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Mini Review

Current advances in adenovirus nanocomplexes: more specificity and less immunogenicity

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An often overlooked issue in the field of adenovirus (Ad)-mediated cancer gene therapy is its limited capacity for effective systemic delivery. Although primary tumors can be treated effectively with intralesional injection of conventional Ad vectors, systemic metastasis is difficult to cure. Systemic administration of conventional naked Ads leads to acute accumulation of Ad particles in the liver, induction of neutralizing antibody, short blood circulation half-life, non-specific biodistribution in undesired organs, and low selective accumulation in the target disease site. Versatile strategies involving the modification of viral surfaces with polymers and nanomaterials have been designed for the purpose of maximizing Ad anti-tumor activity and specificity by systemic administration. Integration of viral and non-viral nanomaterials will substantially advance both fields, creating new concepts in gene therapeutics. This review focuses on current advances in the development of smart Ad hybrid nanocomplexes based on various design-based strategies for optimal Ad systemic administration. [BMB reports 2010; 43(12): 781-788]

INTRODUCTION

Human cancer gene therapy has significantly progressed with the advent of adenovirus (Ad) vectors. Since 1993, numerous clinical trials involving over 392 clinical protocols have been conducted using Ad vector as a delivery vehicle (http://www.wiley.co.uk/genmed/clinical/). Ads have several technically useful attributes, including efficient nuclear entry mechanism, high gene transfer efficiency in both dividing and non-dividing cells, easy production of high-titer Ad stocks, low risk of insertional mutagenesis, and induction of oncolysis by viral replication. Gene delivery vehicles possessing these versatile molecular biological attributes are ideal for use in cancer virotherapy.

One emerging modality of cancer therapy is replication-competent oncolytic Ads that inherently multiply, lyse infected cancer cells, and spread to surrounding cancer cells, resulting in improved efficacy over non-replicating Ads (Fig. 1). Recent studies have reported the tumor-selective replication of oncolytic Ad, guaranteeing enhanced selective anti-tumor activity without killing normal healthy cells (1, 2). Moreover, combining the cell killing effect of tumor-selective replicating oncolytic Ad with gene therapy based on virus-mediated delivery and expression of transgenes will potentially have a significant impact on the efficacy of therapeutic tools for cancer treatment (3-5). Most importantly, the amplification and propagation of therapeutic genes using replicating viruses in infected neighboring cells highlights the potential of replicating virus-based therapy for the treatment of heterogeneous human tumors. In addition, oncolytic Ads express therapeutic genes in a cancer cell-specific manner (6-8), resulting in minimal expression of unwanted genes in normal cells and high expression of therapeutic genes in selected cancer cells.

For virus-based gene therapy of metastasizing cancer to be successful, the virus must have easy access to the tumor cells,

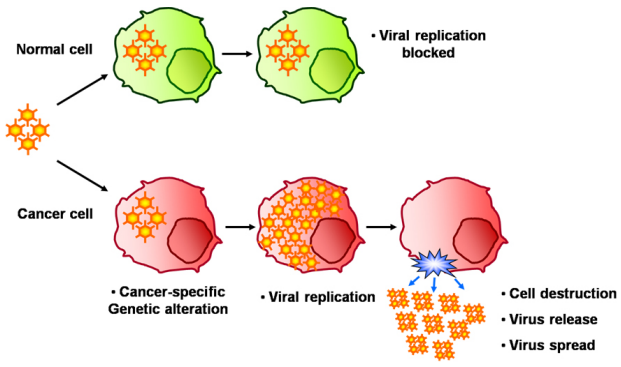

Fig. 1. Schematic diagram of the cancer-selective killing of oncolytic Ads. Oncolytic Ads can specifically kill tumor cells by cancer cell-specific viral replication while sparing normal cells. Replicating viral agents in tumors may lead to improved efficacy over non-replicating Ads due to their inherent ability to multiply, lyse infected cancer cells, and spread to surrounding cells.

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which is made possible by a new delivery system characterized by extended circulation time in plasma (9). Circulation of naked Ad in the blood causes acute viral accumulation and hepatocytosis in the liver, resulting in undesired gene expression and an inappropriate innate immune response. To avoid activating immune responses and hepatotoxicity, an effective Ad nanocomplex delivery system for systemic tumor therapy has been used for minimal hepatic distribution and high localization in tumor cells (10-12). This review describes the recent developments in the design of cancer-targeted Ad nanocomplexes to overcome limited systemic delivery efficiency and inappropriate triggering of immune responses, thereby enhancing the efficacy and safety of anti-cancer therapeutic strategies.

EFFORTS TO DEVELOP SYSTEMIC Ad DELIVERY

Thus far, Ad-mediated cancer gene therapy has been attempted only by intratumoral administration (13, 14) in order to accumulate a high concentration of Ad in the local tumor site. However, systemic administration would make possible the treatment of disseminated metastatic tumors in addition to primary tumors, enabling Ads to access and infect widely distributed cancer cells. The most significant obstacle for Ad systemic administration is the induction of strong innate immune responses. Kupffer cells (KCs) in the liver and spleen cells immediately uptake naked Ad, resulting in immune cell secretion of inflammatory cytokines such as interleukin (IL)-6 and IL-12, which cause local acute inflammation (15). The expression of these pro-inflammatory cytokines combined with the sequestration of intravenously administered Ad by KCs in the liver induces hepatotoxicity. Outside of the liver, Ad transduction of cells in other organs also attributes to potential Ad side effects. Another hurdle for Ad systemic administration is the generation of Ad-specific neutralizing antibody, which masks viral capsid proteins and inhibits Ad infection. This neutralizing antibody may reduce the level of therapeutic gene expression as well as the efficacy of repeated Ad administration.

Although attempts to resolve these difficulties have been made using various molecular biology techniques such as genetic manipulation of Ad capsid protein and substitution of Ad serotypes, very little *in vivo* progress has been made. Therefore, to overcome these hurdles, other strategies involving the chemical and physical modification of Ad vectors have been employed with the goals of attaining preferential tumor targeting, prolonged systemic circulation, minimal side effects, pharmacological analysis, and feasible but optimized clinical potential. Advances in Ad modification now involve smart nanocomplexes, which possess advantages related to specificity, sensitivity, and charge reversibility.

Ad NANOCOMPLEXES FOR SYSTEMIC DELIVERY

The need for systemic administration of Ad therapeutics has been recognized with recent advances in nanotechnology and pharmaceutics, making it possible to engineer numerous chemical modifications of the Ad surface using polymeric materials (16, 17). Originally, systemic and targeted delivery of Ads was performed by a versatile polymeric design and depot system, or by self-assembly in the pharmaceutical field. The need to unite virus and systemic delivery carriers has been recognized for systemic virotherapy (16, 18, 19). Customized strategies can be exploited to direct carriers to a specific disease site, prolong systemic circulation, and modulate the cellular microenvironment. These virus nanocomplex-based therapy strategies utilize passive accumulation based on enhanced permeation and retention (EPR) effects at the tumor site, active targeting with coordination of targeting moieties, and site-specific activatable targeting. Additionally, integral research studies have shown synergistic effects, taking advantage of both non-viral and viral gene carriers for systemic delivery.

Polymer complexes with polyethylene glycol (PEG) and poly-N-(2-hydroxypropyl) methacrylamide (pHPMA)

In general, chemical modification of Ad is carried out by cross-linking of poly-N-(2-hydroxypropyl) methacrylamide (pHPMA) and PEGylation via amine functional groups on the Ad surface. The exterior of the Ad surface has approximately 1,800 exposed lysine molecules located on the hexon, penton, and fiber proteins (20). PEGylation and pHPMA conjugation of Ad is easily applied via amine-mediated simple chemistry. O'Riordan *et al*. performed the first study on hybrid Ad complexes in 1999, proving that PEGylation of amino groups on the Ad surface reduces innate immune responses and shields the Ad surface from neutralization by antibodies. These non-specific modifications of the Ad surface with biopolymers reduce undesired interactions between plasma proteins in the blood stream. In addition, hybrid Ad complexes modified with biocompatible PEG and pHPMA help maintain Ad bioactivity *in vivo* protecting against deterioration by proteolysis, resulting in a prolonged half-life in blood (21). Moreover, PEG acts as a shield on the Ad surface, resulting in reduced hepatic uptake and adsorption of neutralizing antibody. Masking of molecular patterns on the Ad capsid by PEGylation was previously evidenced by a significant reduction in innate immune responses. After 6 hr of intravenous administration of PEGylated Ad, the plasma level of IL-6, which is the main indicator of an innate immune response against Ad, was reduced by 95% compared to that of naked Ad (22).

Previous studies have shown that the molecular weight of PEG determines the degree by which the innate immune responses against Ad are reduced (23). Yao *et al*. compared the biodistribution and reduction of immune responses against Ad after systemic administration of PEG [20K/45% modification ratio]-Ad and PEG [5K/90%]-Ad (24). Administration of Ad PEGylated with high molecular weight PEG, PEG [20K/45% modification ratio]-Ad, resulted in the desired biodistribution, including high Ad accumulation in tumor cells and plasma as

well as low distribution in the liver. The increased size of the Ad complex is attributed to the conjugation of Ad with longer chain PEG, resulting in avoidance of liver sinusoid fenestrae, which would lead to excretion of the Ad. Adequate shielding of the Ad capsid by longer chain PEG also enables the Ad to elude non-specific uptake by macrophages and KCs, which are responsible for innate immune responses (25-28). In addition, PEG can hide the Ad capsid and thus obscure recognition by the highly expressed Ad receptors coxsackievirus and adenovirus receptor (CAR) and heparin sulphate, which are both expressed on KCs and hepatocytes (29, 30), resulting in significantly augmented half-life in blood. This enhanced half-life of PEGylated Ad in the blood consequently promotes highly effective accumulation of Ad in tumor tissue via passive accumulation based on the EPR effect (24, 26, 31). A previously published study quantitatively analyzed the biodistribution of PEGylated Ad using a bioluminescent reporter gene visualized in a metastatic model (24). The gene transfer efficacy of systemically administered PEGylated Ad showed a high tumor-to-liver ratio, proving that PEGylated Ad was highly localized in tumor cells via the EPR effect and resulted in enhanced anti-tumor treatment efficacy.

Another major polymer frequently used for Ad surface modification is pHPMA, which is randomly polymerized on the Ad surface (19). Biocompatible and hydrophilic HPMA copolymers have been used for over 10 years as a drug delivery carrier and depot system, acting as a reservoir for drug storage and preventing protein absorption. Through extensive research, pHPMA was proven to have low toxicity and immunogenicity (19, 32). Affinity moieties on the pHPMA layer are made possible by reaction of amine-reactive 4-nitrophenyl ester (ONp) groups. The side chains of multivalent HPMA copolymers have also been utilized for conjugation with Ad surface amine groups, forming a stable polymeric layer on the Ad surface. In addition, pHPMA harbors numerous amine-reactive groups, which makes it possible to easily add hydrophilic PEG and affinity moieties for generation of a hydrophilic, stable, and dense polymeric layer on the Ad surface. This shell then prevents the binding of neutralizing antibody while enabling tumor-specific targeting.

SMART ENVELOPES ON THE Ad SURFACE

Specificity

PEGylated Ads have prolonged half-lives in the plasma circulation, enhanced EPR effects, and reduced absorption of neutralizing antibodies. However, PEGylated Ads are also characterized by reduced transduction efficacy due to the natural stealth effects of PEG, resulting in avoidance of the CAR-mediated major uptake pathway. Therefore, redirection of PEGylated Ad technology by engineering specific affinities has been extensively explored and proposed as the second generation of Ad nanocomplexes (Fig. 2.1). The outer shell or distal end of PEG in Ad/polymer complexes has been conjugated with active targeting moieties such as antibodies (22), growth factors (31, 33-39), small molecular complexes of peptides (arginine-glycine-aspartic acid; RGD), and ligands (27, 40) in order to direct Ad to a targeted disease site (41). Direction of Ad complexes via affinity tags results in accumulation of a high concentration of Ad nanocomplexes in tumor sites, leading to highly effective gene expression.

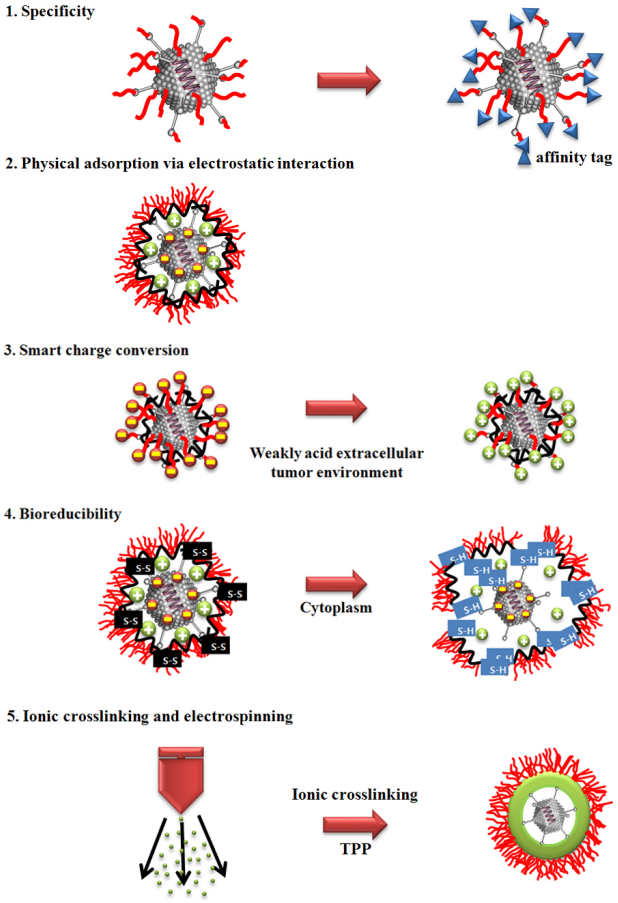

Fig. 2. Diagram of smart Ad nanocomplexes. 1. Specific affinity to targeted tumor. 2. Physical adsorption via electrostatic interaction. 3. Charge-convertible Ad in a weakly acidic extracellular tumor environment. 4. Ad complexes with positively charged bioreducible polymer; recovery of Ad surface by reduction of disulfide backbone. 5. Cross-linking-mediated Ad nanocomplex prepared by electrospinning.

Studies using PEGylated Ad have demonstrated reduced gene expression in CAR-expressing cells due to the masking of Ad capsid protein fiber by PEGylation. Studies by Eto et al. showed that administration of PEGylated Ad modified with RGD peptide motif results in 200-fold higher gene expression than unmodified PEGylated Ad, which lacks a targeting moiety (27). Specifically, this redirection of RGD on the surface of PEGylated Ad enhanced gene expression in both CAR-positive

and CAR-negative cells and indicated that redirection is necessary for efficient infection by PEGylated Ad, which leads to an alternative pathway of cellular uptake. Various macromolecules, such as antibodies, have been conjugated to the end of PEG, as well as small molecules such as folic acid and peptide (27, 40, 42). Ogawara *et al*. reported that E-selectin antibody-conjugated Ad-PEG complexes are selectively targeted to tissue inflamed by delayed type hypersensitivity reaction (36). In this study, systemic administration of E-selectin antibody-conjugated Ad-PEG complexes resulted in accumulation of Ad complexes in inflamed skin, resulting in local expression of the transgene luciferase. This study also showed that systemic administration of Ad tagged with antibodies, which can bind ligands specific to a disease site, can redirect Ad nanocomplexes to the desired target site. In addition, certain growth factors have been conjugated to the end of PEG in order to target specific receptors overexpressed in tumor tissue. Specifically, Park *et al*. published a study in which they conjugated epidermal growth factor (EGF) to PEG, which targets epidermal growth factor receptor (EGFR) (37). EGFR is expressed in tumors of epithelial origin and lung cancer and is involved in various signal cascades. Due to these characteristics, EGF has been employed as a ligand with nanoparticulate and diagnostic motifs. It was found that EGF tethered to the Ad surface resulted in highly efficient gene expression in A431 cells and MCF-7 cells expressing EGFR. These studies utilized a variety of small molecules and macromolecules as targeting moieties and showed that redirection to a target site was an effective approach for achieving high accumulation of Ad, leading to efficient cellular uptake.

Charge conversion

Mok *et al*. utilized new strategies to design Ad nanocomplexes with various surface charges by taking advantage of the unique pH microenvironment of tumors (43) (Fig. 2.3). The surface charges of Ad nanocomplexes in weakly acidic tumor microenvironments can change from negative to positive upon cleavage of the citraconic amide linkage. This conversion to a positive charge increases contacts between the Ad complex surface and the negatively charged cellular membrane, leading to highly improved gene expression. The use of quantum dots as a fluorescence maker has resulted in increased cellular uptake of charge-converted Ad nanocomplexes by direct tracking of Ad complexes. Tumor microenvironment-dependent charge-converted Ad nanocomplexes react only at the tumor site in a weakly acidic extra-tumoral environment ranging from pH 5.8 to pH 7.4. Therefore, self-modification of Ad nanocomplexes at disease sites is a valuable example that signifies the advanced design of Ad surface modifications.

Electrostatic interaction

The surface charge of naked Ad is slightly negative at −4 mV, as measured by a zetapotentiometer (22). Based on this property, Ad complexes were previously constructed using positively charged polymers, such as chitosan, poly-L-lysine, and arginine bioreducible polymer (ABP), via electrostatic interactions (Fig. 2.2). One example of Ad nanocomplexes modified by electrostatic interaction is Ad complexed with PLL-PEG (44). The optimal structure of polymers that interact with Ad was investigated using block copolymers and grafting copolymers while controlling the ratio of PLL and PEG. Ad/PLL-PEG complexes were treated with block and grafting PLL-PEG polymers at three different grafting ratios (1 : 0.035, 1 : 0.084, and 1 : 0.323). Gene expression in cells treated with PLL-PEG block copolymer showed 6-fold greater GFP expression than that induced by Ad treated with PLL-PEG grafting polymer at various ratios. These results indicate that polymer structure and optimal density of PEG may be critical factors for physical adsorption and eventually efficient celluar transduction. Therefore, Ad complexes formed through electrostatic interaction have the potential to enhance gene transduction in a non-specific manner.

Bioreducible surface modification of Ad complexes

An even more advanced design for Ad complexes has been demonstrated through complexation with arginine-grafted bioreducible polymer (ABP), which involves physical adsorption via electrostatic interactions onto the Ad surface (22, 47) (Fig. 2). ABP has reducible disulfide bonds in its backbone and therefore is biodegraded under reducible conditions such as those found in the cytoplasm, which contains ~0.1-10 mM of the reducing molecule glutathione. These reducing conditions lead to the efficient release of Ad from polyplexes as well as a reduction in cytotoxicity. Enhanced transduction efficiency has been observed in cells treated with cationic ABP polymer-coated Ad complexes compared to naked Ad. In addition, administration of ABP-coated Ad complexes produces higher levels of transgene expression than that of the cationic polymer 25K PEI in cells expressing either high or low levels of CAR. These results confirm three major conclusions: (1) cationic polymers can improve gene transfer efficiency of Ad to cells with variable CAR expression; (2) the transduction efficiency of viral complexes formed with ABP is greater than that of complexes formed with 25K PEI cationic polymer; and (3) ABP promotes Ad transduction in both CAR-positive and CAR-negative cells by taking advantage of surface charge. Moreover, ABP-coated Ad complex induces a significantly weaker innate immune response relative to naked Ad, as assessed by IL-6 cytokine release from macrophages. These studies indicate that gene expression and transduction efficacy of Ad complexes can be customized, depending on the function, properties, and design of the polymer used. Two effective examples are the engineering of Ad complexes modified based on electrostatic interaction and the bioreduction of the polymeric layer after administration.

Ionic crosslinking of a chitosan layer processed by electrospinning

Another positive polymer utilized in engineering Ad complexes is PEGylated chitosan (Park *et al*., 2010). Notably, Ad complexes after physical adsorption are stabilized by ionic crosslinking of tripolyphosphate, resulting in a firm polymeric layer. Furthermore, the formation of Ad complex with chitosan polymers has been achieved by electrospinning, making steady production on a large scale possible. Ionic cross-linking of chitosan formed a stable positively charged outer shell around the Ad and allowed for chemical conjugation to the numerous amine groups present (Fig. 2.5). Indeed, PEGylation via the amine groups of chitosan resulted in a weaker immune response as well as increased plasma circulation half-life when compared to naked Ad. As a model tumor-targeting ligand, folic acid (FA) was also attached to the distal end of PEG and then conjugated to the amine groups of the chitosan layer, generating an Ad/chitosan-PEG-FA complex. The targeting ability of FA-conjugated Ad, with the amount of bound FA ligand carefully controlled, was measured using FA receptor-specific transduction. The results of this experiment showed that the transduction efficiency of PEG-conjugated Ad (Ad/chitosan-PEG) was dramatically reduced compared to that of naked Ad in both FA receptor-positive and FA receptor-negative cells. This was due to physical blocking of the Ad capsid protein fiber by PEG. In contrast, the level of GFP expression by Ad/chitosan-PEG-FA was significantly increased compared to that induced by naked Ad, specifically in FA receptor-positive KB cells but not in FA receptor-negative U343 cells. This result demonstrates that entry of FA receptor-targeted Ad/chitosan-PEG-FA is dependent on the expression of the FA receptor on the cell surface. In addition, the transduction efficiency of Ad/chitosan-PEG-FA increased proportionally with the amount of surface-bound FA on Ad nanocomplexes, further supporting FA-mediated viral entry. Surprisingly, electrospinning did not impair the biological activity of Ad nor its ability to induce gene expression compared to that of naked Ad. These results indicate that Ad has considerable latitude, which can be exploited in engineering new Ad nanocomplexes. In summary, these results suggest that targeting of Ad is feasible by conjugating polymers along with an appropriate targeting moiety onto the Ad surface through electrospinning, thereby resulting in an effective method for the mass production of modified Ad nanocomplexes for clinical and animal studies.

ONCOLYTIC Ad WITH NANOCOMPLEXES FOR ANTICANCER THERAPEUTICS

Self-replicable oncolytic virus is recognized as a powerful therapeutic tool that lyses cells, spreads, and then infects neighboring cancer cells. Along with efforts to develop Ad nanocomplexes, oncolytic Ad or replication competent Ad has also been modified with carrier materials (24, 26, 45, 46). Although the advantage conferred by continuous infection of oncolytic viruses potentially impacts virotherapy, naked oncolytic viruses have faced the same hurdles as non-replicating viruses, including issues related to inefficient systemic delivery.

Several groups have studied oncolytic Ads complexed with a polymer as a delivery carrier. Doronic *et al*. demonstrated that PEGylated oncolytic Ad causes reduced transduction of hepatocytes as well as hepatotoxicity after systemic administration, resulting in a higher survival rate compared to naked oncolytic Ad (26). Visual evidence of biodistribution was made possible by the bioluminescence of luciferase-expressing Ad. These experiments showed that the intensity of bioluminescence was reduced 19-90 times in the liver compared to unmodified naked oncolytic Ad. However, the transduction level in cells treated with PEGylated Ad was not significantly different at the tumor site compared to that of naked oncolytic Ad. Although PEGylated oncolytic Ad improved the plasma circulation half-life and reduced biodistribution in undesired organs, the fact that Ad complexes lacked specificity to target tumor cells resulted in a comparable transduction level via the passive EPR effect.

Critical differences in transduction levels at the target tumor site were achieved by Her2/neu-targeted and PEGylated oncolytic Ad, which has a Herceptin Ab at the distal end of PEG (48). Relaxin-expressing oncolytic Ad (DWP418) was chemically conjugated with PEG. Her2/neu-specific Herceptin antibody was then conjugated to the end of biheterofunctional PEG for Her2/neu-targeted cancer gene therapy. DWP418-PEG-HER had Her2/neu-dependent oncolytic activity, indicating that the Herceptin-targeting moiety directed selective entry of this Ad nanocomplex into Her2/neu-positive cells. DWP418-PEG-HER elicited greater anti-tumor activity *in vivo* towards Her2/neu-positive SK-OV3 and MDA-MB435 xenograft tumors than did either naked DWP418 or DWP418-PEG. In contrast, DWP418-PEG-HER had equivalent anti-tumor activity as DWP 418-PEG against Her2/neu-negative MCF7-mot tumors. Thus, the enhanced anti-tumor activity of DWP418-PEG-HER in Her2/neu-positive tumors is based upon targeting of the Ad nanocomplex to the tumor through specific interaction between Herceptin and Her2/neu on the cell surface. Moreover, quantitative PCR assay also resulted in a significant increase in highly localized DWP418-PEG-HER in tumor cells. This increase was as much as 5.8×10^4-fold higher compared to that produced by naked DWP418. In addition, there was a 3.7×10^5-fold reduction in the biodistribution of Ad in the liver when DWP418-PEG-HER was administered compared to naked Ad. Thus, the increase in tumor-to-liver ratio was 10^{10}-fold for DWP418-PEG-HER versus naked Ad. This result critically shows that affinity tags enable highly-specific tumor targeting, resulting in enhanced accumulation of Ad in tumor tissue. Furthermore, successive secondary replication of oncolytic Ad amplifies the high local concentration of therapeutic Ad in tumor tissue, resulting in high anti-tumor activity. No apparent toxicity was noted in animals that received DWP418-PEG or DWP418-PEG-HER during the course of this study. Ad-related

liver toxicity, as measured by serum ALT and AST levels, was absent in PEGylated Ad-treated animals. Taken together, these results demonstrate the potential for effective and safe systemic therapies for the treatment of both primary and metastatic tumors.

FUTURE DIRECTIONS

The first generation of Ad nanocomplexes was modified using simple PEGylation or a variety of polymers such as pHPMA, PLL, and ABP in order to prolong circulation time, reduce toxicity, and enhance the EPR effect. With the development of diverse carrier designs and materials, Ad nanocomplexes have evolved into potent cancer therapeutics tools (48), gene delivery systems (2), and imaging agents (17, 49). The therapeutic effects of these versatile novel Ad nanocomplexes should be characterized *in vivo* in order to validate their practical potential in a clinical setting. Exploitation of the new generation of Ad nanocomplexes may greatly impact the fields of cancer gene therapy, virotherapy, and therapeutic diagnostics.

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