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Hepatoma targeting peptide conjugated bio-reducible polymer complexed with oncolytic adenovirus for cancer gene therapy

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ABSTRACT

Despite adenovirus (Ad) vector's numerous advantages for cancer gene therapy, such as high ability of endosomal escape, efficient nuclear entry mechanism, and high transduction, and therapeutic efficacy, tumor specific targeting and antiviral immune response still remain as a critical challenge in clinical setting. To overcome these obstacles and achieve cancer-specific targeting, we constructed tumor targeting bioreducible polymer, an arginine grafted bio-reducible polymer (ABP)-PEG-HCBP1, by conjugating PEGylated ABP with HCBP1 peptides which has high affinity and selectivity towards hepatoma. The ABP-PEG-HCBP1-conjugated replication incompetent GFP-expressing ad, (Ad/GFP)-ABP-PEG-HCBP1, showed a hepatoma cancer specific uptake and transduction compared to either naked Ad/GFP or Ad/GFP-ABP. Competition assays demonstrated that Ad/GFP-ABP-PEG-HCBP1-mediated transduction was specifically inhibited by HCBP1 peptide rather than coxsackie and adenovirus receptor specific antibody. In addition, ABP-PEG-HCBP1 can protect biological activity of Ad against serum, and considerably reduced both innate and adaptive immune response against Ad. shMet-expressing oncolytic Ad (oAd; RdB/shMet) complexed with ABP-PEG-HCBP1 delivered oAd efficiently into hepatoma cancer cells. The oAd/ABP-PEG-HCBP1 demonstrated enhanced cancer cell killing efficacy in comparison to oAd/ABP complex. Furthermore, Huh7 and HT1080 cancer cells treated with oAd/shMet-ABP-PEG-HCBP1 complex had significantly decreased Met and VEGF expression in hepatoma cancer, but not in non-hepatoma cancer. In sum, these results suggest that HCBP1-conjugated bioreducible polymer could be used to deliver oncolytic Ad safely and efficiently to treat hepatoma.

1. Introduction

The primary goal of gene therapy is development of highly efficient vector or delivery system that can express therapeutic gene at proper target site [1]. To this end, adenovirus (Ad)-based gene delivery system has been extensively evaluated for its therapeutic efficacy and safety in animal models and clinical trials [2,3]. Oncolytic Ad has numerous advantages for cancer gene therapy such as self-propagation, cancer-specific infection, lysis of infected cancer cells, and secondary infection of neighboring cancer cells within the tumor [4]. Oncolytic Ad as a cancer therapeutic agent has been approved for clinical phase III trials [5,6]. However, virotherapy using oncolytic Ad has been strictly limited to local administration [7,8] as systemic injection can induce acute inflammatory and immune response against Ad, resulting in poor therapeutic efficacy and adverse side effects [9].

Generation of hybrid vector system combining viral and non-viral carriers has been extensively studied as a potential solution to immunogenic nature and nonspecific sequestration of systemically administered naked Ad. Non-viral vector has low immunogenicity and therapeutic genes of large size can be delivered efficiently. Various polymeric carriers have been complexed with plasmid DNA to shield and deliver plasmid DNA to targeted cells [10]. However, these plasmid DNA-based hybrid vectors often exhibit polymer-induced cytotoxicity and low transfection efficiency due to inefficient endosomal escape mechanism and non-biodegradable attributes of the polymer [11]. Thus, further modification of polymeric carriers is required for clinically efficacious gene delivery.

Previously, our group developed a bio-reducible poly(cystamine bisacrylamide-diaminohexane, CBA-DAH), which showed significantly higher transfection efficiency and lower cytotoxicity than 25 kDa polyethylenimine (PEI) [12]. Additionally, we synthesized arginine-grafted bio-reducible poly(CBA-DAH) polymer (ABP) using backbone of poly (CBA-DAH) [13]. Arginine has known cell penetrating functions and thus arginine-grafted non-viral carriers should elicit high gene delivery efficiency. In this regard, many studies reported that surface modification of Ad with varying nanomaterials, such as ABP [14], methoxy poly(ethyleneglycol)-b-poly{N-[N-(2-aminoethyl)-2-aminoethyl]-L-glutamate [15,16], mPEG-PEI-g-Arg-S-S-Arg-g-PEI-mPEG [17], bile acid-conjugated PEI [18], and multi-degradable bioreducible PEI [19], through electrostatic interaction resulted in enhanced transfection and

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lowered immunogenicity of Ad. Cationic polymer coated Ad can easily dissociate in the endosomes, effectively releasing the Ads through the proton-sponge effect [20] which enhances transduction efficacy of Ad. However, excess cationic surface charge of Ad and cationic polymer complex shows poor tumor selectivity due to their nonspecific uptake into the normal cells, restricting its clinical application. Moreover, cationic polymer-based delivery vector easily aggregate and nonspecifically interact with serum proteins ultimately reducing delivery efficacy. Thus, development of polymeric vector with cancer-selective targeting function is required. To overcome nonspecific uptake of polymer-coated Ad, cancer-targeting moieties, such as RGD peptide and folate, have been investigated and demonstrated efficient cancer-specific delivery of Ad [21–23]. Furthermore, several biomarkers with high affinity and cancer-selectivity have demonstrated target specific delivery of Ad [9,24,25]. Recently, highly cancer-selective hepatoma targeting peptide (HCBP1), which could discriminate between normal and hepatoma cells both in vitro and in vivo, has been identified by phage display method [26]. Moreover, HCBP1 conjugated chitosan-linked PEI/DNA complex showed high gene transfer efficiency and antitumor efficacy in hepatoma [27].

In this study, we report the development of a novel hepatoma cancer-targeting hybrid delivery system using HCBP1-conjugated polymer and oncolytic Ad. A bioreducible polymer, HCBP1 conjugated PEGylated ABP, was electrostatically complexed with oncolytic Ad generating Ad–ABP-PEG-HCBP1 nanocomplex. The hepatoma-specific cell killing effect and suppression of both c-Met and VEGF expression was investigated using Met-inhibiting oncolytic Ad (RdB/shMet) coated with the ABP-PEG-HCBP1. Furthermore, we demonstrated that the Ad–ABP-PEG-HCBP1 nanocomplex protected Ad against the serum and reduced immunogenicity. In sum, our results show for the first time that the Ad–ABP-PEG-HCBP1 hybrid system can efficiently target hepatoma with higher safety profile than naked Ad.

2. Materials and methods

2.1. Cell lines and preparation of Ad vectors

The human hepato carcinoma (Huh7, and HepG2), human fibrosarcoma (HT1080), human embryonic kidney cell line expressing the Ad E1 region (HEK 293) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GIBCO-BRL) and penicillin/streptomycin (GIBCO-BRL) at 37 °C in a humidified atmosphere containing 5% CO_2. The adenoviral transduction efficiency was examined with a green fluorescence protein (GFP)-expressing replication-incompetent Ad (Ad/GFP). The Ad/GFP was constructed and characterized in our previous study [9,28]. The construction and generation of oncolytic Ad backbone was previously described (RdB) [19,29]. The oncolytic Ad (RdB/shMet, or oAd) was generated and inserted expressing short hairpin RNA (shRNA) against c-Met RNA into the RdB by homologous recombination. The Ad/GFP and RdB/shMet were propagated in HEK293 cells and purified by the CsCl gradient method. Purified viruses were stored at −80 °C until use.

2.2. Synthesis of hepatoma targeting peptide conjugated ABP (ABP-PEG-HCBP1)

The poly (cystaminebisacrylamide-diaminohexane) (CBA-DAH) and arginine-grafted poly (cystaminebisacrylamide-diaminohexane) (ABP) were synthesized as described previously [12,13]. Then, ABP was activated with NHS-PEG_{2K}-Mal (M.W. = 2 kDa, JenKem Technology, Plano, TX). Briefly, ABP was dissolved in 0.1 M phosphate buffered saline (PBS, pH 7.2, 0.15 M NaCl, 2.0 mM EDTA). 2.4 equivalents of NHS-PEG-Mal were added to the ABP solution and stirred for 1 h at room temperature and then the mixture was dialyzed (MWCO = 3500, Spectrum Laboratories, Inc., Rancho Dominguez, CA) and lyophilized. The molecular weight of ABP-PEG was estimated to be 8.85×10^3 Da/mol by gel-permeation chromatography (GPC). For the thiolation of HCBP1 (FQHPSFI, University of Utah HSC Cores Research Facility, Salt Lake City, UT), Peptide was dissolved in 0.1 M PBS (pH 8.0, 0.15 M NaCl, 2.0 mM EDTA). The 2 equivalents of Traut's reagent (Thermo scientific Inc., Rockford, IL) were added to the peptide solution, and the mixture was further reacted for 2 h at room temperature. Then, the mixture was dialyzed and lyophilized. For the conjugation of target peptides with polymer, PEGylated ABP (ABP-PEG2kDa-Mal) was dissolved in 50 mM PBS (pH 7.2 0.15 M NaCl, 10 mM EDTA). The 1.7 equivalents of thiolated peptide per maleimide groups of ABP-PEG 2 kDa-Mal were added to the solution. The mixture was further reacted for 4 h and then the mixture was dialyzed and lyophilized. The molecular weight of ABP-PEG-HCBP1 as determined by GPC was 10.6×10^3 Da/mol its PDI value was 1.41. All reaction was monitored by Thin-Layer Chromatography with ninhydrin staining, UV spectroscopy and ^{1}H NMR (400 MHz, D_2O).

2.3. Cytotoxicity of ABP, ABP-PEG-HCBP1

The ABP or ABP-PEG-HCBP1 polymers were analyzed for cytotoxicity, including 25 kDa branched PEI (Mw 25,000 Da, Sigma-Aldrich, St. Louis, MO). The cell viability determination was performed by measuring conversion of MTT to formazan as a function of time. Huh7, HepG2, and HT1080 cells were grown to 50% confluence in 96 well plates and were then treated with varying polymer concentrations, up to 20 μg/mL. 48 h following polymer treatment, 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 2 mg/mL in PBS; Sigma) in PBS was added to each well and incubated for 4 h at 37 °C. The supernatant was discarded, and the precipitate was dissolved in 200 μL dimethyl sulfoxide (DMSO, Sigma). Plates were read on a microplate reader (Tecan Infinite M200; TecanDeutschland GmbH, Crailsheim, Germany) at 540 nm. The number of living cells in a PBS-treated cell group was analyzed similarly as a negative control.

2.4. Complexation and physical characterization of polymer coated ad

Complexes between cationic polymers and Ad were formed by ABP or ABP-PEG-HCBP1 cationic components and the Ad particles (1×10^{10} VP) in an E-tube using PBS (pH 7.4). The molar ratios of cationic molecules to Ad particle (1×10^5–1×10^6). The diluted cationic polymers were added drop-wise to the solution of diluted Ad particles, mixed by inversion or tapping in a tube diluted to total volume of 100 μl with PBS solution. The hydrodynamic diameters of naked Ad, ABP or ABP-PEG-HCBP1 coated Ad nanocomplex were measured with a dynamic light scattering (DLS). DLS was measured with an argon ion laser set at 488 nm and a fixed 90° scattering angle (Malvern Instruments, Inc., Worcestershire, UK). Surface charge was measured using a zeta potentiometer (Zetasizer 3000HS, 10 mW HeNe laser, 633 nm; Malvern Instruments) at 25 °C. Ad particles (1×10^{10}) were gently added to each polymer (1×10^5–1×10^6 polymer molecules/Ad particle) diluted in PBS for 30 min. The naked Ad, or after formation of Ad–ABP, Ad–ABP-PEG-HCBP1 complexes, HEPES (H0887; Sigma, St. Louis, MO) was added to a final volume at 1 mL before analysis. The sizes and potential values were presented as the average values from five measurements.

2.5. Cellular uptake of Ad/polymers

The synthesis of fluorescein isothiocyanate (FITC, Sigma)-labeled Ad was based on the reaction between the isothiocyanate group of FITC and the primary amine groups of Ad. The Ad (2×10^{11} VPs) was conjugated with FITC (FITC/Ad molar ratio = 1×10^5 in 1 mL PBS in the dark at room temperature for 4 h then Ad–FITC was dialyzed at 4 °C (10 K cut off, Slide-A-Lyzer™ Dialysis Cassettes, Life Technologies, Grand Island, NY) to remove unconjugated FITC and Ad until no fluorescence was detected in the dialysis buffer (PBS). Ad–FITC was sequentially complexed with either ABP or ABP-PEG-HCBP1 for subsequent cellular uptake