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Antitumor effect and safety profile of systemically delivered oncolytic adenovirus complexed with EGFR-targeted PAMAM-based dendrimer in orthotopic lung tumor model

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ABSTRACT

Adenovirus (Ad)-mediated cancer gene therapy has been proposed as a promising alternative to conventional therapy for cancer. However, success of systemically administered naked Ad has been limited due to the immunogenicity of Ad and the induction of hepatotoxicity caused by Ad's native tropism. In this study, we synthesized an epidermal growth factor receptor (EGFR)-specific therapeutic antibody (ErbB)-conjugated and PEGylated poly(amidoamine) (PAMAM) dendrimer (PPE) for complexation with Ad. Transduction of Ad was inhibited by complexation with PEGylated PAMAM (PP) dendrimer due to steric hindrance. However, PPE-complexed Ad selectively internalized into EGFR-positive cells with greater efficacy than either naked Ad or Ad complexed with PP. Systemically administered PPE-complexed oncolytic Ad elicited significantly reduced immunogenicity, nonspecific liver sequestration, and hepatotoxicity than naked Ad. Furthermore, PPE-complexed oncolytic Ad demonstrated prolonged blood retention time, enhanced intratumoral accumulation of Ad, and potent therapeutic efficacy in EGFR-positive orthotopic lung tumors in comparison with naked Ad. We conclude that ErbB-conjugated and PEGylated PAMAM dendrimer can efficiently mask Ad's capsid and retarget oncolytic Ad to be efficiently internalized into EGFR-positive tumor while attenuating toxicity induced by systemic administration of naked oncolytic Ad.

1. Introduction

Lung cancer is one of the most commonly diagnosed and malignant types of cancer. It was estimated that 408,808 people in the United States were living with lung and bronchus cancer in 2014 [1]. According to cancer statistics 2014 from the NIH, the prognosis of lung cancer with conventional therapies, such as chemical and radiological treatments, is ineffective in eradication of lung cancers and five-year survival rates are low at 15% with an 8–9 month predicted median survival for front-line stage IIIB/IV patient [2]. Therefore, there is a substantial need for further research into development of novel and targeted therapeutics.

In order to overcome non-specificity of conventional chemotherapeutics, molecular mechanism behind lung carcinogenesis was extensively studied and subsequently lung cancer-targeted therapeutics were developed [3–5]. Activation of receptor tyrosine kinase (RTK), such as epidermal growth factor receptor (EGFR) and tyrosine kinase receptor for hepatocyte growth factor (c-Met), induces cellular proliferation, differentiation, migration, and angiogenesis, ultimately contributing to lung cancer oncogenesis [6–9]. Due to high prevalence of aberrant EGFR expression in lung cancer, EGFR inhibitors have been extensively developed [10,11]. The EGFR inhibitor, Erbitux (ErbB) is a well-documented and efficacious anti-EGFR monoclonal antibody (Ab) that has a high specificity toward EGFR [12–14]. ErbB elicits potent growth inhibition of EGFR-expressing lung cancer cells and antitumor effect in preclinical lung cancer models [15,16], and now it is widely used to treat lung cancer patients [17,18].

Oncolytic adenovirus (Ad) is widely regarded as a novel and promising alternative to traditional cancer therapy as it exhibits tumor-selective replication, high rate of viral production, and potent cytopathic effect [19,20]. Further, "armed" oncolytic Ad expressing a therapeutic transgene have been extensively investigated to maximize the potency of oncolytic Ad. Advantages of this approach are subsequent infection to neighboring cancer cells following lysis of cells and cancer-selective amplification of therapeutic gene by conditionally replicative Ad [21]. Among many candidates of therapeutic transgenes for oncolytic Ad, genes targeting RTK could be a promising candidate for targeted lung cancer therapy. To this end, we have previously reported oncolytic Ad

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expressing c-Met-specific short-hairpin RNA (shMet) to induce cancer-specific downregulation of c-Met signaling, resulting in induction of autophagy and tumor growth inhibition [22].

Despite the many advantages of armed oncolytic Ad, as a monotherapeutic agent, its efficacy when administered intratumorally is inadequate against disseminated tumors, resulting in poor clinical outcomes. Systemic administration of naked Ad can induce severe hepatotoxicity due to its native tropism which contributes to nonspecific liver sequestration [23–25]. Furthermore, highly immunogenic viral capsid of naked Ad triggers rapid induction of innate and adaptive immune responses against Ad by the host [26,27], resulting in rapid blood clearance and potentially fatal inflammatory response [28,29]. These drawbacks of systemically administered Ad can be overcome by chemical modification of Ad surface with non-immunogenic nanomaterials such as polymers, liposomes, and peptides [30–32]. Synthetic polycationic dendritic poly(aminoamine) (PAMAM) has been reported to efficiently bind with anionic viral capsid to enhance cellular uptake of Ad as well as reducing Ad's immunogenicity [33,34]. However, highly cationic PAMAM dendrimer has narrow clinical applications due to PAMAM's poor biocompatibility and biodegradability which causes nonspecific internalization into non-targeted cells and severe toxicity [35,36].

In the present study, we have generated oncolytic Ad complexed with ErbB-conjugated and PEGylated PAMAM (PPE) to reduce the toxicity of the PAMAM dendrimer while improving specificity and therapeutic efficacy to EGFR-expressing lung cancer. Oncolytic Ad was constructed to co-express decorin (DCN) and shMet to maximize the therapeutic efficacy against lung cancer by enhancing viral distribution through the tumor tissues and inhibiting RTK signaling pathway, respectively [37–39]. We specifically aimed to demonstrate that systemically administered PPE-complexed oncolytic Ad (oAd/DCN-shMet/PPE) can efficiently target EGFR-overexpressing orthotopic lung tumor, which enhances tumor accumulation and antitumor efficacy of oAd/DCN-shMet/PPE. Further, PP- or PPE-coating's effect on immunogenicity, blood retention time, and systemic toxicity of oAd/DCN-shMet was extensively analyzed in vivo.

2. Materials and method

2.1. Cells and chemical materials

The human embryonic kidney cells (HEK293), EGFR-positive human lung cancer cells (A549, H322, H358, H460, H1299, and H1975), EGFR-negative breast cancer cells (MCF7), and EGFR-negative normal cells (HDF) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in high glucose Dulbecco's Modified Eagle's Media (DMEM; Gibco BRL, Waltham, MA) containing 10% fetal bovine serum (FBS; Gibco BRL) in an incubator at 37 °C with 5% CO_2. Methoxyl PEG succinimidyl carbonate NHS was purchased from Nanocs (New York, NY). G4 PAMAM cystamine core dendrimer (10 wt.% in MeOH), N-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma chemical Co. (St Louis, MO). Erbitux® (anti-EGFR antibody) was purchased from Merck (Kenilworth, NJ).

2.2. Construction and preparation of Ads

The EGFR-dependent transduction efficiency was determined using green fluorescent protein (GFP)-expressing replication-incompetent Ad (dAd-GFP) [29]. For the generation of oncolytic Ad co-expressing DCN and shMet, the HRE enhancer was first inserted into pDE1sp1B/Rd19 Ad E1 shuttle vector [40] to increase viral replication in hypoxic condition, resulting in pDE1sp1B/HRE-Rd19 Ad E1 shuttle vector. To insert the DCN expression cassette, the DCN gene was isolated from pCA14/DCN [41] using *Bgl*II, and then ligated into pDE1sp1B/HRE-Rd19 E1 shuttle vector, thus generating a pDE1sp1B/HRE-Rd19/DCN. For homologous recombination, *Xmn*I-treated pDE1sp1B/HRE-Rd19/DCN Ad E1 shuttle vector was co-transformed into *Escherichia coli* BJ5183 with the linearized Ad dE1-k35 [42], generating a HRE-Rd19-k35/DCN oncolytic Ad plasmid. To express shMet in the E3 region of Ad, shMet-expressing E3 shuttle vector (pSP72dE3-U6-shMet4; [22]) was linearized and co-transformed with a DCN-expressing oncolytic Ad vector (HRE-Rd19-k35/DCN) in *E. coli* BJ5183, generating an HRE-Rd19-k35/DCN/shMet oncolytic Ad plasmid. The proper homologous recombinant Ad plasmid DNA was digested with *Pac*I and transfected into 293 cells to generate an HRE-Rd19-k35/DCN/shMet oncolytic Ad (oAd/DCN-shMet). Replication-deficient dAd-GFP and replication-competent oAd/DCN-shMet were propagated in 293 and A549 cells, respectively, and purified by CsCl gradient centrifugation. The numbers of viral particles (VP) were calculated from optical density measurements at 260 nm (OD_{260}) where an absorbance of 1 ($OD_{260} = 1$) was equivalent to 1.1×10^{12} VP/mL [43]. Purified viruses were stored at −80 °C until use.

2.3. Synthesis of ErbB-conjugated and PEGylated PAMAM dendrimer

For the generation of PEGylated PAMAM (PP), conjugation of mPEG with G4 PAMAM was performed according to the previously described procedure [44]. Briefly, PAMAM dendrimer (0.56 μM) dissolved in PBS buffer was mixed with mPEG-NHS (18 μM), and resulting reaction mixture was stirred at room temperature for overnight. The dendrimer product was purified by dialysis (MWCO 3.5 kDa) with double distilled water for 1 day and lyophilized to yield PEGylated PAMAM. Chemical structure of mPEG-PAMAM was analyzed by ^{1}H NMR in D_2O (Varian 600 MHz spectrometer; Varian Inc., Palo Alto, CA; Fig. S1). Conjugation of PEG on PAMAM was confirmed by the appearance resonance peaks at 3.2 ppm and 3.4–3.6 ppm corresponding to the protons of terminal —OCH_3 group of PEG and —OCH_2–CH_2 repeating units of PEG, respectively. Further, the degree of PEGylation was estimated by the proton integration method as described in the previous report [44]. The feed ratio was 1:32 (PAMAM:mPEG). The observed ratio from ^{1}H NMR was 1:24 (PAMAM:PEG), and the molecular weight of PEGylated PAMAM was calculated as 63.3 kDa.

For the generation of ErbB-conjugated PP (PPE), EGFR-specific Ab (ErbB; 0.1 mg/mL in 250 μL) was mixed with EDC (5.0 mg/mL in 75 μL) and NHS (5.0 mg/mL in 50 μL) in PBS, and then resulting solution was incubated at room temperature for 30 min to activate the carboxylic acid groups of the Ab. Then, the PP solution (6 mg/mL in 250 μL) was added to above solution and incubated at room temperature for 2 h. After the initial incubation period, the reaction mixture was further incubated at 4 °C overnight. Finally, unreacted reagents were removed by dialysis [45,46].

2.4. Preparation and characterization of dendrimer-coated Ad

For complexation of Ad with either PP or PPE, both polymers were prepared in 1× PBS at 1.7×10^{-1} pM concentration. Ad:polymer (PP or PPE) molar ratio of 1×10^6 was prepared by gently mixing 10^8 VP of Ad (100 μL) and polymers (100 μL). For lower molar ratio samples, 1.7×10^{-1} pM polymer solutions were diluted by factor of 5, 10, 100, or 1000 in 1× PBS, and 100 μL of diluted polymers were reacted with 10^8 VP of Ad (100 μL), generating polymer-coated Ad at 5×10^5, 1×10^5, 1×10^4, or 1×10^3 molar ratio, respectively. The mixtures were allowed to electrostatically interact to form Ad/dendrimer polyplex at room temperature for 30 min. For physiochemical characterization of each Ad formulation, the average particle size and surface charge of naked dAd-GFP, PP-complexed dAd-GFP (dAd-GFP/PP), or PPE-complexed dAd-GFP (dAd-GFP/PPE) were determined using the Zetasizer 3000HS (Malvern Instrument Inc., Worcestershire, UK) with a He–Ne laser beam (633 nm, fixed scattering angle of 90°) at room temperature [25,29]. The average particle size and surface charge were computed as the average value of three independent