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Anti-angiogenic inhibition of tumor growth by systemic delivery of PEI-*g*-PEG-RGD/pCMV-sFlt-1 complexes in tumor-bearing mice

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Abstract

Vascular endothelial growth factor (VEGF) is an endogenous mediator of tumor angiogenesis. Blocking associations of the VEGF with its corresponding receptors (Flt-1, KDR/flk-1) have become critical for anti-tumor angiogenesis therapy. Previously, we synthesized PEI-*g*-PEG-RGD conjugate and evaluated as an angiogenic endothelial polymeric gene carrier. In this study, PEI-*g*-PEG-RGD/pCMV-sFlt-1 complexes are evaluated in terms of tumor growth inhibition in vivo. Complexes were repeatedly injected systemically via tail vein into subcutaneous tumor-bearing mice. As a result, tumor growth was inhibited in the PEI-*g*-PEG-RGD/pCMV-sFlt-1 injected group. However, this effect was not identified in PEI-*g*-PEG/pCMV-sFlt-1 or PEI-*g*-PEG-RGD/pCMV-GFP control groups. Moreover, the survival rate increased in the PEI-*g*-PEG-RGD/pCMV-sFlt-1 group compared with the controls group. These results suggest that delivery of pCMV-sFlt-1 using PEG-*g*-PEG-RGD may be effective for anti-angiogenic gene therapy.

Keywords: Systemic injection; Anti-angiogenesis; Tumor therapy; Soluble Flt-1; Targeted gene delivery

1. Introduction

Angiogenesis, the development of new blood vessels from the endothelium of a pre-existing vasculature, is a critical process required by most solid tumors to support their growth and metastasis [1–4]. Therefore, anti-angiogenic therapy has been demonstrated to be an attractive strategy for cancer treatment. Among the known angiogenic growth factors and cytokines implicated in the modulation of normal and pathological angiogenesis, the VEGF [3] family (VEGF-A, VEGF-B, VEGF-C, VEGF-D) and their corresponding receptor tyrosine kinases [VEGF-R1 (Flt-1), VEGF-R2 (KDR/flk-1) and VEGF-R3 (Flt-4)] play a paramount and indispensable role in regulating the angiogenic processes, as well as the induction of vascular permeability and inflammation [1,2,5–7]. Increased expression of VEGFs by tumor cells, VEGF-R1 (Flt-1) and VEGF-R2 (KDR/flk-1), by the tumor-associated vasculature is a hallmark of a variety of tumors in vivo and correlate with tumor growth rate, microvessel density/proliferation, tumor metastatic potential and poorer patient prognosis in a variety of malignancies [1–6].

The soluble form of VEGF-R1 (sFlt-1), an alternatively spliced form of Flt-1, is a potent endogenous agent for anti-angiogenic therapy. The sFlt-1 sequesters VEGF produced by tumor cells and forms a heterodimeric complex with a wild type VEGF receptor in a dominant negative fashion, inhibiting its signal transduction [8–11]. Recently, several reports have been published on gene therapy with the soluble VEGF receptors. These include ex vivo transfection of cancer cells with a plasmid encoding sFlt-1 receptor [12], regional administration of an adenovirus-mediated sFlt-1 cDNA [13], systemic administration of an adenovirus-mediated sFlt-1 cDNA [14] and intraperitoneal transduction of a sFlt-1 cDNA using HVJ-cationic liposomes [15]. However, the efficient delivery of anti-angiogenic genes to tumors remain a major obstacle. Especially in the case of the

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systemic administration, where targeted delivery of sFlt-1 cDNA may increase the efficiency and specificity without systemic side-effects.

We previously reported an angiogenic endothelial cell-targeted polymeric gene carrier, PEI-*g*-PEG-RGD. This targeted carrier was developed by the conjugation of the $\alpha\nu\beta3/\alpha\nu\beta5$ integrin-binding RGD peptide (ACDCRGDCFC) to the cationic polymer, branched polyethylenimine (bPEI) with a hydrophilic polyethylene glycol (PEG) spacer [16,17]. In vitro transfection showed that PEI-*g*-PEG-RGD efficiently transferred therapeutic pCMV-sFlt-1 gene to angiogenic endothelial cells, but not to the non-angiogenic cells. Moreover, PEI-*g*-PEG-RGD/pCMV-sFlt-1 complexes inhibited proliferation of endothelial cells in vitro by blocking the binding of VEGF to the membrane bound Flt-1 receptor [17]. In this report, we injected PEI-*g*-PEG-RGD/pCMV-sFlt-1 complexes systemically in tumor bearing mice. The expression of sFlt-1 and its therapeutic effects were evaluated. The results showed that the delivery of the therapeutic plasmid using PEI-*g*-PEG-RGD was effective in the suppression of tumor growth.

2. Materials and methods

2.1. Materials

Branched PEI (bPEI, average molecular weight 25 kDa, average degree of polymerization 580), terrific broth, ampicillin and Rosewell Park Memorial Institute (RPMI 1640) medium were purchased from Sigma-Aldrich (Milwaukee, WI). *N*-Hydroxy-succinimide-vinyl sulfone polyethylene glycol (NHS-PEG-VS; molecular weight 3400) was purchased from NEKTAR (Huntsville, AL). RGD peptide, ACDCRGDCFC, was purchased from the Genemed Synthesis, Inc. (San Francisco, CA). After synthesis, peptides were purified via reverse phase high performance liquid chromatography (HPLC) and then analyzed by mass spectrometry performed using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer. Plasmid DNA (pDNA) was propagated in chemically competent DH5α strain (GibcoBRL) and prepared from overnight bacterial cultures by alkaline lysis and column purification with a Qiagen plasmid Maxi kit (Qiagen, Valencia, CA).

2.2. Polymer synthesis and construction of pCMV-sFlt-1 gene

The PEI-*g*-PEG-RGD conjugate (Fig. 1A) was synthesized and purified as reported earlier [16,17]. Briefly, in the first reaction step, RGD peptide was conjugated to NHS-PEG-VS in anhydrous DMF containing 4 molar excess of TEA. Then in the second reaction step, two molar excess of RGD-PEG-VS conjugates were mixed with bPEI solution in pH 9.0 sodium carbonate buffer and incubated at room temperature overnight. The final product, PEI-*g*-PEG-RGD, was purified by dialysis and lyophilized. The composition of PEI-*g*-PEG-RGD conjugates was analyzed by ^{1}H-NMR, both at the reaction step of peptide with NHS-PEG-VS and at the conjugation step of PEI with RGD-PEG-VS. ^{1}H-NMR spectra were obtained on a Varian Inova 400 MHz NMR spectrometer (Varian, Palo Alto, CA) using standard proton parameters. Chemical shifts were referenced to the residual HDO resonance at approximately 4.7 ppm.

pDNA encoding sFlt-1 (Fig. 1B) was constructed and confirmed as previously reported [17]. Human soluble Flt-1 was amplified from human placenta cDNA (Spring Bioscience) using primers modified to allow ligation into the pCI plasmid (Promega Corp, Carlsbad, CA) at the *Xho*I and *Sal*I restriction sites (in bold): forward 5′-CGT **GAA TTC** GCT CAC CAT GGT CTA-3′ and reverse 5′-GCT **GTC GAC** CTG CTA TCA TCT CCG AAC TC-3′. The PCR reaction was ran at 94 °C for 5 min, 10 cycles at 94 °C for 15 s, 55 °C for 60 s and 72 °C for 180 s followed by 25 cycles at 94 °C for 15 s, 60 °C for 60 s, 72 °C for 180 s and an extension for 10 min at 72 °C. The length of the expected product was 2214 bp and was confirmed by running on a 1% agarose gel. Following digestion, ligation and transformation of DH5α cells, positive colonies were selected by endonuclease digestion. Positive colonies were then sequenced for final confirmation.

2.3. Preparation of polymer/pDNA complexes

The polymer/pDNA complexes were prepared by mixing equal volumes of aqueous solution of cationic polymer and pDNA dissolved in 5% glucose solution to give a net charge ratio (N/P ratio) of 10. The electrostatic interactions between cationic polymers and pDNA resulted in the formation of polyelectrolyte complexes with an average particle size distribution of ~150 nm, as reported previously [17].

2.4. Tumor cell line

CT-26 colon adenocarcinoma cell line was grown and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin (Gibco-BRL, Gaithersburg, MD), and maintained at 37 °C under humidified atmosphere.

2.5. Mice

Five-week-old female BALB/c mice were purchased from Simonsen Laboratories (Gilroy, CA) and housed in the Animal Care Facility, Biomedical Polymers Research Building, University of Utah. Mice were maintained on ad libitum rodent feed and water at room temperature, 40% humidity. All mice were acclimated for at least 1 week before tumor implantation. All studies were performed in accordance with the approved animal protocol.

2.6. Tumor implantation and treatment

To generate tumors, 4–5-week-old female BALB/c mice were injected subcutaneously in the middle of the right flank with 100 μl of a single cell suspension containing 1×10^6 CT-26 cells. Tumor size was measured using a digital vernier caliper across its longest (a) and shortest diameters (b) and its volume was calculated using the formula $V=0.5ab^2$. Treatment of the tumors was