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Bioreducible polymer-conjugated oncolytic adenovirus for hepatoma-specific therapy via systemic administration

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ABSTRACT

Systemic administration of adenovirus (Ad) vectors is complicated by host immune responses and viral accumulation in the liver, resulting in a short circulatory virus half-life, low efficacy, and host side effects. Ad surface modification is thus required to enhance safety and therapeutic efficacy. An arginine-grafted bioreducible polymer (ABP) was chemically conjugated to the Ad surface, generating Ad-ΔE1/GFP-ABP. A hepatocellular carcinoma [HCC]-selective oncolytic Ad complex, YKL-1001-ABP, was also generated. Transduction efficiency of Ad-ΔE1/GFP-ABP was enhanced compared to naked Ad-ΔE1/GFP. YKL-1001-ABP elicited an enhanced and specific killing effect in liver cancer cells (Huh7 and HepG2) expressing α-fetoprotein (AFP). Compared with naked Ad, systemic administration of ABP-conjugated Ad resulted in reduced liver toxicity and interleukin (IL)-6 production *in vitro* and *in vivo*. Ad-ΔE1/GFP-ABP was more resistant to the neutralizing effects of human serum compared to naked Ad-ΔE1/GFP. ABP conjugation extended blood circulation time 45-fold and reduced anti-Ad Ab neutralization. Moreover, systemic administration of YKL-1001-ABP markedly suppressed growth of Huh7 hepatocellular carcinoma. These results demonstrate that chemical conjugation of ABP to the Ad surface improves safety and efficacy, indicating that ABP-conjugated Ad is a potentially useful cancer therapeutic agent to target cancer via systemic administration.

1. Introduction

In the past few years, cancer gene therapy has been evaluated with the aim of developing a targeted therapeutic delivery system. Delivery vehicles are categorized into two systems: viral and non-viral. Both DNA and RNA viruses have been evaluated as possible gene carriers [1]. Among the viral vectors that have been investigated, adenoviruses (Ads) are the most promising for the dissemination of therapeutic agents into tumors. Although the benefits of Ads for cancer treatment have been reported, some limitations exist that complicate the use of these viruses as therapeutic vehicles. Following intravenous delivery, Ads are inactivated and cleared by immune cells. Additionally, they interact with platelets and erythrocytes leading to toxicity and possibly severe side effects [2,3]. Systemic delivery of Ads is also hampered by accumulation in the liver, which can cause liver toxicity, and very short half-life in circulation. Furthermore, tumor cells have low coxsackie and Ad receptor (CAR) expression, limiting the amount of CAR-mediated tumor cell transduction [4–6]. Therefore, current clinical treatment using Ad is limited to localized delivery into solid tumors by intratumoral administration. The development of a successful gene therapy targeting both primary and metastatic cancers requires systemic administration of Ads and surmounting low tumor cell transduction efficiency. Thus, strategies need to be devised to efficiently deliver Ads systemically to avoid immune clearance, non-specific liver uptake, and poor CAR-mediated viral transduction in a various cancer cell types.

One strategy to overcome Ad limitations is modification of the Ad surface using a non-viral system in order to generate hybrid vectors that combine the advantages of viral and non-viral elements. Non-viral systems are beneficial because they are minimally immunogenic, highly reproducible, and involve a simple

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quality control process. Lipid and polymer components of non-viral systems have been employed as an alternate approach to generate improved and more effective Ad vectors. For example, polyethylene glycol (PEG) covalently conjugated to the viral capsid was used to reduce the immunogenicity of Ad and protect the virus from immune clearance [7–9]. Cationic lipids and polymers, including poly-L-lysine (PLL) and polyethylenimine (PEI), have been tested in Ad-mediated gene delivery systems to enhance transduction efficiency and transgene expression in various cancer cells [10,11]. However, PLL and PEI induced severe toxicity, significantly limiting their usefulness [12].

Both viral and non-viral vectors have certain useful and complementary characteristics. Viral vectors efficiently deliver therapeutic genes intracellularly, while non-viral vectors are less immunogenic. Enhancement of Ad infectivity with cationic polymers remains a promising approach, but polymer-modified Ad demonstrating high infectivity and low toxicity are needed for optimal results. We have recently reported the effects of modifying the Ad surface with an arginine-grafted bioreducible polymer (ABP) as a model of viral/non-viral combination system [13]. In the previous report, ABP-coated Ad complexes were formed using physical charge interactions, and enhanced transduction efficiency, minimal cytotoxicity, and lower immunogenicity were observed. Unfortunately, this complex cannot be injected systemically because the size of complex is far larger (around 500 nm) than the maximum dimensions (<200 nm) required for accumulation in target tumor cells through enhanced permeability and retention (EPR) effects following systemic administration. Furthermore, vectors generated through charge interactions are prone to aggregation, leading to widely variable and inherently unstable complexes that are difficult to translate to a clinical setting. Therefore, a method to generate more stable complexes *in vivo* is required.

In this study, we developed an Ad complex chemically conjugated with ABP that may be used for systemic delivery.

2. Materials and methods

2.1. Cell lines and adenoviruses

The following cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA): HEK293, a human embryonic kidney cell line, transformed with Ad5 E1; A549, a non-small cell lung carcinoma cell line; Huh7 and HepG2, liver cancer cell lines; MCF7, a breast carcinoma cell line; PC-3, a prostate cancer cell line; HT1080, a fibrosarcoma cell line; and C2C12, a mouse myoblast cell line. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (FBS; Gibco-BRL) and penicillin/streptomycin (Gibco-BRL) at 37 °C and 5% CO_2.

Three Ad types were used: Ad-ΔE1/GFP, YKL-1001, and Ad-ΔE1/IX-GFP. Ad-ΔE1/GFP virus is an E1-deleted Ad expressing green fluorescent protein (GFP) under the control of the cytomegalovirus promoter in the E1 region. YKL-1001 is a hepatoma-targeted oncolytic Ad, which encodes the E1A gene driven by the α-fetoprotein (AFP) promoter [14]. Ad-ΔE1/IX-GFP is an E1-deleted Ad expressing GFP on pIX of the viral capsid.

Ads were propagated in 293 cells and purified by CsCl equilibrium centrifugation. The numbers of viral particles (VP) were calculated from optical density measurements at 260 nm (OD_{260}), and one absorbency unit was considered equivalent to 10^{12} VP/mL.

2.2. Cationic reagents and materials

Hyperbranched polyethylenimine (bPEI, 25 kDa), dithiothreitol (DTT), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO) and 3,3′-dithiobis(sulfosuccinimidyl-propionate) (DTSSP) was purchased from Pierce (Rockford, IL). LysoTracker® Red DND-99 was purchased from Invitrogen (Carlsbad, CA). The characterization and synthesis of ABP cationic polymers were performed and validated as previously described [15].

2.3. Preparation of Ad complexes chemically conjugated with a cationic polymer

Ad-ΔE1/GFP and YKL-1001 oncolytic Ad (1×10^{10} VP) were chemically conjugated with a cationic polymer, ABP. Ads were pre-activated with the DTSSP cross-linker at a 1:5 $\times$ 10^5 molar ratio (Ad:DTSSP) in phosphate buffered saline (pH 7.4) (PBS; Gibco-BRL). After 30 min at room temperature, ABP polymers were added to the solution at molar ratios from 1:1 to 1:10 (DTSSP:ABP polymer). After the reaction was incubated for 1 h at room temperature, the product was purified by ultrafiltration with centricon Ultracel YM-10 columns (Millipore, M_W cutoff: 10,000, Billerica, MA) to remove a free linker and ABP polymer residues. The purified ABP-conjugated Ad complex was stored at −20 °C until use. The scheme for synthesis of the chemically-conjugated complex is shown in supplementary S1.

2.4. Size and zeta potential measurements and characterization of naked Ad and chemically-conjugated Ad complexes

The average size and zeta potential of ABP-conjugated Ad complex were measured with a Zetasizer 3000HS (Malvern Instruments, Inc., Worcestershire, UK) with a He–Ne laser beam (633 nm, fixed scattering angle of 90°) at 25 °C. Ad complexes conjugated with ABP polymers were prepared as described above. After the formation of complexes at each ratio, PBS (pH 7.4) was added to final volume of 3 mL before analysis. The dissociation of ABP-conjugated Ad complex in the presence of DTT and its relation to particle sizes was assessed. Ad complexes at a 1:2 ratio were treated with 20 mM DTT, and particle size was measured after 24 h. The sizes and potential values were presented as the average values from three measurements.

2.5. Cell transduction measurements

Human cancer cells (A549, PC-3, MCF7, and HT1080) were maintained at 37 °C in a humidified incubator at 5% CO_2. Cells (4×10^4 cells/well) were first plated at approximately 60–70% confluence in a 24-well plate. At 24 h before transduction, the medium in each well was exchanged with fresh serum-free medium. Cells were transduced with naked Ad-ΔE1/GFP, Ad-ΔE1/GFP-DTSSP, or Ad-ΔE1/GFP-ABP at multiplicity of infections (MOIs) of 100 (A549), 150 (PC-3), 250 (MCF7), or 60 (HT1080). After incubation for 6 h, the virus solution was removed and fresh 10% FBS-containing medium was added. After 2 days, cells were observed by fluorescence microscopy (Olympus BX51; Olympus Optical, Tokyo, Japan) using the MetaMorph Imaging System (Molecular Devices, Inc., Sunnyvale, CA).

2.6. Flow cytometry

GFP expression in Ad complex-transduced cells was quantitatively measured by flow cytometry. Cells were seeded in 24-well plates (4×10^4 cells/well) one day before transduction with naked Ad-ΔE1/GFP, Ad-ΔE1/GFP-DTSSP, or Ad-ΔE1/GFP-ABP. Cells were raised with cell dissociation solution (Sigma) at 48 h post-transduction and washed 3X with PBS. PBS (400 μl) was added to resuspend the cells before flow cytometric analysis. To assess the induction of apoptosis and nuclear damage in the cells treated with the YKL-1001-ABP complex, Huh7 hepatoma cells were plated in 12-well plates (2×10^5 cells/well) for 24 h, after which cells were treated with naked YKL-1001, YKL-1001-DTSSP, or YKL-1001-ABP. Cells treated with 1 mM cobalt ($CoCl_2$; Sigma) were used as a positive control. After 72 h post-treatment, both attached and floating cells were processed according to the manufacturer's instructions for the ApoAlert Annexin V-FITC apoptosis kit (BV-K101-3; Medical & Biological Laboratories co., LTD, Woburn, MA). Cells were analyzed on a FACScan flow cytometer (Beckton-Dickinson, Sunnyvale, CA). Data from 10,000 events were collected for further analysis using CellQuest software (Beckton-Dickinson).

2.7. MTT assay

The cytotoxicity of chemically-conjugated Ad complexes was determined by measuring the conversion of the tetrazolium salt MTT to formazan. After 48 h of treatment with naked Ad-ΔE1/GFP, Ad-ΔE1/GFP-DTSSP, Ad-ΔE1/GFP-ABP, or Ad-ΔE1/GFP-25KPEI, the medium was removed, and fresh medium containing 2 mg/mL MTT was added to each well. The cells were then incubated for 4 h at 37 °C in the dark. After removing the MTT solution, the remaining crystals were solubilized with 1 mL dimethyl sulfoxide for 20 min, after which the absorbency was measuring with a microplate reader at 540 nm. Cell viability is expressed as a percentage of control uninfected cells. Each experiment was repeated at least three times.

2.8. Viral production assay

Liver cancer Huh7 cells were seeded in a 12-well plate at approximately 60–70% confluence. Twenty-four hours before infection, the medium in each well was exchanged with fresh serum-free DMEM, and cells were then incubated with naked YKL-1001, YKL-1001-DTSSP, or YKL-1001-ABP at an MOI of 25. At 72 h post-infection, both supernatant and cells were collected for virus titration analysis. Cell lysates underwent three cycles of freezing and thawing, and serial dilutions of supernatant and lysates were administered to 293 cells. Infectious viral titers were determined by limiting dilution assay [16].