Cancer-targeting Gene Therapy Using Tropism-modified Adenovirus

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Abstract. Gene therapy has the potential to provide highly selective, curative cancer treatments without inducing systemic toxicity. Adenoviral vectors have been extensively used for cancer gene therapy because of their relatively high efficacy of gene transfer. However, gene transduction to cancer cells is limited by the necessity of using adenoviral type 5 vectors. This is because these vectors have a low transduction efficiency due to weak expression of the adenovirus receptor, coxsackie-adenovirus receptor (CAR), on cancer cells. Moreover, there may be side-effects to the treatment as normal cells also express CAR. In order to eradicate cancer cells without side-effects, the development of a targeting-vector is therefore crucial. In this review, the recent targeting strategies of adenoviral vectors for cancer gene therapy are summarized.

Cancer gene therapy represents one of the most rapidly developing areas in pre-clinical and clinical cancer research. However, the principal obstacles to the use of gene therapy are related to either the inefficient delivery of the ‘therapeutic’ gene to the cancer cells or the side-effects due to undesirable gene transfer into normal cells. This has led to unsatisfactory outcomes in clinical trials because of the recurrence of cancer in tumor cells missed by the delivery system. A pressing issue of gene therapy is the improvement of current treatment modalities based on better selectivity for cancer cells.

In order to develop more effective gene therapy, the therapeutic gene must be inserted into a vector adjacent to a promoter that induces transcription. The construct must be delivered to specific target cells, and transcribed and expressed at a high enough concentration to have an effect.

The transfer of the gene constructs into the target cells can be accomplished using appropriate vectors. Adenoviruses have linear double-stranded DNA. The adenoviral genome can accommodate up to a 30 kb DNA insert (1). The viral genome does not normally integrate into the host genome but instead replicates as episomal elements in the nucleus of the host cells with no risk of insertional mutagenesis. Moreover, they can be produced in high titers and can infect many different cell types. Therefore, adenovirus (Ad) vectors are now widely used for gene transfer into tumor cells (2). The adenovirus of type 5 (Ad5) (Figure 1) is most frequently used in several types of cancer gene therapy, such as gallbladder, brain, colon, rectum, lung, prostate, liver, stomach, ovary, esophagus and peritoneum (3-16). Ad5 infection is mediated by the high-affinity binding of the fiber knob to the coxsackie-adenovirus receptor (CAR) (17-19), followed by internalization mediated by the binding of arginine-glycine-aspartic acid (RGD) motifs that contains five Arg-Gly-Asp sequences, in the penton base to integrins αvβ3 and αvβ5 on the cell surface (20, 21) (Figure 2). However, the widespread application of Ad vectors for cancer gene therapy is hampered by a lack of specificity for malignant cells, as the CAR is expressed on both normal and malignant cells (17). Therefore, the development of tropism-modified, tumor-targeted adenoviral vectors is a critical issue in further developing the cancer gene therapy approach. In this review, we focus on three strategies for increasing the tumor specificity of adenovirus vectors.

Incorporation of Specific Ligands against Receptors on Cancer Cells (Figure 3A)

In order to specifically target cancer cells, specific sequences have been inserted into the Ad5 fiber protein. High-affinity peptide ligands have been inserted into the HI-loop or on to the C-terminus of the fiber. McDonald, Wickham et al. compared the gene transfer of an Ad fiber protein that
Ad vectors containing the RGD peptide in the HI-loop of knob showed levels of gene transfer similar to those of the fiber knob, depending on the cell type. The Ad vectors containing both peptides in the HI-loop or C-terminus of the fiber knob showed the highest levels of gene transfer and a broader tropism. For gene transfer into tumor cells, the Ad vectors containing the RGD peptide were the most efficient.

**Fiber Replacement (Figure 3B)**

There are more than 50 serotypes of human adenoviruses. Group B adenovirus serotypes (Ad3, 7, 11, 14, 16, 21, 34, 35 and 50) use a receptor other than CAR. Several studies have documented that mutations which abolish CAR and integrin interactions are not sufficient to eliminate liver transduction (25-28). Smith et al. showed that a KKTK motif in the Ad5 fiber shaft binds hepatic heparan sulfate proteoglycans (HSPGs), thus resulting in liver transduction (29). Furthermore, Ad vectors containing short fiber shafts (lacking the KKTK motif) derived from Ad35 (30) do not efficiently transduce liver cells, these data indicate that for liver uptake the KKTK motif pathway does not function in short shafted Ads. Vectors containing both the shaft and the knob of Ad35 have become increasingly popular as gene transfer vectors because they are able to transduce a number of important target tissues that are relatively refractory to Ad5 infection, including human hematopoietic stem cells, dendritic cells, and malignant tumor cells. CD46 has been identified as a cellular receptor that is used by most B-group adenoviruses (31-33). The expression of CD46 is greatly up-regulated in malignant tumor cells, including those of breast, colon, liver and endometrial carcinomas (34-36). This makes CD46-targeting vectors potential tools for tumor gene therapy. In order to target tumor cells through CD46, Ni et al. used vectors possessing fibers derived from serotype 35 (37). They demonstrated the in vivo properties of Ad5-based vectors that contained the Ad35 fiber (Ad5/35) (Figure 3Ba) in transgenic C57Bl/6-mice (38) that express CD46 in a pattern and at a level similar to humans. Upon intravenous and intraperitoneal injection, the Ad5/35 vector did not efficiently transduce the normal tissue, but was able to target the metastatic or intraperitoneal tumors that express CD46 at levels comparable to human tumors. Another advantage for Ad35 is low human immunogenicity. Cellular immunity to Ad5 may severely limit the use of Ad5-based vectors for gene therapy applications. Seshidhar Reddy et al. (39) have identified Ad35 as an alternative adenoviral serotype to which the majority of humans do not have neutralizing antibodies.

Adenovirus serotype 40 (Ad40) contains two distinct long and short fibers, the short fiber is unable to recognize CAR, while the long fiber binds to CAR. Ad5 vectors have a flexible long (22-β-repeat) fiber shaft (40-42), but Ad40 possess shafts formed by six or seven β repeats (43). Nakamura et al. (43) have generated Ad5-based mutants with chimeric Ad40-derived fibers, which were composed of either long or short
shafts together with CAR binding or non-binding knobs (Figure 3Bb). They examined the capacity of these Ad mutants for both in vitro and in vivo gene transfer to liver cells. The high transduction efficiency observed in the liver and spleen following intravenous administration of the Ad vector was dramatically reduced by both the ablation of the fiber–CAR interaction and the use of a replaceable short fiber. In other tissues displaying a low level of transduction, no significant differences in transduction efficiency were observed among the Ad vector mutants. The natural tropism of the adenovirus in vivo is influenced not only by the fiber–CAR interaction but also by the fiber shaft length. Furthermore, their strategy may also be useful for retargeting adenoviruses to particular tumors and tissue types with specific receptors.

**Combination Approaches (Figure 3C)**

Bispecific antibody to fiber knob and TAA (Figure 3Ca). Haisma et al. (44) and Heideman et al. (45) have demonstrated tumor-specific gene transfer via an Ad vector targeted to an epithelial cell adhesion molecule (EpCAM). An anti-fiber knob Fab’ antibody conjugated to an anti-EpCAM Fab’ antibody was created so that it could target the Ad to the EpCAM antigen present on the tumor cells. The EpCAM antigen was chosen as the target because this antigen is highly expressed on a variety of adenocarcinomas of different origins such as breast, ovary, colon and lung. In these studies, the EpCAM-targeted Ad vector was shown to specifically infect the EpCAM-expressing cancer cell lines of a different origin. Gene transfer was blocked by an excess of anti-EpCAM antibody and it was thus dramatically decreased in the EpCAM-negative cell lines, thus showing the specificity of the EpCAM-targeted Ad vector. Importantly, the infection with the targeted Ad vector was independent of CAR, since blocking of CAR with recombinant fiber knob did not affect the infection with the targeted Ad. Apart from the cancer cell lines, the efficacy of a targeted viral infection was studied in freshly isolated primary human colon cancer cells. As colon cancer predominantly metastasizes to the liver, and adenoviruses have a high tropism for hepatocytes, they determined whether the EpCAM-targeted Ad vector showed a reduced infectivity to human liver cells. The bispecific antibody could successfully mediate gene transfer to primary human colon cancer cells, whereas it almost completely abolished the infection of the liver cells. Therefore, chemically prepared bispecific antibodies are versatile tools, but the production and purification of the conjugates pose problems of heterogeneity and are time consuming.
Nettelbeck et al. (46) have reported the retargeting of an adenoviral infection to melanoma by combining the genetic ablation of native tropism with a recombinant bispecific single-chain diabody (scDb) adapter that binds to the fiber knob and high molecular weight melanoma-associated antigen (HMWMAA). This strategy combines the genetic ablation of native adenoviral tropism with the redirected viral binding to melanoma cells via a bispecific adapter molecule, a bacterially expressed single-chain diabody, scDb MelAd, which binds to both the adenoviral fiber knob and to HMWMAA. The results showed specific and strong binding of the bispecific adapter scDb MelAd to the melanoma cells. In the adenoviral infection experiments, they demonstrated i) substantially reduced infectivity of capsid mutant adenoviruses, ii) restored, CAR-independent and HMWMAA-mediated infectivity of these mutant viruses by scDb MelAd specifically in melanoma cells, and iii) higher levels of transgene expression in melanoma cells by fiber mutant virus complexed with scDb MelAd, relative to a vector with wild-type fibers. Hence, the HMWMAA-targeted Ad vector lacking native tropism exhibited both enhanced specificity and augmented infectivity of gene transfer to melanoma cells, thus suggesting that it may be feasible to use this vector to improve gene therapy for malignant melanoma.

**Fusion protein of sCAR and scFv antibody to cell receptor (Figure 3Cb).** Kashentseva et al. (47) proposed the use of the soluble CAR (sCAR) ectodomain fused with a ligand to block CAR-dependent native tropism and simultaneously achieve infection through a novel receptor overexpressed in target tumor cells. In order to confer Ad vector-targeting capability on cancer cells expressing the HER-2 oncogene, they engineered a bispecific adapter protein, sCARIC6.5, which consisted of sCAR, phage T4 fibritin polypeptide, and the C6.5 scFv antibody against the HER-2 oncoprotein. They demonstrated that the sCARIC6.5 protein bound to the cellular HER-2 oncoprotein and mediated efficient adenovirus vector targeting via a CAR-independent pathway. The targeted Ad vector, complexed with the sCARIC6.5 adapter protein, provided a significant enhancement of gene transfer in comparison to the Ad vector alone and the untargeted Ad vector complexed with sCAR control protein. Therefore, the use of recombinant trimeric sCAR-scFv adapter proteins may augment Ad vector potency for targeting cancer cell types.

**Immunoglobulin-binding domain inserted fiber-knob protein (Figure 3Cc).** Recently, we have developed adenovirus vectors (Adv-FZ33) in which an IgG-binding domain was inserted into the fiber protein (48). A synthetic 33 amino acid IgG-binding domain (Z33), derived from staphylococcal protein A, was inserted into the HI-loop of the knob protein (49, 50). The modified fiber knob on Adv-FZ33 bound to immunoglobulins and allowed an antibody to redirect the vector to a new target molecule on the cell surface. In this strategy, the carcinoembryonic antigen (CEA) as the target molecule for cancer gene therapy was selected and the extent of retargeting toward and therapeutic effectiveness against CEA-positive gastric carcinomas was evaluated using the fully human CEA antibody (C2-45) complexed with the modified vector both in vitro and in vivo.

First, we constructed Ax3CAEGFP-FZ33, which carried the enhanced green fluorescent protein (EGFP) gene driven by the chicken beta-actin (CA) promoter, and then determined the specificity of the C2-45 antibody for CEA by flow cytometric analysis. Ax3CAEGFP-FZ33 alone or with a control mAb showed poor transduction of the CEA-CHO cells (CEA transfectant). However, Adv-FZ33 with C2-45 showed a strongly enhanced rate of transduction. In contrast, no significant effect on transduction efficiency was observed when the CHO cells (CEA non-transfectant) were exposed to Ax3CAEGFP-FZ33 with or without the antibody. In order to evaluate the ability of Adv-FZ33 with anti-CEA mAb to mediate tumor-specific gene therapy against established tumors, we generated Ax3CAUP-FZ33 (UP-
FZ33), which is an Adv-FZ33 expressing therapeutic gene, and the E. coli uracil phosphoribosyltransferase (UPRT), which converts 5-FU directly to 5-fluorouridine monophosphate. The sensitivity of human CEA-positive (MKN-45) and -negative (MKN-74) gastric cancer cell lines to 5-FU was evaluated using UP-FZ33. When the cells were infected with UP-FZ33 alone, the IC$_{50}$ of 5-FU did not change in the MKN-45 and MKN-74 cells. In MKN-45, UP-FZ33 with C2-45 significantly increased the sensitivity of these cells to 5-FU, with a 10.5-fold difference in IC$_{50}$ (0.081 µM with C2-45 versus 0.85 µM with the IgG4 antibody). The transduction of the UPRT gene restored drug sensitivity to cancer cells that show resistance to 5-FU. However, this approach may be unstable because when they are administrated systemically into the blood, Adv-FZ33 can infect target cells without reconstruction of the vector simply by changing the antibody against the specific targets.

**Conclusion**

Highly improved vector systems for adenovirus-based gene delivery have now been realized via tumor specific targeting strategies. However, to date, the biosafety of the modified vectors is still disputable. Further development of the targetable and injectable vectors will therefore determine the success of these gene therapy systems.

**References**


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