in dichloromethane), 100 μl Fe_3O_4 (20 nm, 10 mg/ml stock solution in dichloromethane) or 10 μl Dir (20 mg/ml stock solution in dichloromethane) was additionally added to dichloromethane containing copolymers before primary emulsification and the obtained nanoparticles were subjected to a 1.5 × 20 cm sepharose CL-4B column and eluted with 0.05M HEPES buffer pH 7.0 containing 0.15M NaCl to remove the unentrapped coumarin6, Fe_3O_4 or Dir. In addition, the preparation of NP-Fe_3O_4 should be avoided of magnetic stirring.

2.3. Modification of nanoparticles with EGFP-EGF1 fusion protein

The EGFP-EGF1 fusion proteins were obtained and purified according to our previous work with major modification [7]. In brief, the proteins were harvested from *E. coli* strain BL21 and applied to a Ni^{2+}-NTA affinity chromatography. The non-purified proteins were eluted by 300 mM imidazole (pH 8.0) after non-specific having been eluted by 60 mM imidazole (pH 8.0). Then the proteins were purified by taking advantage of the Sephacryl S-100 HR chromatography, and the elution products were collected in 5 different time periods on the protein purification machine (AKTAprimer, GE healthcare, USA) for SDS-PAGE. Finally, the most purified proteins were confirmed by Western blotting according to our previous report [7]. The protein was then thiolated using 2-iminothiolane (Traut's reagent) [20]. Ellman's reagent was used to determine the extent of thiolation [19].

The thiolated EGFP-EGF1 was then mixed with nanoparticles at a thiolated EGFP-EGF1: maleimide ratio of 1:3. The volume of mixture was 2 ml and the conjugation of the EGFP-EGF1 to the nanoparticles was performed 8 h on a rotating plate set at a low speed. The reaction mixture was then applied to a 1.6 × 20 cm Sepharose CL-4B column and eluted with 0.01 M PBS buffer (pH 7.4). The milky EGF1-EGFP-NP fractions were visually identified and collected, and the nanoparticles concentration was determined by turbidimetry with UV2401 spectrophotometer at 350 nm (Shimadzu, Japan) [20]. The average number of EGFP-EGF1 molecules conjugated per nanoparticle was determined by the Thermo Scientific Pierce BCA Protein Assay Kit and calculated by dividing the number of EGFP-EGF1 by the average number of nanoparticles using the methods described by Oliver et al. [21].

2.4. Characterization of EGF1-EGFP-NP

2.4.1. Morphology, particle size and surface charge

EGF1-EGFP-NP and NP were observed by transmission electron microscope (H-600, Hitachi, Japan) following negative staining with sodium phosphotungstate solution. The mean diameters and zeta potential of NPs were determined by dynamic light scattering (DLS) using a Zeta Potential/Particle Sizer NICOMPTM 380 ZLS (Santa Barbara, USA) with He–Ne laser at 632.8 nm.

2.4.2. Transmission electron microscopy (TEM) of gold-labeled EGF1-EGFP-NP

For the confirmation of EGFP-EGF1 binding on the surface of nanoparticles, EGF1-EGFP-NP was examined under a transmission electron microscope after sequential incubation with antibodies. There were six histidine (His6) sequences in the N-terminus of the EGF1-EGFP fusion protein, and the rabbit anti-histag polyclonal antibody was employed as the primary antibody. Firstly, EGF1-EGFP -NP (1 mg) was incubated with 500 μl 10 μg/ml rabbit anti-histag polyclonal antibody overnight at 4 °C in 0.01M phosphate buffered saline, and then applied to a 1.6 × 20 cm sepharose CL-4B column and eluted with PBS to remove the unbounded primary antibody. The nanoparticles were incubated with 50 μl Goat anti-rabbit IgG/Gold Colloid for 1.5 h at 37 °C. After that, the nanoparticles were deposited on a 200 mesh formvar-coated copper grid and stained with 1% (w/v) phosphotungstic acid solution, and finally examined by TEM. The EGF1-EGFP-NP (1 mg) without the addition of the primary antibody and the NP (1 mg) were used as the negative controls.

2.4.3. Drug loading efficiency and in vitro release of coumarin6 or Dir

The coumarin6 loading capacity of NPs was determined by HPLC analysis after dissolving them in 20 times volume of methanol. A 20 μl diluted sample was injected in the Agilent 1200 (Agilent Technologies, USA) which consist of a fluorescence detector (G1321A FLD, Ex 465 nm/Em 502 nm). With a Dikma Diamonsil C18 (5 μm, 200 mm, 4.6 mm) column, the separations were achieved in methanol:deionized water (96:4) mobile phase with a flow rate of 1.2 ml/min and column temperature of 30 °C.

To determine the content of Dir, nanoparticles were dissolved in 20 times volume of methyl cyanides and measured the absorbance by UV 2401 spectrophotometer at 758 nm. The release of Dir and coumarin6 from both nanoparticles and EGFP-EGF1 conjugated ones were incubated in rat plasma at 37 °C on a shaker at 100 rpm. Periodic samples were subject to centrifugation at 14,000 rpm for 45 min and the supernatant was analyzed for the released of coumarin6 or Dir as described above. The samples were protected from light throughout the experimental procedure.

2.5. Primary culture and characterization of rat brain capillary endothelial cell (BCEC)

The method of the primary culture was adopted and modified according to previously described techniques [22–25]. In brief, cortexes of 3-week-old SD rats were dissected and gray matter was minced into small pieces. The homogenate was digested by collagenase type 2 (1 mg/ml) and collagenase/dispase (1 mg/ml) sequentially, and then the microvessel endothelial cell clusters were separated on a 50% continuous Percoll gradient. Microvessel and BCEC cultures were maintained in Medium 131 supplemented with MVGS, streptomycin (100 μg/ml) and penicillin