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Linearized oncolytic adenoviral plasmid DNA delivered by bioreducible polymers

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Abstract

As an effort to overcome limits of adenovirus (Ad) as a systemic delivery vector for cancer therapy, we developed a novel system using oncolytic Ad plasmid DNA with two bioreducible polymers: arginine-grafted bioreducible poly(disulfide amine)polymer (ABP) and PEG5k-conjugated ABP (ABP5k) in expectation of oncolytic effect caused by progeny viral production followed by replication. The linearized Ad DNAs for active viral replication polyplexed with each polymer were able to replicate only in humancancer cells and produce progeny viruses. The non-immunogenic polymers delivering the DNAs markedly elicited to evade the innate and adaptive immune response. The biodistribution ratio of the polyplexes administered systemically was approximately 99% decreased in liver when compared with naked Ad. Moreover, tumor-to-liver ratio of the Ad DNA delivered by ABP or ABP5k was significantly elevated at 229- or 419-fold greater than that of naked Ad, respectively. The ABP5k improved the chance of the DNA to localize within tumor versus liver with 1.8-fold increased ratio. In conclusion, the innovative and simple system for delivering oncolytic Ad plasmid DNA with the bioreducible polymers, skipping time-consuming steps such as generation and characterization of oncolytic Ad vectors, can be utilized as an alternative approach for cancer therapy.

Keywords

oncolytic adenovirus; adenoviral replication; bioreducible polymer; cancer gene therapy

1. Introduction

The potential use of adenovirus (Ad) vectors for gene therapy has been focused on the therapeutic applications for cancer treatment since the clinical trials using Ad vectors have been approved worldwide and has taken the first ranking (more than 24% (414 protocol numbers) of all cases including viral & non-viral vectors)

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(<http://www.wiley.co.uk/genmed/clinical>). Although Ad vectors have been utilized to transfer tumor suppressor genes, suicide genes, immune-stimulator genes or small interfering RNA (siRNA) to kill tumor cells [1–6], ultimately, conditionally cancer-fighting Ad (oncolytic Ad) vectors have been more highlighted in the field due to their enhanced transgene expression by multiplied viral genomes including transgene cassette and tumor-selective killing effect by themselves [7, 8]. However, *in vivo* applications of Ad vectors by systemic administration have been strictly limited because of induction of serious liver toxicity by inherent Ad tropism, fast serum clearance by innate & adaptive host immune systems, and side effects by localization of the Ad particles to unwanted organs [9, 10] and thus, has not been sufficient to eradicate fast-growing tumors [11].

A multitude of technologies for masking the tropism of Ad vectors with non-viral vector systems to reduce host immune responses have been introduced on account of their properties such as less-toxicity, non-immunogenic and biocompatibility [12–17]. Among these polymers, polyethylene glycol (PEG) and poly-N-(2-hydroxypropyl)methacrylamide (pHPMA), have been chiefly utilized to shield the surface of Ad vectors to evade the immune responses and reduce viral toxicities [12, 18, 19]. The promising outcomes of the polymers shielding the outer surface of therapeutic Ad vectors, such as extended blood circulation time and reduced liver toxicity, are still plagued by the significant cytotoxicity due to the poor biocompatibility and non-degradability of the polymers. In order to overcome these problems, we have introduced the bioreducible polymers poly(cystaminebisacrylamide-diaminohexane) [poly(CBA-DAH)] and arginine-grafted bioreducible poly(CBA-DAH) (ABP), that are more stable than ester bonds in the extracellular environment and rapidly degraded to small & non-toxic molecules by glutathione and thioredoxin reductase in cytoplasm after internalization [20–22]. Although these bioreducible polymers have been shown the promising outcomes with the enhanced transduction efficiency of Ad vectors, additional modification such as PEGylation on these cationic polymers is required for controlling *in vivo* pharmacokinetics to increase blood circulation time while maintaining the bioactivity of the complex.

To generate infectious recombinant Ad vectors for *in vitro* and *in vivo* assay as a high titer, many time-consuming steps are needed [23, 24]. The procedures can be briefly classified into the following sequential steps: (1) transfection of Ad plasmid DNA to 293 cells to produce initial viral stock; (2) amplification of the Ad stock as maxi-scale; (3) cesium chloride-gradient ultracentrifugation to get a pure and high-concentrated Ad vector; (4) desalting by dialysis and storage at deep freezer; (5) determination of viral titer depending on physical or biological particles. Contamination of wild type E1 region by recombination of 293 genomes should be considered for generating Ad that is limiting factor for producing Ad. Recently, we introduced the potential systemic delivery of the oncolytic Ad DNA complexed with the cationic liposomes (DOTAP/DOPE) [25]. The results demonstrated that systemic delivery of therapeutic Ad DNA polyplexed with liposomes can elicit potent antitumor efficacy in orthotopic lung tumor xenograft models.

It has been known that the replication of Ad DNA occurs by a strand displacement mechanism initiated at both ends of the virus genome [26]. The presence of inverted terminal repeated (ITR) sequences on the single-stranded DNA molecules can permit formation of a double-stranded region (called as panhandle) that is utilized as the template for the initiation of DNA replication [27, 28]. Thus, the initiation of Ad replication takes place at either end of the Ad genome, indicating that circular Ad plasmid DNA is inactive to produce infectious Ad particle [29]. With reference to these strategies, we demonstrated the viral replication and oncolytic ability of the Ad DNA polyplexed with the bioreducible polymers (ABP or PEG5k-conjugated ABP) in human cancer or normal cells whether the plasmid DNA was intact or linearized. ABP polymer is reducible in cytoplasm by breaking

disulfide bonds in polymers. Innate and adaptive immune response caused by foreign pathogens was determined and *in vivo* biodistribution of Ad DNA/polymers delivered via systemic administration was examined. In summary, this simple approach for delivering oncolytic Ad DNA can be utilized as an alternative way for treatment of metastatic and disseminated cancers with improved safety profiles by potential bioreducible polymers and more effective cancer-eradicating ability by linearization of Ad plasmid DNA for active replication of the Ad genome.

2. Materials and methods

2.1. Cell lines, adenoviral plasmid DNAs and adenoviruses

Human embryonic kidney cell line expressing Ad E1 proteins (HEK293) and liver cancer cell lines (Hep3B and Huh7) were purchased from American Type Culture Collection (Mannas, VA). Human normal fibroblast cell line (HDF) was purchased from the Gelatinis (Telesis Court, San Diego, CA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and maintained in 37°C incubator with 5% CO₂. Replication-incompetent Ad (pAd-ΔE1/GFP) or oncolytic Ad (pAd-ΔB7) plasmid were constructed as described previously [8]. The production, amplification and titration methods of replication defective Ad (Ad-ΔE1/GFP) expressing GFP proteins and oncolytic Ad (Ad-ΔB7) were introduced in our previous results [7, 8].

2.2. Polymer and Ad plasmid DNA digestion

The synthesis and characterization of arginine-grafted bioreducible poly(disulfide amine) polymer (ABP) was shown by our previous publications [30]. For PEG5k conjugation to ABP, 0.1 equivalent of methoxy PEG 5K-NHS was added to the solution (pH 7.4, 0.1 M PBS (0.15 M NaCl, 2 mM EDTA)) of ABP having about 10 residues of arginine. The reaction was carried out in a room temperature for 2 h. After the reaction, the resulting crude product was precipitated to remove the unreacted and excess reagents with cold ethyl ether. The collected sample was purified using a dialysis membrane and then lyophilized. The conjugation of PEG was confirmed with proton NMR and size-exclusion chromatography (SEC, Superdex 75 column, calibrated with standard poly[N-(2-hydroxypropyl)-methacrylamide] (pHPMA)) using AKTA FPLC system. All Ad plasmid DNA was obtained using Endotoxin-free plasmid DNA purification kit (NucleoBond Xtra Maxi Plus EF, Macherey-Nagel Inc., Bethlehem, PA). To prepare linearized Ad plasmid DNAs, each Ad plasmid DNA was digested with Pac I restriction enzyme for 1 h at 37°C and precipitated with ethanol including 1/10 volume of sodium acetate (3 M NaOAc, pH 5.2). After DNA pellet rinsed with 70% ethanol was dissolved in DNase/RNase free dH₂O, the concentrations of DNA were determined by Nanodrop spectrophotometer measurements. Circular- or linear-type of Ad plasmid DNA polyplexes with ABP derivatives (ABP or ABP5k) or branched poly(ethylenimine) (bPEI, 25 kDa) were formed at various weight ratios (polymer/DNA) ranging from 5:1 to 40:1 for ABP or ABP5k or 1:1 for bPEI, respectively [31].

2.3. Dynamic light scattering (DLS) assay

After circular or linearized Ad plasmid DNA was polyplexed with each polymer with different concentrations (0.5:1 to 40:1 or 1:1 for bPEI) in 100 μL HEPES buffer (HEPES 20 mM, 5% glucose, pH 7.4) for 30 min incubation at room temperature, the polyplex solution was 6-fold diluted with deionized/filtered water. To determine the biodegradation ability of the Ad DNA/polymer polyplexes, the complexes were further incubated with 5 mM DTT for 2 h at 37°C. Average particle sizes and surface charges were measured using Zetasizer 3000HS (Malvern Instrument Inc., Worcestershire, UK) with a He-Ne Laser beam (633 nm,

fixed scattering angle of 90°) at room temperature. The obtained sizes were presented as the average values of 5 runs [32, 33].

2.4. Quantification of plasmid DNA or GFP protein expression

The condensing ability of the polymers and viral plasmid was examined by DNA quantification assay using Quant-iT PicoGreen assay kit (Molecular Probes Inc., Willow Creek Rd, Eugene, OR). Each polyplex was prepared in HEPES buffered saline (10 mM HEPES, 1 mM NaCl, pH 7.4) at various weight ratios (0.5, 1, 2, 5, 10, 20 or 40 µg, w/w). The level of GFP expression in each cell type transfected with polyplexes was measured by Qubit Fluorometer (Molecular probes Inc.).

2.5. Transfection of the Ad DNA with the polymers in 293 cells

293 cells were seeded on 24-well plate at 80% of confluence 1 day before transfection. The 293 cells were transfected with each polyplex solution of circular or linear Ad plasmid DNA (1 µg of pAd-ΔE1/GFP or pAd-ΔB7)/polymers (ABP, ABP5k or bPEI) at variable weight ratios (5 to 40 µg, w/w) at 37°C incubator. Fresh media containing 10% FBS were exchanged after incubation for 4 h, the treated cells were further incubated for 2, 4 or 7 days and photographed under optical or fluorescence microscopy.

2.6. Quantification of Ad replication by PCR or progeny viral production

To assess the viral replication from each cell type (293, Hep3B or Huh7), real-time quantitative PCR was performed. The cells transfected with circular or linearized Ad plasmid DNA polyplexed with ABP, ABP5k or bPEI were harvested at 2, 4 or 7 days. Each genomic DNA including the introduced Ad genome was purified using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The copy number of Ad genomes was measured by real-time quantitative PCR (Taqman PCR detection; Applied Biosystems, Foster City, CA). A fluorogenic probe (FAM-5'-CCGCCGYYCAGCC-3'-NFQ) was designed to anneal to the target sequence primer between the sense primer (5'-GGAACGCCGTTGGAGACT-3') and the antisense primer (5'-GGAAAGCAAAGTCAGTCACAATCC-3') in the IX gene of the Ad genome [33]. Samples were amplified for 40 cycles in an ABI 7500 sequence detection system (Applied Biosystems) with continuous fluorescence monitoring. All samples were analyzed in triplicate and data were processed by the SDS 19.1 software package (Applied Biosystems). To determine the progeny viral production of circular or linearized Ad DNA with the polymers, the cells transfected with each DNA type polyplexed with each polymer were harvested at 2, 4 or 7 days. The generated infectious Ad particles (plaque forming unit: PFU) were determined by the end-point dilution assay as shown previously [34].

2.7. Transfection efficiency and viral replication of the polyplexes in cancer cells

Hep3B, or Huh7 cells were seeded on 24-well plate at 70% of confluence 1 day before transfection. The cells were treated with polyplex solutions of circular or linear Ad plasmid DNA (1 µg of pAd-ΔE1/GFP)/polymers (ABP, ABP5k or bPEI) at variable weight ratios (5 to 40 µg, 1 µg for bPEI, w/w). Fresh media containing 10% FBS were exchanged after incubation for 4 h at 37°C. The treated cells were further incubated for 2 days and monitored GFP expression under fluorescence microscopy. To assess viral replication of the introduced oncolytic Ad DNA(pAd-ΔB7) with the polymers, each type of DNA was transfected to Hep3B, Huh7 or HDF with the same condition as above. After harvesting the cell lysate at 8 days, the lysate was treated to 293 cells for 4 h. At 4 days, generated infectious Ad particles were determined by the end-point dilution assay.

2.8. Secretion of Interleukin-6

Murine RAW264.7 macrophage cells were seeded on a 6-well plate at a cell density of one million/well containing DMEM supplemented with 10% FBS. After 24 h, PBS, naked Ad (1×10^9 particles), Ad plasmid DNA (1 μ g) polyplexed with ABP (10 μ g), ABP5k (10 μ g) or bPEI (1 μ g) suspended in serum-free media was treated for 4 h. The treated cells were incubated with fresh DMEM containing 10% FBS for 24 h after removal of the treated solutions. The cell free supernatants were collected and analyzed for the presence of Interleukin (IL)-6 by the mouse ELISA kit (R&D Quantikine; M6000B, Minneapolis, MN).

2.9. Protection from neutralization by anti-Ad antibody

Balb/c mouse was received with naked Ad at a single dose of 2×10^{10} viral particles (VP) intravenously. Mouse serum immunized with or without naked Ad was harvested at 14 days after 2nd injection and inactivated at 56°C for 45 min to inactivate blood complement and stored at -20°C for neutralization protection assay. Naked Ad or Ad DNA/polymer polyplex was exposed to the serum including Ad-specific neutralizing antibody (Ab) or not, for 30 min at 37°C and then treated to human cancer cells.

2.10. Biodistribution

Athymic female nude mice bearing Hep3B tumor were established by injecting 5×10^6 cells subcutaneously into their flank regions. When tumor reached about 80 mm³ in volume, mice were randomized in to 5 groups and injected intravenously with PBS, naked oncolytic Ad (1×10^{10} VP), naked/linearized Ad DNA (10 μ g) or linearized Ad DNA (10 μ g)/polymer (ABP or ABP5k, 100 μ g). Each organ was harvested 24 h after systemic administration. The genomic DNAs were extracted from the organs (liver, tumor or spleen) of the treated mice using the QIAamp DNA mini kit (Qiagen) and determined the copy numbers of viral genomes by real-time quantitative PCR method, as mentioned above [33].

2.11. Statistical analysis

The data are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical comparisons were performed using the Mann-Whitney test. The criterion for statistical significance was p values < 0.05 .

3. Results and discussions

3.1. Characterization of polyplex

The Ad plasmid DNA including Ad genome and bacterial portions such as ori site and antibiotic resistant gene (β -lactamase) is circular-typed for replicating efficiently in *Escherichia coli*. However, for effective viral replication in mammalian cells, both ITR regions should be exposed to each other because the replication of Ad is actively initiated at the ends of the viral genome followed by producing progeny Ad particles, while the circular DNA can rarely replicate in cells as shown in supplementary data 1 [26–29]. To further examine the ability of viral replication and progeny viral production of Ad plasmid DNA, the DNA was prepared with a circular- or linear-type by Pac I restriction enzyme to investigate the influence of the shape of Ad plasmid DNA on the efficiency of viral production and oncolysis.

We have previously reported that non-viral vectors can be utilized to deliver not only genetic materials, but also therapeutic Ad vectors with less toxicity and higher transductional efficiency [20–22, 32, 33, 35, 36]. For efficient delivery of oncolytic Ad plasmid DNA to host nucleus, the Ad plasmid DNA was polyplexed with ABP at variable weight ratios to determine the optimal conditions for generating the complex. Since

conjugation of PEG has been known to be able to prolong the blood circulation time and alter the tissue biodistribution of the conjugates, ABP conjugated with PEG5k molecules (ABP5k) were also synthesized and polyplexed with the Ad DNA to evaluate the effect on the experiments for controlling the further *in vivo* pharmacokinetics of the Ad DNA/polymers.

To examine the average sizes and the surface-charge values of the DNA/polymers polyplexes, DLS assay was accomplished. Since positively-charged cationic polymers are easily interact with negatively-charged plasmid DNA to form tight complex via electrostatic interaction, all polyplexes of polymers (ABP or ABP5k) with the circular or linearized DNA were stably formed at below 100 nm in size with weight ratios above 10:1 (Fig. 1A). According to our previous result, ABP polyplex with typical pDNA (pCMV-Luc, 5.6 kb) displayed the average size between 150 to 200 nm at all weight ratios used. Interestingly, in the case of the Ad DNA (about 33 to 36 kb), the average sizes of the polyplexes reached 70 to 80 nm. Two-times decreased sizes of the polyplexes of the Ad DNAs with the polymers indicate much smaller and more compact than the polyplex with pCMV-Luc plasmid, which would take a great advantage for systemic delivery. Each polydispersity index (PDI) value of the polyplex at weight ratios above 10 indicated evenly distributed complex formation between the polymers and the Ad DNAs whether circular or linearized (Supplementary data 2). Also, zeta-potential values of the complexes were measured to be about +20 mV, indicating that negatively charged Ad plasmid DNAs (circular or linearized) were completely condensed with cationic ABP or ABP5k at weight ratios above 10 (Fig. 1B). To confirm the biodegradable characteristic of ABP or ABP5k under reductive conditions, each polyplex was preincubated in 5 mM of DTT solution for 2 h at 37°C after complexing with Ad DNAs for 30 min at room temperature. In contrast, there was no difference in detectable Ad DNA content for the Ad DNA/bPEI complex with or without DTT treatment, the bioreducible polymers harboring disulfide bonds were rapidly degraded and released free Ad plasmid DNA (Fig. 2) by measuring the exposed DNAs released from each polymer using PicoGreen assay kit. The electrostatic formation and biodegradability of the polymers were also visualized by electrophoretic mobility shift assay in Supplementary data 3. These results demonstrated that the polymers are able to form very compact polyplex with circular or linearized Ad plasmid DNA, demonstrating the bio-reducibility of the polymer under reductive environment.

3.2. Viral production of linear-typed Ad plasmid by the polymers in 293 cells

To assess the ability with regard to replication and progeny viral production of the Ad plasmid in transfected cells, circular- or linear-typed plasmid DNA of replication-incompetent Ad (pAd Δ E1/GFP) encoding GFP was polyplexed with ABP or ABP5k polymer at weight ratio of 20, and transfected to 293 cells complementing Ad E1 proteins. At 2 days post-transfection, the 293 cells transfected with circular or linearized pAd Δ E1/GFP by each polymer strongly expressed GFP in all experimental groups (Fig. 3A). However, the zone of cytopathic effect (CPE) showing increased GFP expression by viral production through lytic pathway was slightly monitored at 4 days and clearly monitored in and spreaded to the cells transfected with linearized pAd Δ E1/GFP after 7 days post-transfection under fluorescence microscopy, while the GFP expression of the cells transfected with the circular DNA was getting decreased as day goes by. The CPE zones showing densely packed GFP expression by producing progeny Ad particles expressing GFP were getting increased and expanded to the surrounding cells only transfected with the linearized Ad plasmid DNA. As we expected, the same results of CPE formation in the 293 cells after transfection were acquired when oncolytic Ad plasmid polyplexed with each polymer (Fig. 3B). Only the 293 cells transfected with the linearized oncolytic Ad DNA/polymers (ABP or ABP5k) also showed the clear CPE zone after 4 days post-transfection. Although the 293 cells transfected with

circular Ad plasmid showed CPE formation rarely, the chance was very occasionally monitored (data not shown). These results indicate that Ad plasmid should be linearized to expose both ITR regions for having the characteristics to produce progeny infectious Ad particles.

To confirm that the CPE formation in the 293 cells transfected with the linearized Ad plasmid DNA (pAd-ΔE1/GFP or pAd-ΔB7) is exactly correlated with viral replication and generation, the 293 cells transfected with each type of the Ad DNA with the polymers (ABP or ABP5k) were harvested and analyzed Ad viral genome copies by real-time quantitative PCR (Fig. 4A) and progeny viral production by the end-point dilution assay determining plaque forming unit (PFU) (Fig. 4B). The copy number of the viral genomes showed significant time-dependent increase, only in the cells transfected with the linearized Ad DNA (pAd-ΔE1/GFP or pAd-ΔB7). At 7 days after transfection, the viral genome copy of pAd-ΔE1/GFP or pAd-ΔB7 with ABP or ABP5k was dramatically increased 119-, 115-fold or 222- or 178-fold, respectively. The production of the progeny viruses was quantified by measuring infectious viral particles (also referred as PFU) in the cells transfected with each polyplex. Corresponding to the result of viral replication, real-infectious Ad particles were produced only in the cells introduced with linearized Ad DNA delivered by each polymer, while no infectious Ad particles were detected in the cells treated with circular DNA by each polymer (data not shown). The viral production (PFU) by pAd-ΔE1/GFP with ABP (4.8×10^6 PFU) or ABP5k (2.5×10^6 PFU) was 14.7- or 10.7-times lower than that by pAd-ΔB7 with ABP (7.1×10^7 PFU) or ABP5k (2.6×10^6 PFU) at 7 days after transfection. The reduced viral production of the cells transfected with the DNA expressing GFP might be caused by the toxicity effect of GFP itself [37]. There was no considerable difference on the transfection efficiency by ABP or ABP5k. Taken together, we demonstrated that Ad plasmid DNA is needed to expose both ITRs for efficient viral replication and production in the transfected cells.

3.3. Transfection efficiency and viral production in human cancer cells

The transfection efficiency of linearized Ad plasmid DNA with the polymers at different weight ratios (10 or 20) was evaluated in human cancer (Hep3B or Huh7) or normal (HDF) cells (Fig. 5A). The efficiency, by measuring GFP expression of pAd-ΔE1/GFP with ABP or ABP5k, was increased polymer-dose dependently in all cells used. Whereas the transduction efficacy by ABP5k was continuously increased dose dependently even at the weight ratio of 40, the efficiency by ABP was slightly decreased at the highest weight ratio of 40 (data not shown). The reduced efficiency would be caused by the toxicity of polymer/Ad complex. As shown in supplementary data 4, the toxicity of ABP was little stronger although the cell viability of all polymers was greater than 80% in both cell lines at the weight ratios (10 to 40) used. bPEI polyplexed with linearized Ad DNA showed weaker transfection efficiency than other experimental groups.

The oncolytic Ad (Ad-ΔB7), which has deletion of E1B 19/55 kDa genes and mutation of E1A at pRb binding sites, was technically designed to selectively replicate in and ultimately kill cancer cells harboring p53 and pRb defective pathway [7, 8]. To check cancer-selective viral replication of the oncolytic Ad DNAs delivered by ABP or ABP5k in human cancer cells depending on the status of DNA type, while sparing normal cells, the linearized pAd-ΔB7 DNA/polymer polyplex was introduced to human cancer (Hep3B or Huh7) or normal (HDF) cells (Fig. 5B). At 8 days post-transfection, the treated cells were harvested and the supernatants from each cell lysate were treated to 293 cells. Only the cancer cells (Hep3B and Huh7) treated with linearized oncolytic Ad DNA delivered by ABP or ABP5k showed CPE zones (data not shown) and were detected by Q-PCR to check the increased the quantity of Ad genomic DNA. Since oncolytic Ad (Ad-ΔB7) showed cancer-specific killing effect as reported previously [8], the viral replication was restricted in normal cells (HDF) as

shown Fig. 5B. These results demonstrated that Ad plasmid DNA can be successfully delivered by the bioreducible polymers to human cells with expressing reporter proteins. Furthermore, delivery of linearized oncolytic Ad plasmid DNA by the polymers can elicit viral replication and production of progeny viruses in cancer-dependent manner.

3.4. Evading innate and adaptive immune response

When naked Ad particles are administered systemically, most of them are immediately extinguished within 2 min because of accumulation into liver and biological defense mechanisms of host. There are two major gateways of host immune response to inactive foreign pathogens. The first response is mediated by innate immune response by macrophages and dendritic cells, secreting proinflammatory cytokines and chemokines like IL-6, tumor necrosis factor- α and interferon- γ inducible protein-10 [9, 38, 39]. For inactivating the injected Ad particles, a more specific interaction for inactivating the Ad particles is by widespread anti-Ad neutralizing Ab produced in individuals who have previously been exposed to Ad [40]. Therefore, variable efforts to overcome the limit of therapeutic Ad delivery have been tried and achieved with promising results by combining with non-viral vectors to mask the surface of the Ad vectors.

Since secretion of IL-6 represents induction of innate immune response, murine macrophage cells (RAW264.7) were incubated with naked Ad or Ad plasmid DNA polyplexed with each polymer (ABP, ABP5k or bPEI) to determine the level of secreted IL-6 (Fig. 6A). While the amount of secreted IL-6 from the cells treated with naked Ad was 8.9-fold increased when compared to that with PBS, there was no significant induction of IL-6 from the cells treated with other experimental groups. In contrast with naked Ad vector, this result demonstrates that ABP or ABP5k delivering Ad DNA had no effect on the secretion of the cytokines from the macrophage cell lines, indicating evasion of innate immune response.

To evaluate the adaptive immune response induced by the experimental groups, the heat-inactivated serum from Balb/c mouse immunized twice with naked Ad particles for 14 days was treated with naked Ad, naked Ad DNA or each Ad DNA/polymer polyplex. After incubation of each group with the serum including Ad-specific neutralizing Ab for 30 min, naked Ad or each linearized Ad DNA with or without the polymers was treated to the cancer cells to evaluate the transgene expression of each vector, whether the activity of each group was eliminated by the Ad-specific neutralizing Ab (Fig. 6B). While the transduction efficiency of naked Ad particles were significantly reduced by 92.1% compared to that by the heat-inactivated serum without the Ab, the cancer cells treated with Ad DNA/polymers (ABP, ABP5k or bPEI) expressed the similar amount of GFP levels whether the polyplex was exposed to the serum with or without Ad-specific neutralizing Ab, resulting in 2.0, 1.1 or 8.0% reduced GFP expressions, respectively. Thus, these data demonstrate that ABP or ABP5k delivering Ad DNA showed markedly reduced innate immune response mediated by secretion of cytokines from splenocytes and adaptive immune response related with existing Ad-specific neutralizing Ab.

3.5. Biodistribution

One goal of therapeutic-Ad delivery is to minimize accumulation in untargeted organs in order to decrease side effects. The highest responsive organ of naked Ad administered systemically has been known as a liver [41]. The accumulation of naked Ad in liver causes not only liver toxicity but also much reduced therapeutic efficacy. Thus, we examined the *in vivo* biodistribution of naked oncolytic Ad or oncolytic Ad DNA polyplexed with ABP or ABP5k in Hep3B-tumor bearing mice (Fig. 7A). As we anticipated, most of the Ad genomic DNA was detected in the liver of the mice injected with naked Ad particles, indicating the strong liver- accumulation. However, the Ad genome content in the livers of the mice

treated with Ad DNA/ABP or Ad DNA/ABP5k was considerably lower than that of mice treated with naked Ad, showing 99.1% or 98.4% reduced liver accumulation, respectively. In addition, the biodistribution of Ad DNA/ABP or Ad DNA/ABP5k within tumor showed 2.1- or 6.6-fold increase compared with that of naked Ad. Especially, the DNA amount of the tumor versus liver in the mice injected with Ad DNA/ABP5k was shown 1.8-fold increased level of viral genomes, in contrast to the copy number of Ad genome delivered by ABP was 0.7-fold decreased at tumor/liver ratio. As an important therapeutic index, the tumor-to-liver ratio of the mice injected with Ad DNA/ABP or Ad DNA/ABP5k was markedly elevated by 229- or 419-fold when compared with the result of naked Ad (Fig. 7B). As it has been known that covalent conjugation of PEG molecules delivering therapeutic materials can prolong the plasma half-life, and alter the tissue distribution of the conjugates for controlling *in vivo* pharmacokinetics [42], the tumor-localization efficiency of the Ad DNA delivered by the ABP5k was 3.2-fold higher than that by ABP. In addition, variable modification ratios of PEG molecules and attachment of targeting moiety on the end of PEG linked to ABP should be considered for systemic cancer therapy against metastases [43]. Taken all together, the PEG-conjugated bioreducible polymer delivering linearized Ad DNA with the abilities of active replication and oncolytic ability can be useful as a novel and potential vector for cancer gene therapy.

4. Conclusion

To develop a more convenient vector system of oncolytic Ad for cancer gene therapy, the Ad plasmid DNA was polyplexed with ABP or PEG5k-conjugated ABP, and transduced to human cells. Linearized Ad plasmid DNA by Pac I digestion was performed since initiation of Ad DNA replication takes place on both ends of ITR region. Only the human cancer cells (Hep3B or Huh7) transfected with the polyplex of the linearized oncolytic Ad DNA polyplexes showed cancer-selective replication and progeny viral production, while sparing normal cells (HDF). Naked Ad induced the secretion of high IL-6 by innate immune response and inactivated by adaptive immune response mediated with Ad-specific neutralizing Ab. The Ad DNA polyplexes, which have no immunogenic Ad capsid proteins, had no effects on any immune responses. The biodistribution results demonstrated that oncolytic Ad DNA delivered by ABP5k significantly accumulated within tumors than naked Ad or ABP. Although Ad vector system with a variety of advantages has been highlighted on the field of cancer gene therapy, this convenient and simple delivery system of oncolytic Ad plasmid DNA by the bioreducible polymers can be utilized for *in vivo* application such as metastasis and disseminated cancer via systemic administration, accompanying with the effects of tumor-specific viral replication of the Ad genome and generation of progeny viruses, spreading to neighbored cancer tissues as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Characterization of circular- or linearized Ad DNA with the polymers at different weight ratios. (A) Average size (nm) measurements of each type of Ad DNA polyplexed with ABP, ABP5k or bPEI. (B) Zeta-potential value (mV) measurements of each polyplex.

Fig. 2. Bioreducible activity of ABP or ABP5k polymers polyplexed with Ad DNA (circular or linearized) under normal condition (left 2 panel) or reductive condition (5 mM DTT treatment for 2 h at 37°C) (right 2 panel). The lower 2 boxes show that of low weight ratios (less than 3) of polymer/Ad DNA to check more the bioreducible activity clearly.

Fig. 3. Viral production of Ad DNA polyplexed with the polymers (ABP or ABP5k) in 293 cells. Replication-incompetent Ad DNA (pAd-ΔE1/GFP) (A) or oncolytic Ad DNA (Ad-ΔB7) (B) linearized by Pac I restriction enzyme was complexed with each polymer. The polyplexes were transfected to 293 cells for 4 h at 37°C incubator. At 2, 4 or 7 days after transfection, GFP expression or CPE formation was monitored under fluorescence or optical microscopy (White bars represent 400 μm, magnification × 100).

Fig. 4.

Viral replication and production of pAd-ΔE1/GFP or pAd-ΔB7 polyplexed with the polymers in 293 cells. (A) Viral replications of each polyplex in 293 cells were examined by real time quantitative PCR at 2, 4 or 7 days after transfection. (B) The production of infectious progeny Ad viral particles (PFU) was also measured by end-point dilution assay at 2, 4 or 7 days. * $p < 0.01$ versus the cells transfected with circular Ad DNA with each polymer.

Fig. 5. Transfection efficiency and viral replication efficacy of linearized Ad DNA delivered by the polymers in human cancer (Hep3B or Huh7) or normal (HDF) cells. (A) Transfection efficiency of linearized pAd- Δ E1/GFP polyplexed with the polymers was measured by GFP expression levels in the cells. (B) Viral replication of linearized oncolytic Ad DNA polyplexed with the polymers was examined by real-time quantitative PCR. * $p < 0.01$ versus the cells transfected with circular Ad DNA with each polymer.

Fig. 6.

Measurements of innate and adaptive immune response. (A) The secretion of IL-6 from murine RAW264.7 macrophage cells treated with naked Ad, naked Ad DNA or each polyplex was determined by mouse IL-6 ELISA kit. (B) Neutralization effect of Ad-specific Ab from immunized mouse with naked Ad (2 times of 1×10^{10} VP) on transduction efficiency of naked Ad (Ad- Δ E1/GFP) Ad DNA (pAd- Δ E1/GFP) or each polyplex. After incubation of the experimental groups with untreated serum or antiserum including Ad-specific Ab for 30 min at 37°C, each group was treated to Hep3B or Huh7 cells to check GFP expression.

Fig. 7. Biodistribution of naked Ad, Ad DNA, Ad DNA polyplexed with ABP or ABP5k in tumor-bearing mice. Naked Ad (1×10^{10} VP) or Ad DNA (10 μ g) or each polyplex (10 μ g DNA plus 100 μ g polymer) was systemically injected to the mice. At 24 h after single injection, liver and tumor of the mice were harvested. Each genomic DNA was extracted from the tissue and quantified viral genomic copy numbers using real-time quantitative PCR. Data are expressed as mean $\pm$ SE and $n=3$ for each experimental condition. (B) The tumor-to-liver ratios of viral genomes harvested from the experimental groups were acquired after calculating with the normalized value of that injected with naked Ad. * $p < 0.05$ versus Ad DNA/ABP or Ad DNA/ABP5k.