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ORIGINAL ARTICLE

Engineering conditionally replication-competent adenoviral vectors carrying the cytosine deaminase gene increases the infectivity and therapeutic effect for breast cancer gene therapy

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We constructed a conditionally replication-competent adenoviral vector Ad.Lp-CD-IRES-E1A(control) in which the expression of both the prodrug-activating cytosine deaminase gene and the viral replication E1A gene were driven by the L-plastin tumor-specific promoter. In order to overcome the low infectivity of the adenoviral vectors for breast cancer cells, and to increase the safety and efficacy for cancer gene therapy, this vector was further modified on a transductional level by simultaneously ablating the native tropism of the vector to the primary CAR receptor and inserting a RGD-4C peptide into the HI loop of the fiber, which allows the vector to use the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors as alternative receptors. The resulting vector was named Ad.Lp-CD-IRES-E1A(MRGD). The transduction efficiency of the vector for breast cancer cell lines which have low expression level of CAR was increased both *in vitro* and *in vivo*. The Ad.Lp-CD-IRES-E1A(MRGD) vector produces a higher vector particle yield and a greater cytotoxic effect in tumor cells which have a low expression level of CAR, than did the Ad.Lp-CD-IRES-E1A(control) vector. Intratumoral injection of the Ad.Lp-CD-IRES-E1A(MRGD) vector following the intraperitoneal injection of 5FC into xenotransplanted human breast cancer cell lines which have low expression level of CAR led to greater degree of tumor regression *in vivo* than did the intratumoral injection of control adenoviral vectors not so modified.
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Introduction

Cancer gene therapy is often carried out by the introduction of specific genes into cancer host cells that destroy cancer or that will enhance the immune response against cancer cells. Viruses are frequently chosen as gene therapy vectors for this purpose based on their unparalleled ability to deliver and express genetic elements. A number of important biological features such as the stability of viral particles and the high infection efficiency for quiescent as well as dividing cells make the adenoviral vector one of the best gene delivery vectors developed so far.[1]

For safety reasons, the first generation of adenoviral vectors used clinically were replication deficient. However, the success of these replication-deficient vectors for cancer gene therapy is limited by their inability to spread and infect tumor cells subsequent to the initial infection. The recognition of this gene transfer limitation as a major problem for clinical gene therapy has led to the development of replication-competent adenoviral vectors for clinical trials.[2] The greatest challenge of developing replication-competent adenoviral vectors is to deliver therapeutic genes to the sites of the disease and replicate them in a safe and efficient manner. In this regard, conditionally replication-competent adenoviral (CRAD) vectors are considered very promising.

There are two main strategies used for constructing CRAD vectors: (1) targeting the vector on the transcriptional level such that vector replication and transgene expression will occur only in cancer tissue, and (2) targeting the vector on the level of cellular binding and infection (transduction), which allows the vector to bind selectively to a tumor-specific cell surface by modifying the viral attachment protein.[3] Targeting on a transcriptional level can be achieved by using a tumor-specific promoter which drives critical viral genes so that they can be expressed only in tumor cells.[4]

The primary receptor for adenovirus serotypes 2 and 5 is the coxsackie-adenovirus receptor (CAR),[5] which is widely expressed in normal tissues. However the expression level of CAR is highly variable and often low on the surface of cancer cells, including breast cancer cells. Targeting on a cell transductional level can be accomplished by simultaneously ablating the native viral element which binds to CAR and redirecting the viral-binding protein to alternative cellular receptors which are

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present at high levels on cancer cells, like $\alpha_v\beta_3$ integrin. The use of a transductional-targeted vector, which binds only to cancer cells may generate greater therapeutic benefit because the gene delivery to normal tissues is reduced. This decreases the viral dose required, the immune response to the vector, and reduces the toxicity of the vector to noncancerous tissues.

Many strategies have been used for generating transductional-targeted adenoviral vectors: (1) bifunctional antibodies which block the vector's native tropism to CAR and redirect the vector to other cellular receptors;[6] and (2) genetic modification of the vector to eliminate native receptor interactions and redirect the vector to a novel receptor. The genetic modification method may be better than the bifunctional antibody approach since the vector is more stable *in vivo* and easier to produce on the large scale needed for therapy. Moreover, only genetically modified vectors still contain the necessary modifications in further rounds of infection. However, it is often difficult to engineer the fiber protein of adenoviral vectors with large molecules without decreasing the packaging efficiency of the virus.

Suicide/prodrug gene therapy holds great promise for the treatment of some forms of cancer.[7,8] The introduction of suicide genes into CRAD vectors produces a therapeutic tumor suppressive effect which is greater than vectors with either viral replication genes or suicide genes alone.[9–11] The combination of transcriptional and transductional targeting approaches may generate a safer vector which displays a higher degree of specificity.[12–14] We have previously demonstrated that a truncated form of the L-plastin promoter can confer tumor-specific patterns of expression on replication-incompetent adenoviral vector reporter genes and therapeutic transcription units.[15,16] We also constructed the Ad.Lp-E1A CRAD vector in which the E1A expression was driven by the L-plastin promoter. The Ad.Lp-E1A vector was shown to be cytotoxic to established human breast cancer cell lines but was not toxic to normal human mammary epithelial cells. We then further improved this CRAD vector by adding a therapeutic cytosine deaminase (*CD*) prodrug activation transcription unit to the Ad.Lp-E1A vector. The resulting vector Ad.Lp-CD-IRES-E1A encodes the *CD* prodrug activation transcription unit and the E1A gene, the expression of which is driven by the L-plastin promoter. These modifications have been shown to increase the therapeutic effect over that seen with the replication-deficient Ad.Lp-CD vector without increasing toxicity.[17]

In the study reported in the present paper, we investigated whether the combination of transductional targeting and transcriptional targeting in one CRAD vector, which carries a prodrug activation gene as well as the viral E1A gene under control of the L-plastin promoter, could improve the safety and therapeutic effect of the vector in breast cancer cell lines. To circumvent the low level of CAR on the plasma membranes of many different types of cancer cells so as to obtain a higher infection efficiency, we modified the fiber protein of the L-plastin series of CRAD vectors by inserting the RGD-containing peptide into the HI loop of the fiber knob domain. In addition, to obtain truly targeted CRAD vectors, we ablated the CAR-binding ability of the L-plastin series of CRAD vectors by introducing a two-site mutation in the AB loop of the fiber. This dual targeted vector was shown to increase the transduction efficiency and specificity for breast cancer cell lines and to exert a greater suppressive effect on the growth of breast cancer in human tumor xenograft models.

Materials and methods

Cell lines

The 293 human embryonic kidney cell line, the MDA-MB-468 and the MCF-7 human breast cancer cell line were all purchased from the American Type Culture Collection (ATCC, Manassas, VA). The MDA-MB-435 human breast cancer cell line was kindly provided by Dr Erkki Ruoslahti of the Burnham Institute (San Diego, CA). Normal human mammary epithelial cells (HMEC) were purchased from Clonetics (Walkersville, MD). The HEK-293 and MDA-MB-435 cell lines were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) purchased from Hyclone (Logan, UT). The MCF-7 cell line was grown in EMEM (ATCC) supplemented with 0.01 mg/ml insulin and 10% FBS. The HMEC was grown in mammary epithelial basal medium supplemented (Clonetics) with hydrocortisone, insulin, epidermal growth factor and bovine pituitary extract. All the cell lines were maintained in a 5% CO_2, humidified tissue culture incubator at 37°C.

Plasmids and viruses

The plasmids pShuttleCMV-Luciferase, pAd(RGD), pCR-5.9, pCR-BE and pCR-1.1(RGD) were constructed as described previously.[18] pCR-1.1(RGD) contained a complete sequence from nt 31950 to nt 33125 of the Ad5 genome and an additional RGD-4C sequence CDCRG DCFC in the HI loop. PCR-1.1(RGD) was used as a template to further modify the fiber. Primer 1: 5′ CAACA AAGGCCTTTACTTGTTTACAGCTTCA 3′ and primer 2: 5′ GTTAGCCTCTGGAGCTGGTGTGGTC 3′ were used to amplify the 321 bp PCR1 fragment from pCR-1.1; primer 3: 5′ CCAGAGGCTAACTGTAGACT AAATGC 3′ and primer 4: 5′ TGGACACGACATGAA CTTAAGTGAGCTG 3′ were used to amplify the 800 bp PCR2 fragment from pCR-1.1. PCR1 and PCR2 were gel purified, mixed and joined by PCR using primers 1 and 4. The pCR-1.1(MRGD) plasmid was generated by inserting the new 1.1 kb PCR band into the pCR-Blunt vector. The pCR-1.1(MRGD) contained the same knob sequence of Ad5 as pCR-1.1(RGD) except that mutations from TCTCCT to GAGGCT (underlined above) which generated amino-acid mutation S408E and P409A were introduced.

The *Stu*I/*Afl*II fragment of the pCR-5.9 plasmid was replaced by the *Stu*I/*Afl*II fragment of the pCR-1.1(MRGD) plasmid. The resulting plasmid pCR-5.9(MRGD) was digested by *EcoR*I and the 5.9 kb band

generated was cloned into the *EcoR*I digested pCR-BE plasmid, which generated plasmid pCR-BE(MRGD). The 11.7 kb *BamH*I band from the pCR-BE(RGD) plasmid was ligated with a 21.7 kb *BamH*I fragment of pAdeasy-1.[19] The resulting 33.4 kb plasmid was named pAd(MRGD). It contained the pAdeasy-1 sequence plus mutations from TCTCCT (nt 32263 to nt 32268) to GAGGCT, and an additional RGD-4C sequence in the HI loop (between nt 32679 and nt 32680).

The pShuttleLp plasmid was constructed by inserting the 2.4 kb *Not*I/*Xho*I fragment from pShuttleLp-CD-IRES-E1A into pShuttle.[19] The 1.9 kb *Xho*I/*Sal*I fragment from the pGL3-Basic vector (Progema, Madison, WI) was inserted into pShuttleLp. The resulting vector was named pShuttleLp-Luciferase. The pShuttleLp-Luciferase and pShuttleCMV-Luciferase plasmids were used for homologous recombination with the pAd(MRGD) in the bacterial strain BJ5183. The recombinant plasmids were digested with *Pac*I and transfected, respectively, into 293 cells. The resulting viral vectors were named Ad.Lp-Luciferase(MRGD) and Ad.CMV-Luciferase(MRGD). Ad.CMV-Luciferase(control), Ad.CMV-Luciferase(RGD) and Ad.CMV-E1A vector were constructed as described previously.[18]

The shuttle plasmids for generating the Ad.Lp-E1A (pShuttleLp-E1A) and Ad.Lp-CD-IRES-E1A (pShuttleLp-CD-IRES-E1A) vectors were constructed as described previously.[17] Ad.Lp-E1A(control), Ad.Lp-E1A(RGD) and Ad.Lp-E1A(MRGD) vectors were obtained by homologous recombination between the pShuttleLp-E1A and the pAdeasy-1, the pAd(RGD) or the pAd(MRGD) plasmids, followed by *Pac*I digestion and transfection into 293 cells. The Ad.Lp-CD-IRES-E1A(control), Ad.Lp-CD-IRES-E1A(RGD) and Ad.Lp-CD-IRES-E1A(MRGD) vectors were obtained by the same method except that the homologous recombination was carried out between the pShuttleLp-CD-IRES-E1A and the pAdeasy-1, the pAd(RGD) or the pAd(MRGD) plasmids.

All the vectors were amplified and purified following the methods described previously.[18] The titers of different vectors were tested by the BD Adeno-X™ Rapid Titer Kit (BD biosciences, Palo Alto, CA). The viral DNA was isolated from purified stocks and PCR was used for detecting wild-type virus and modified sequence according to methods previously described.[18] All the PCR products were sequenced.

Flow cytometry analysis of cell receptors

The mouse anti-CAR monoclonal antibody RmcB (Upstate Biotechnology, Lake Placid, NY), anti-$\alpha_v\beta_3$ monoclonal antibody LM609 (Chemicon International, Temecula, CA), anti-$\alpha_v\beta_5$ monoclonal antibody P1F6 (Chemicon International), and fluorescein-conjugated sheep anti-mouse IgG antibody (Amersham Biosciences, Piscataway, NJ) were used to detect the density of the human CAR, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors on the test cells following the method described previously.[18] Normal murine IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) served as control.

Ad-mediated in vitro *gene transfer assay*

The Ad.CMV-Luciferase(control), Ad.CMV-Luciferase(RGD), Ad.CMV-Luciferase(MRGD) and Ad.Lp-Luciferase(MRGD) vectors were used to infect cell lines. The infectivity of the vectors with modified or wild-type fibers in different cell lines was determined by measuring luciferase activity. In brief, subconfluent cell monolayers in 24-well plates were exposed to the Ad.CMV-Luciferase(control), Ad.CMV-Luciferase(RGD) or Ad.CMV-Luciferase(MRGD) vectors, respectively, at MOI 5 (ifu/cell) for 2 h at 37°C, followed by 48 h incubation. After that, cells were washed once with PBS. The cells were then lysed and a luciferase assay was performed with the Luciferase Assay System (Promega, Madison, WI) following the manufacturer's protocol. The protein concentration of the cell lysates was determined by using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA) to normalize the value of the relative light units by the protein concentration for each sample.

Competitive inhibition assay

MDA-MB-468 or MDA-MB-435 cells were cultured in 24-well plates at a density of 5×10^4 cell/well. The second day after cells were plated, Ad.CMV-Luciferase(control) or Ad.CMV-Luciferase(MRGD) vectors were used to infect cells at MOI 5 for competitive inhibition assay. Vectors were preincubated with 2 μg purified human integrin $\alpha_v\beta_3$ protein (Chemicon International, Temecula, CA) for 30 min at room temperature, respectively, then used to infect cells for 2 h at 37°C, followed by 48 h incubation. After that, the cells were processed for luciferase assay as described above. Vectors without preincubation were used to infect same cell lines at the same MOI at the same time as control.

Ad-mediated in vivo *gene transfer assay*

Tumors of the MDA-MB-468 and MDA-MB-435 cell lines were established by subcutaneously injecting, respectively, 2×10^6 MDA-MB-468 or 2×10^6 MDA-MB-435 human breast cancer cells into nude mice (Harlan Sprague Dawley Company, Indianapolis, IN) aged 5–6 weeks. The Ad.CMV-Luciferase(control), Ad.CMV-Luciferase(RGD), Ad.CMV-Luciferase(MRGD) or Ad.Lp-Luciferase(MRGD) vectors (1×10^9 ifu in each case) were injected intratumorally in a total volume of 50 μl when the subcutaneous tumor nodules reached approximately 60 mm^3 in size. The tumors were harvested and snap-frozen 2 days later. The tumors were ground to frozen powder and luciferase activity was analyzed as before.[18] The mean background luciferase activity from tumors without vector injection was subtracted from all the experimental results from tumor injected with the luciferase vectors.

Cytotoxicity assay

Different tumor cell lines were seeded in 96-well plates (5×10^3 cells/well) and infected, respectively, with vectors at MOI 5 (ifu/cell). At 24 h after vector infection, sufficient 5-fluorocytosine (5-FC) (Sigma, St Louis,

MO) was added to each well of the cells infected with the Ad.Lp-CD-IRES-E1A series of vectors to generate a 0.5 mM concentration. Then the cells were incubated for another 7 days. Cell survival was determined using the MTS cytotoxicity assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay, Promega) according to the manufacturer's instructions. The percentage of surviving cells is calculated as the ratio of the absorbance at 490 nm in the test culture to that of the culture without added vectors. All experiments were carried out in triplicate.

Viral yield assay

Different cell lines grown as monolayers in 12-well plates were infected with a series of vectors at MOI 1. At day 7, the cell-free supernatant medium was collected. The titer of vectors in the supernatant medium was then determined by using the BD quick titer kit on 293 cells.

Tumor models and intratumoral injections

Two nude mouse models were used to evaluate the antitumor effect of Ad.Lp-CD-IRES-E1A(MRGD) vector *in vivo*. The subcutaneous MDA-MB-468 and the MDA-MB-435 human breast cancer tumor models were established by the method mentioned above. Tumor nodules were allowed to grow subcutaneously to approximate 60 mm^3 in size. In each tumor model, the mice were randomly divided into four groups. The Ad.Lp-Luciferase(MRGD), Ad.Lp-CD-IRES-E1A(control) or Ad.Lp-CD-IRES-E1A(MRGD) vectors (1×10^9 ifu in each case) were injected intratumorally into four mice in a total volume of 50 μl into each treatment group, and with phosphate-buffered saline (PBS) for the control group. The second day after vector injection, 500 mg/kg of 5-FC was injected into the peritoneal cavity each day for 7 days. The tumor size was measured every 5 days and the tumor volume was calculated according to the formula: tumor volume = length $\times$ width^2/2.

Results

Construction of adenoviral vectors

The 2.4 kb truncated form of the L-plastin promoter, from nt -2265 of the 5′ region of the L-plastin promoter to nt $+18$ from the transcription initiation site of the L-plastin gene,[15] was used to replace the original E1A promoter. The resulting conditionally replication-competent vector was named Ad.Lp-E1A(control) which contained wild-type fiber. The Ad.Lp-E1A(RGD) vector has the same structure as the Ad.Lp-E1A(control) vector except for the presence of a RGD-4C peptide inserted into the HI loop of the fiber region. The Ad.Lp-E1A(MRGD) has the same structure as the Ad.Lp-E1A(RGD) vector except for two mutations: S408E and P409A in the AB loop of the fiber. The introduction of these two mutations ablated the CAR-binding ability of the vector.

The Ad.Lp-CD-IRES-E1A(control) vector was a replication-competent vector with a wild-type fiber in which the expression of both the *CD* and *E1A* gene was driven by the L-plastin tumor-specific promoter. The Ad.Lp-CD-IRES-E1A(RGD) vector has the same structure as the Ad.Lp-CD-IRES-E1A(control) vector except for a RGD-4C peptide inserted into the HI loop of the fiber protein. The Ad.Lp-CD-IRES-E1A(MRGD) vector has the same structure as the Ad.Lp-CD-IRES-E1A(RGD) vector except for two mutations: S408E and P409A in the AB loop of the fiber region.

The Ad.CMV-Luciferase(control), Ad.CMV-Luciferase(RGD) and Ad.CMV-Luciferase(MRGD) vectors were replication-deficient adenoviral vectors in which the reporter gene luciferase was under the control of the CMV promoter each with a different fiber gene. The Ad.Lp-Luciferase(MRGD) vector was a replication-deficient adenoviral vector in which the luciferase gene was driven by the L-plastin promoter and the fiber was modified by inserting the RGD-4C peptide into the HI loop and creating mutations in the AB loop that ablated the CAR-binding ability of the vector. The characteristics of all of these different vectors are described in Table 1.

Table 1 The characteristics of different vectors

Vectors	*Promoter*	*Foreign gene*	*Fiber*	*Replication potential*
Ad.Lp-E1A(control)	L-plastin	E1A	Wild type	RC
Ad.Lp-E1A(RGD)	L-plastin	E1A	Knob(+)RGD(+)	RC
Ad.Lp-E1A(MRGD)	L-plastin	E1A	Knob(−)RGD(+)	RC
Ad.Lp-CD-IRES-E1A(control)	L-plastin	CD+E1A	Wild type	RC
Ad.Lp-CD-IRES-E1A(RGD)	L-plastin	CD+E1A	Knob(+)RGD(+)	RC
Ad.Lp-CD-IRES-E1A(MRGD)	L-plastin	CD+E1A	Knob(−)RGD(+)	RC
Ad.CMV-E1A	CMV	E1A	Wild type	RC
Ad.Lp-Luciferase(MRGD)	L-plastin	Luciferase	Knob(−)RGD(+)	RD
Ad.CMV-Luciferase(control)	CMV	Luciferase	Wild type	RD
Ad.CMV-Luciferase(RGD)	CMV	Luciferase	Knob(+)RGD(+)	RD
Ad.CMV-Luciferase(MRGD)	CMV	Luciferase	Knob(−)RGD(+)	RD

RC = replication competent; RD = replication deficiency.
Knob(+): with CAR-binding ability; Knob(−): without CAR-binding ability.
RGD(+): with RGD-4C peptide inserting.

The presence of the modified sequences and the absence of contamination with Ad5 wild-type virus were confirmed by PCR and by DNA sequencing.

Analysis of the expression level of the adenoviral vector cell surface attachment receptor CAR and the $\alpha_v\beta_3$, $\alpha_v\beta_5$ internalization receptors in breast cancer cell lines

Adenoviral vector-mediated transducing efficiency depends on the presence of the primary receptor CAR,[5] which mediates the attachment of the vector to the target cell, and the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors. These integrin receptors mediate the internalization of the vector and the release of the vector from the endosome.[20] We analyzed the expression level of CAR, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in three human breast cancer cell lines MDA-MB-468, MDA-MB-435 and MCF-7, in normal human mammary epithelial cells (HMEC), and the 293 cell line by flow cytometry. The expression level of CAR was low in the MDA-MB-435 and MCF-7 breast cancer cell lines, whereas the MDA-MB-468 breast cancer cell line, the HMEC and the 293 cell line had a high level of expression of the CAR (see Table 2). Almost all of the tested breast tumor cell lines, with the exception of MDA-MB-468, expressed both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ at relatively high levels. Both the HMEC and the 293 cell line have a relatively high expression level of CAR and low expression level of $\alpha_v\beta_3$ (see Table 2).

The transductional efficiency of adenoviral vectors with different fiber modifications or with L-plastin promoter in breast cancer cell lines

We hypothesized that the differential expression levels of the CAR, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors would correlate with infectivity by adenoviral vectors. Cell lines with different expression levels of CAR were infected by Ad.CMV-Luciferase(control) at MOI 5. Luciferase activity was measured 48 h after infection. The luciferase expression level correlated with the expression level of the CAR in Ad.CMV-Luciferase(control)-infected cell lines (see Table 2 and Figure 1).

We then evaluated whether modification of the fiber can change the transduction efficiency in cell lines. The Ad.CMV-Luciferase(RGD) vector that contained a RGD-4C peptide in the HI loop of the fiber uses both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors and CAR to infect cells. As expected, the Ad.CMV-Luciferase(RGD) vector increases transduction efficiency in almost all the cell lines tested. The luciferase activity in cell lines infected by the Ad.CMV-Luciferase(RGD) vector was 24-, 5-, 1.2- and 1.1-fold higher in MDA-MB-435, MCF-7, MDA-MB-468 and HMEC cells, respectively, than was the case when the Ad.CMV-Luciferase(control) vector infected the same cells at the same MOI (see Figure 1).

The Ad.CMV-Luciferase(MRGD) vector contained both a RGD-4C peptide in the HI loop and S408E and P409A mutations in the AB loop. Thus, this vector uses $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors exclusively to infect cells but could not use CAR to infect cells. As shown in Figure 1, the luciferase activity was 22-fold and fivefold higher, respectively, following infection at MOI 5 with the Ad.CMV-Luciferase(MRGD) vector than was the case of the infection at MOI 5 with the Ad.CMV-Luciferase(control) vector in the MDA-MB-435 and MCF-7 cell lines, which contained a low expression level of CAR and a high expression level of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors (see Table 2). The luciferase activity in the HMEC and MDA-MB-468 cells, which had a high expression level of CAR, and a low expression level of the $\alpha_v\beta_3$ integrin receptor, was 6.7- and 2.9-fold lower compared with that seen with the Ad.Luciferase(control) vector at the same MOI (see Figure 1 and Table 2). Thus, the expression level of primary receptor CAR appears to be the major factor determining the efficiency of Ad-mediated gene transfer in

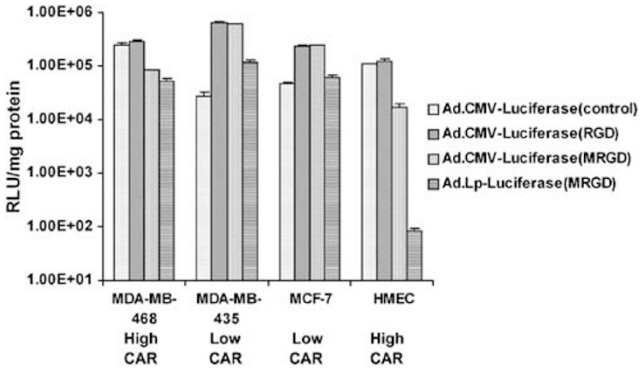


Figure 1 Transduction efficiency of adenoviral vectors with different fiber modifications in established tumor cell lines. At 48 h after *in vitro* infection at MOI 5 (ifu/cell), the transduction efficiency was determined by measuring luciferase activity, which is expressed as relative light units (RLU) normalized for total protein concentration. The data shown represent the mean ± s.d. of three experiments.

Table 2 Expression of CAR, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in cell lines and luciferase level after exposure to the Ad.CMV-luciferase(control) vector

Cell lines	*CAR (%)*	*$\alpha_v\beta_3$ (%)*	*$\alpha_v\beta_5$ (%)*	*Luciferase level (RLU/mg protein)*
MDA-MB-468	83.28 ± 8	1.36 ± 1	81 ± 7	$2.5 \times 10^5 \pm 33\,918$
MDA-MB-435	0.26 ± 1	59.61 ± 4	79.09 ± 3	$2.73 \times 10^4 \pm 4489$
MCF-7	1.04 ± 1	5.56 ± 2	79.72 ± 5	$4.8 \times 10^4 \pm 1781$
HMEC	47.44 ± 6	3.21 ± 3	27.09 ± 4	$1.3 \times 10^5 \pm 25\,463$
293	69.76 ± 5	1.07 ± 1	73.98 ± 8	Not done

Correlations between the percentage of CAR, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptor-positive cells and the luciferase level after exposure to the Ad.CMV-Luciferase(control) vector. The data shown represents the mean ± s.d. of three experiments.

cells with a high level of expression of CAR, whereas RGD modification of the fiber in the adenoviral vector increases the transduction efficiency of cancer cells which have high expression level of $\alpha_v\beta_3$ integrin receptor. Most importantly, the combination of inserting the RGD peptide into the fibrillar protein and mutational inactivation of the binding of the fibrillar protein to the CAR receptor creates a vector which is selective for cells that have a low expression level of CAR but a high expression level of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors. The transduction efficiency was decreased in the HMEC and MDA-MB-468 cell line that have a high expression level of CAR (Figure 1). Since breast cancer cells often have a low CAR and a high $\alpha_v\beta_3$ expression level, the MRGD vector may be an ideal one for breast cancer gene therapy in human subjects.

We then evaluated L-plastin promoter activity in normal cells and in breast cancer cells. The Ad.Lp-Luciferase(MRGD) vector was used to infect MDA-MB-435, MDA-MB-468, MCF-7 breast cancer cell lines and HMEC. The luciferase activity in cell lines infected by the Ad.Lp-Luciferase(MRGD) vector was 5.1-, 4.0-, 1.6- and 195-fold lower in MDA-MB-435, MCF-7, MDA-MB-468 and HMEC cells, respectively, than was the case following infection of the same cells with the Ad.CMV-Luciferase(MRGD) vector at the same MOI (see Figure 1). Although the L-plastin promoter is not as strong as CMV promoter in breast cancer cell lines, it has a very high selectivity for breast cancer cell lines, as measured by induction of gene expression.

Human integrin $\alpha_v\beta_3$ protein inhibits Ad.CMV-Luciferase(MRGD) mediated gene transfer in vitro

We next investigated if the levels of expression of the luciferase gene inserted in breast cancer cell lines following their exposure to the Ad.CMV-Luciferase(MRGD) vector can be inhibited by adding to the vector the human integrin $\alpha_v\beta_3$ protein prior to adding the vector to the cultures of the human breast cancer cell lines. We preincubated Ad.CMV-Luciferase(MRGD) or Ad.CMV-Luciferase(control) vectors with 2 μg of human integrin $\alpha_v\beta_3$ protein and then used these vectors in a gene transfer assay which was evaluated by luciferase activity. The results are shown in Figure 2. Preincubation with human $\alpha_v\beta_3$ did not affect the transduction efficiency of the Ad.CMV-Luciferase(control) in the MDA-MB-468 human breast cancer cell line but it caused a 2.6-fold decrease of luciferase activity in the MDA-MB-435 cell line compared with that without $\alpha_v\beta_3$ preincubation (see Figure 2). Preincubation with $\alpha_v\beta_3$ decreased the transduction efficiency of the Ad.CMV-Luciferase(MRGD) vector in both tumor cell lines: luciferase activity was 9.0- and 7.5-fold lower in Ad.CMV-Luciferase(MRGD)-infected MDA-MB-468 and MDA-MB-435 cell lines, respectively, than was the case when the Ad.CMV-Luciferase(MRGD) vector infected the same cells at the same MOI without $\alpha_v\beta_3$ preincubation (see Figure 2). These results show that the Ad.CMV-Luciferase(MRGD)-mediated transduction is more dependant on the interaction of RGD peptide with integrin receptors than the Ad.CMV-Luciferase(control) vector.

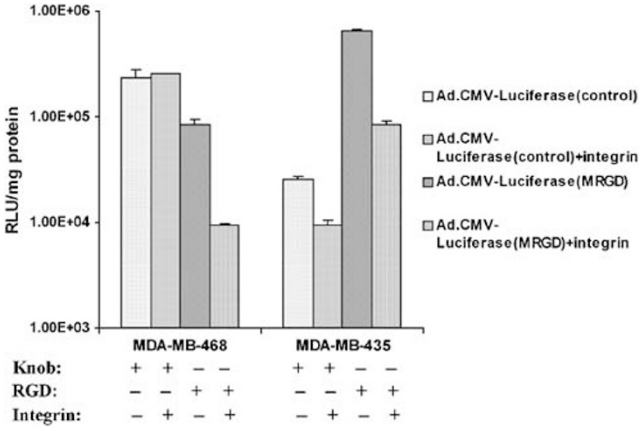


Figure 2 Competitive inhibition assays with human $\alpha_v\beta_3$ protein in tumor cell lines. MDA-MB-468 (high CAR) and MDA-MB-435 (low CAR) tumor cell lines were infected *in vitro* by Ad.CMV-Luciferase (control) or Ad.CMV-Luciferase(MRGD) vectors in the absence or presence of human integrin $\alpha_v\beta_3$. The transduction efficiency was measured by luciferase activity. The data shown represent the mean $\pm$ s.d. of three experiments.

The in vivo *transduction efficiency of adenoviral vectors with different fiber modifications*

To evaluate whether modifying the fiber of adenoviral vectors can change the efficiency of gene delivery *in vivo* and whether the L-plastin promoter can induce expression of transgenes efficiently in tumor cells *in vivo*, the subcutaneous MDA-MB-468 and the MDA-MB-435 breast cancer human tumor xenograft models were used to test the transduction efficiency of adenoviral vectors carrying different fiber genes *in vivo*. The Ad.Lp-Luciferase(MRGD), Ad.CMV-Luciferase(control), Ad.CMV-Luciferase(RGD) and Ad.CMV-Luciferase (MRGD) vectors were injected intratumorally into established subcutaneous tumors. The tumors were harvested 2 days later and the luciferase activity was analyzed. As shown in Figure 3, the Ad.CMV-Luciferase(RGD), Ad.CMV-Luciferase(MRGD) and Ad.Lp-Luciferase(MRGD) vectors increased the transduction efficiency in MDA-MB-435 (low CAR) tumor cell line as compared with the Ad.CMV-Luciferase(control) vector. In contrast, the *in vivo* transduction efficiency of the Ad.CMV-Luciferase(MRGD) vector was decreased 2.4-fold compared with that of the Ad.CMV-Luciferase(control) vector in MDA-MB-468 tumor model. The *in vivo* transduction efficiency of the Ad.Lp-Luciferase(MRGD) vector was decreased 2.4-fold compared with the Ad.CMV-Luciferase(MRGD) vector in the MDA-MB-468 (high CAR) tumor cell line (see Figure 3). These results, which show that the transduction efficiency is increased for the Ad.CMV-Luciferase(MRGD) vector vs the Ad.CMV-Luciferase(control) vector in cell lines which have low levels of CAR, are consistent with the results of *in vitro* experiments (see Figure 1 and Table 2).

Cytotoxicity of different CRAD vectors in cell lines in vitro

A series CRAD vectors [Ad.Lp-E1A(control), Ad.Lp-E1A(RGD), Ad.Lp-E1A(MRGD), Ad.Lp-CD-IRES-E1A(control), Ad.Lp-CD-IRES-E1A(RGD), Ad.Lp-CD-IRES-E1A(MRGD) and Ad.CMV-E1A] was used for *in vitro* infection of the MDA-MB-435, MCF-7 and MDA-MB-468 human breast cancer cell lines as well as the infection of HMEC at MOI 5. Cytotoxicity was detected by the MTS cytotoxicity assay, which reflects the number of living cells at a specific time after infection relative to the uninfected control. The data are shown in Table 3.

In the absence of 5FC, the direct oncolytic effect of the Ad.Lp-CD-IRES-E1A series vectors was less than the Ad.Lp-E1A series vectors in the tested breast cancer cell lines. In contrast, when cell lines were incubated in medium supplemented with 0.5 mM 5FC after exposure to the Ad.Lp-CD-IRES-E1A series vectors, the cytotoxic effect of these vectors was increased. The RGD modification of the fiber can increase the cytotoxicity of the adenoviral vectors for breast cancer cells. As shown in Table 3, the MRGD series CRAD vectors have a higher cytotoxic effect *in vitro* compared with that of the CRAD vectors, which have the same transcription units but with a wild-type fiber in breast cancer cell lines which have a low expression level of CAR (MDA-MB-435 and MCF-7). The MRGD series CRAD vectors also have the comparable cytotoxic effect in the MDA-MB-468 cell line, which has a high expression level of CAR. All of the conditionally replicate-competent adenoviral vectors, which contain the L-plastin tumor-specific promoter have little cytotoxic effect in HMEC whereas the Ad.CMV-E1A vector in which E1A gene was driven by the CMV nonspecific promoter has a very strong cytotoxic effect in HMEC (see Table 3). The percentage of surviving cells after exposure to the Ad.CMV-Luciferase(control) vector or 5FC alone was more than 92% (data not shown).

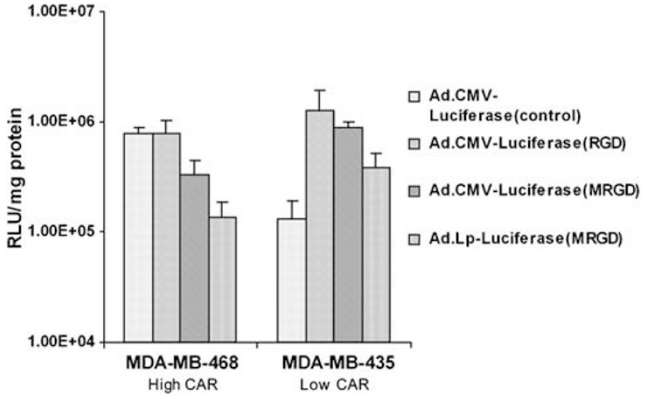


Figure 3 Transgene expression level in human breast cancer xenograft model. Subcutaneous human breast cancer MDA-MB-468 (high CAR) and MDA-MB-435 (low CAR) tumor cell lines were injected intratumorally with 1×10^9 ifu of the Ad.CMV-Luciferase (control), Ad.CMV-Luciferase(RGD), Ad.CMV-Luciferase(MRGD) and Ad.Lp-Luciferase(MRGD) vectors. Luciferase activity was analyzed. The relative light units (RLU) were normalized for protein concentration. The data presents the mean of RLU of three animals $\pm$ s.d.

Viral yield of different CRAD vectors in breast cancer cell lines in vitro

To test the replication ability of the Ad.Lp-E1A(control), Ad.Lp-E1A(RGD), Ad.Lp-E1A(MRGD), Ad.Lp-CD-IRES-E1A(control), Ad.Lp-CD-IRES-E1A(RGD) and Ad.Lp-CD-IRES-E1A(MRGD) vectors, tumor cells were infected by each test vector at MOI 1, and the cell-free supernatant medium was titered on 293 cells 7 days after infection. The results are shown in Figure 4. The viral yield of the Ad.Lp-CD-IRES-E1A series CRAD vectors is less than that of the Ad.Lp-E1A series CRAD vectors. The reason for this may be in part due to the fact that the L-plastin promoter drives both the *CD* and *E1A* gene expression in these vectors and the E1A gene is 3′ to the *CD* gene and the IRES. The expression level of *E1A* on

Table 3 *In vitro* cytotoxicity test

Vectors	*Percentage of tumor cells alive after treatment*			
	MDA-MB-468	*MDA-MB-435*	*MCF-7*	*HMEC*
Ad.Lp-E1A(control)	65% $\pm$ 0.03	76% $\pm$ 0.03	79% $\pm$ 0.03	89% $\pm$ 0.01
Ad.Lp-E1A(RGD)	52% $\pm$ 0.01	62% $\pm$ 0.02	71% $\pm$ 0.01	92% $\pm$ 0.02
Ad.Lp-E1A(MRGD)	66% $\pm$ 0.03	68% $\pm$ 0.01	71% $\pm$ 0.03	90% $\pm$ 0.02
Ad.CMV-E1A	47% $\pm$ 0.03	63% $\pm$ 0.01	57% $\pm$ 0.01	49% $\pm$ 0.04
Ad.Lp-CD-IRES-E1A(control)	68% $\pm$ 0.01	81% $\pm$ 0.04	86% $\pm$ 0.03	88% $\pm$ 0.01
Ad.Lp-CD-IRES-E1A(RGD)	67% $\pm$ 0.05	76% $\pm$ 0.01	79% $\pm$ 0.01	89% $\pm$ 0.01
Ad.Lp-CD-IRES-E1A(MRGD)	73% $\pm$ 0.03	76% $\pm$ 0.01	76% $\pm$ 0.04	92% $\pm$ 0.06
Ad.Lp-CD-IRES-E1A(control)+5FC	41% $\pm$ 0.04	59% $\pm$ 0.01	51% $\pm$ 0.03	90% $\pm$ 0.02
Ad.Lp-CD-IRES-E1A(RGD)+5FC	38% $\pm$ 0.05	36% $\pm$ 0.02	44% $\pm$ 0.04	91% $\pm$ 0.02
Ad.Lp-CD-IRES-E1A(MRGD)+5FC	39% $\pm$ 0.01	37% $\pm$ 0.02	42% $\pm$ 0.02	92% $\pm$ 0.01

Different tumor cell lines were seeded in 96-well plates and were infected by a series of CRAD vectors at MOI 5. The survival of cells was measured by MTS assay 7 days later. The data represents the average of three determinations.

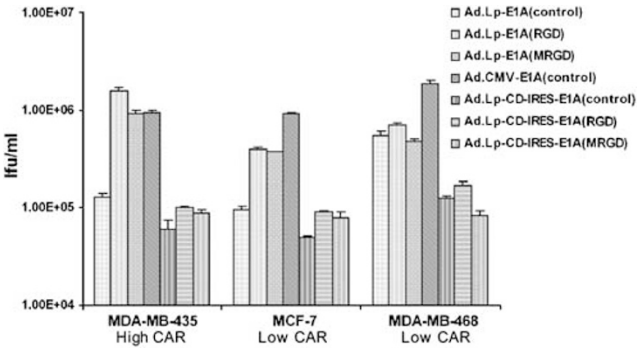


Figure 4 Viral yield. Cells were plated in 12-well plates and infected by the L-plastin series of CRAD vectors at MOI 1(ifu/cell), respectively. After 7 days, the supernatant medium was collected and tested for virus production by the BD quick titer kit on 293 cells following the manufacturer's protocol. Viral titers are shown in ifu/ml. The results are presented as a mean of duplicate experiments. The bars represent the s.d.

the 3′ end of the IRES is usually weak compared with the expression level of *E1A* gene that is directly driven by L-plastin promoter. The RGD modification increased the viral yield of all the tested vectors in all of the cell lines. The MRGD series of vectors has an increased viral yield compared with that of the wild-type control vector in cell lines that have a lower expression level of CAR, such as the MDA-MB-435 and MCF-7 breast cancer cell lines. In contrast, the MRGD series of vectors generates a decreased viral yield in the human breast cancer cell line MDA-MB-468, which has a high expression level of CAR as expected (see Figure 4).

Antitumor efficiency of the Ad.Lp-CD-IRES-E1A(MRGD) vector in vivo

Based on the cytotoxicity assay results, the Ad.Lp-CD-IRES-E1A(MRGD) vector, which contained transcriptional level targeting and transductional level targeting seems to be the ideal vector since it shows an increased cytotoxic effect *in vitro* with 5FC and shows a dramatically reduced level of toxicity for normal cells. We carried out animal experiments to test if the Ad.Lp-CD-IRES-E1A(MRGD) vector suppressed the growth of the breast cancer cell lines *in vivo*. Subcutaneous tumor nodules derived from the MDA-MB-468 (high CAR) or the MDA-MB-435 (low CAR) human breast cancer cell lines growing in nude mice were subjected to intratumoral injection of 1×10^9 ifu of the Ad.Lp-CD-IRES-E1A(MRGD) vector, Ad.Lp-CD-IRES-E1A(control) vector or Ad.Lp-Luciferase(MRGD) vector. The second day after vector injection, 5FC was injected intraperitoneally each day for 7 days. As shown in Figure 5a (MDA-MB-468) and in Figure 5b (MDA-MB-435), at 25 days after injection, the Ad.Lp-CD-IRES-E1A(MRGD) vector suppressed the growth of subcutaneous tumor nodules derived from both MDA-MB-468 and MDA-MB-435 cancer cell lines compared with PBS control ($P<0.01$). In contrast, the intratumoral injection of an equivalent number of the Ad.Lp-Luciferase(MRGD) vector had no antitumor effect. As shown in Figure 5a

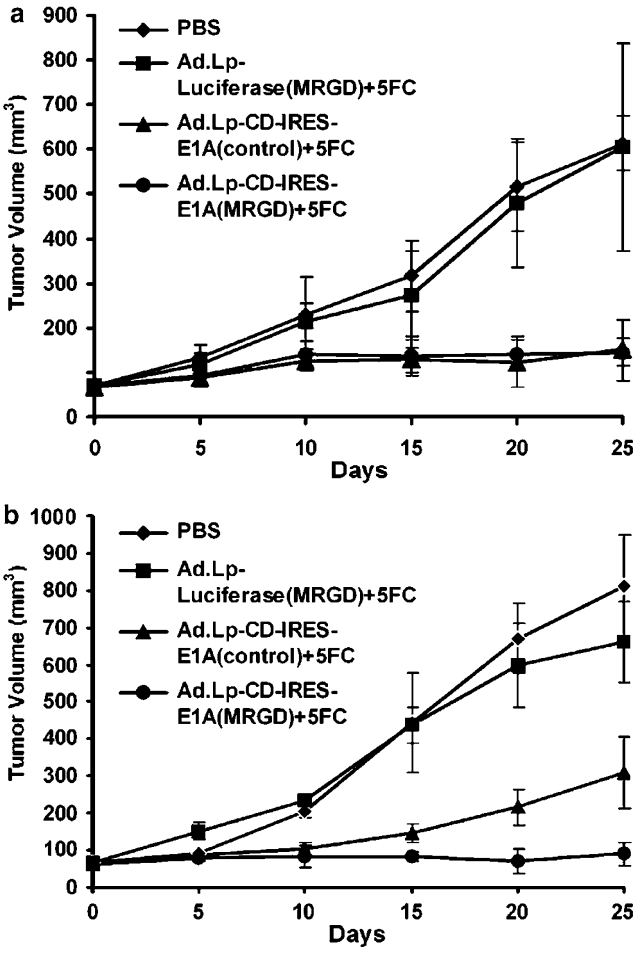


Figure 5 (**a**) Growth of the MDA-MB-468 human breast cancer cells *in vivo* following intratumoral injection of the Ad.Lp-CD-IRES-E1A(MRGD) vectors. Subcutaneous tumor nodules derived from MDA-MB-468 human breast cancer cell line (high CAR) were injected intratumorally with 1×10^9 ifu of the Ad.Lp-CD-IRES-E1A(MRGD) vector, Ad.Lp-CD-IRES-E1A(control) vector, the Ad.Lp-Luciferase(MRGD) vector or PBS. The second day after vector injection, 500 mg/kg of 5-FC was injected into the peritoneal cavity each day for 7 days. Tumor volumes were measured every 5 days. The data represents the mean of four animals. The bars represent the s.d. (**b**) Growth of the MDA-MB-435 human breast cancer cells *in vivo* following intratumoral injection of the Ad.Lp-CD-IRES-E1A(MRGD) vectors. Subcutaneous tumor nodules derived from the MDA-MB-435 human breast cancer cell line (low CAR) were injected intratumorally with 1×10^9 ifu of the Ad.Lp-CD-IRES-E1A(MRGD) vector, Ad.Lp-CD-IRES-E1A(control) vector, Ad.Lp-Luciferase(MRGD) vector or PBS. The second day after vector injection, 500 mg/kg of 5-FC was injected into the peritoneal cavity each day for 7 days. Tumor volumes were measured every 5 days. The data represents the mean of five animals. The bars represent the s.d.

and b, the Ad.Lp-CD-IRES-E1A(control) vector has the same antitumor effect in MDA-MB-468 (high CAR) tumor nodules as compared with the Ad.Lp-CD-IRES-E1A(MRGD) vector, but has less antitumor effect in MDA-MB-435 tumor nodules (low CAR) compared with the Ad.Lp-CD-IRES-E1A(MRGD) vector ($P<0.01$).

Since the MDA-MB-435 tumor cell has low expression level of CAR (see Table 2), the transduction efficiency of adenoviral vector with fiber modification (MRGD vector) will be increased compared with the adenoviral vector with wild-type fiber, thereby generating an advanced therapeutic effect of the vector.

Discussion

The successful clinical application of Ad-mediated cancer gene therapy requires the development of replication-competent adenoviral vectors that can specifically bind to cancer cells in tumor tissue, can express the therapeutic gene in tumor cells but not in normal cells and can replicate selectively within the target cancer cells. Replacement of the promoter of the adenoviral E1A gene by the tissue-specific promoter is one of the most promising methods to generate tissue-specific replication-competent adenoviral vectors.[21]

The L-plastin gene belongs to a family of genes which encode actin-binding proteins.[22] These proteins are present in $>90\%$ of epithelial neoplastic cells and are not found in normal epithelial cells. The previous work in our lab demonstrated that reporter genes driven by the L-plastin promoter were expressed in ovarian cancer cells, but not in normal mesothelial peritoneal cells.[15] The CRAD vector Ad.Lp-E1A in which the *E1A* gene was driven by the L-plastin promoter was cytotoxic to the breast cancer cell lines but was not toxic to normal human mammary epithelial cells.[23]

The prodrug activation gene therapy is currently one of several new approaches to cancer treatment. Generation of CRAD vectors that carry a suicide gene transcription unit may be even more successful. Our laboratory has created the Ad.Lp-CD-IRES-E1A vector in which both the prodrug activation *CD* gene and the *E1A* viral replication gene were driven by L-plastin promoter.[17] The cytotoxic effect of this vector was greater than the vector which contained only the *CD* gene both *in vitro* and *in vivo*.

In this study, we compared the replicative ability and cytotoxic effect of the Ad.Lp-CD-IRES-E1A vector with that of the Ad.Lp-E1A vector in different breast cancer cell lines. The results showed that the infection with the Ad.Lp-CD-IRES-E1A vector produces less vector particles than is seen with the Ad.Lp-E1A vector (Figure 4). We hypothesized that the lower replication ability shown by Ad.Lp-CD-IRES-E1A vector compared with Ad.Lp-E1A vector may due to the expression level of the E1A gene. When one promoter is used to drive two different genes, the expression level of downstream gene on the 3′ end of the IRES is usually weaker than the expression level of upstream gene. As a consequence, the direct oncolytic effect of the Ad.Lp-CD-IRES-E1A vector in the absence of 5FC was less than that of the Ad.Lp-E1A vector in all the tumor cell lines tested (Table 3). As expected, the cytotoxic effect was greatly increased after adding 5FC to the cell lines exposed to the Ad.Lp-CD-IRES-E1A vector and was greater than that generated by the Ad.Lp-E1A vector. Ad.Lp-CD-IRES-E1A did not cause cytotoxic effect in HMEC (Table 3).

The level of CAR, the key receptor for adenoviral vector infection, appears to determine the extent of the tumor-suppressive effect. It is quite common to see low levels of CAR expression in aggressive populations of cancer cells. In contrast, the expression of CAR is uniformly high in normal tissues. Unless the tropism of the adenoviral vector is modified in a way that increases its transduction efficiency for cancer cells, very high particle numbers would be required for generating a therapeutic effect and such high levels of vectors would increase the risk of side effects. Since high-affinity binding of adenovirus to the CAR is mediated by the knob domain of the fiber region,[24] we had proposed to genetically modify the knob by ablating its natural tropism and by incorporating alternative targeting peptide into the knob in order to generate a more stable and safer vector for gene delivery.

Analysis of the crystal structure of the Ad5 fiber knob has revealed that the HI loop of the knob domain is an ideal site for incorporating alternative peptides.[25] Dmitriev *et al*.[26] succeeded in inserting the RGD-4C motif into the HI loop of the adenoviral fiber domain, thus allowing the virus to use integrin as an alternative receptor in addition to CAR. We constructed adenoviral vectors carrying the luciferase reporter gene in which the RGD-4C peptide was inserted into the HI loop. We evaluated the efficiency of the adenoviral vector-mediated gene transfer both *in vitro* and *in vivo* by testing luciferase activity. The results showed that RGD modification of the fiber increased the *in vitro* gene transfer efficiency (see Figure 1), especially in tumor cell lines which have low expression level of CAR. RGD modification of adenoviral vectors also increases the *in vivo* transduction efficiency in established subcutaneous nodules derived from breast cancer cell lines (see Figure 3). The RGD-modified L-plastin series of CRAD vectors generate a higher viral yield and a greater cytotoxic effect in established breast cancer cell lines than that seen with the L-plastin series of CRAD vectors with wild-type fiber (see Table 3 and Figure 4).

Previous work has shown that the CAR-binding site is located in residues from the AB loop, the B β sheet and the DE loop in fiber.[27] The mutations of S408E and P409A in the AB loop demonstrated the greatest reduction in CAR-dependent gene transfer ability.[28] We constructed several adenoviral vectors in which the RGD peptide was inserted into the HI loop and in which the S408E, P409A mutations were generated in the AB loop simultaneously (MRGD vector). Preincubation of the vector with human $\alpha_v\beta_3$ protein inhibits the transduction efficiency of the MRGD vector in both tumor cell lines tested (MDA-MB-468 and MDA-MB-435). Preincubation of the vector with human $\alpha_v\beta_3$ protein does not inhibit the transduction efficiency of the adenoviral vector with wild fiber in MDA-MB-468 cell line (high CAR) but has inhibition effect in the low CAR MDA-MB-435 cell line (see Figure 2). MDA-MB-435 cell line has a low expression level of CAR and a high expression level of

$\alpha_v\beta_3$, so preincubation with human integrin $\alpha_v\beta_3$ protein has higher inhibition effect than in the MDA-MB-468 cell line (high CAR) when infected with Ad.CMV-Luciferase(control) vector. The transduction efficiency of MRGD vector was eight-fold lower in normal human mammary epithelia cells compared with adenoviral vector with wild-type fiber (see Figure 1). The transduction efficiency of the Ad.CMV-Luciferase(MRGD) vector was higher than that of the Ad.CMV-Luciferase(control) vector in established human breast cancer cell line MDA-MB-435 which has a low expression level of CAR both *in vitro* and *in vivo*, but decreased in MDA-MB-468 cell line which has a high expression level of CAR (see Figures 1 and 3). The CAR level has been reported to correlate inversely with the aggressiveness of the tumor,[29] and the low expression level of CAR was seen in many primary cancer cells and in established cancer cell lines.

We tested whether the safety and specificity of the vector can be increased by the combination of transcriptional targeting (using the L-plastin tumor-specific promoter) with transductional targeting (simultaneously ablating the ability of CAR binding and redirecting the vector to the integrin receptors). The Ad.Lp-Luciferase(MRGD) vector was constructed to test this question. The luciferase acitivity of tumor cells infected by Ad.Lp-Luciferase(MRGD) is lower than that infected by Ad.CMV-Luciferase(MRGD) vector (Figure 1). We also injected Ad.Lp-Luciferase(MRGD) intratumorally and tested the luciferase activity. As shown in Figure 3, the transduction efficiency of the Ad.Lp-Luciferase(MRGD) vector in subcutaneous deposits of tumors derived from established cancer cell lines as measured indirectly by the luciferase activity is less than that seen with the Ad.CMV-Luciferase(MRGD) vector in which the luciferase gene was driven by the CMV promoter. This indicated that L-plastin promoter is not as strong as the CMV promoter. The transduction efficiency of the Ad.Lp-Luciferase(MRGD) vector increased 4.4-fold in MDA-MB-435 cell line and 1.3-fold in MCF-7 cell line over that seen with the Ad.CMV-Luciferase(control) vector, but decreased 1302.2-fold in normal HMEC (see Figure 1). These results indicate that the combination of L-plastin tumor-specific promoter with transductional targeting further increases the specificity of the vector. The MRGD-modified L-plastin series of CRAD vectors generate a higher viral yield and cytotoxic effect in established human breast cancer cell lines which have a low expression level of CAR (see Table 3 and Figure 4).

Based on these experiments, the Ad.Lp-CD-IRES-E1A(MRGD) vector, a CRAD vector carrying the CD prodrug activation gene and a modification of the fiber seems to be the most useful vector of all the CRAD vectors we have constructed. *In vivo* animal experiments were carried out using this vector. This vector successfully suppressed the growth of subcutaneous tumor nodules derived from established breast cancer cell lines (see Figure 5a and b). In MDA-MB-468 breast cancer tumor model, which has high expression level of CAR, Ad.Lp-CD-IRES-E1A(MRGD) vector has the same antitumor effect as Ad.Lp-CD-IRES-E1A(control) vector. However, in experiments involving the MDA-MB-435 breast cancer tumor model that has a low expression level of CAR, the Ad.Lp-CD-IRES-E1A(MRGD) vector has superior antitumor effect compared with that of the Ad.Lp-CD-IRES-E1A(control) vector (see Figure 5a and b). Our laboratory will carry out additional preclinical studies to test the feasibility of taking this vector system into the phase I clinical testing.

Acknowledgements

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References

1 Zhang W. Development and application of adenoviral vectors for gene therapy of cancer. *Cancer Gene Ther* 1999; **6**: 113–138.
2 Kirn D, Martuza RL, Zwiebel J. Replication-selective virotherapy for cancer: biological principles, risk management and future directions. *Nat Med* 2001; **7**: 781–787.
3 Wickham TJ. Targeting adenovirus. *Gene Therapy* 2000; **7**: 110–114.
4 Kurihara T, Brough DE, Kovesdi I, Kufe DW. Selectivity of a replication-competent adenovirus for human breast carcinoma cells expression the MUC1 antigen. *J Clin Invest* 2000; **106**: 763–771.
5 Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS *et al*. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* 1997; **275**: 1320–1323.
6 Kashentseva EA, Seki T, Curiel DT, Dmitriev IP. Adenovirus targeting to c-erbB-2 oncoprotein by single-chain antibody fused to trimeric form of adenovirus receptor ectodomain. *Cancer Res* 2002; **62**: 609–616.
7 Chen SH, Shine HD, Goodman JC, Grossman RG, Woo SL. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*. *Proc Natl Acad Sci USA* 1994; **91**: 3054–3057.
8 Lawrence TS, Rehemtulla A, Ng EY, Wilson M, Trosko JE, Stetson PL. Preferential cytotoxicity of cells transduced with cytosine deaminase compared to bystander cells after treatment with 5-flucytosine. *Cancer Res* 1998; **58**: 2588–2593.
9 Oliver W, Michael B, Jogn CM. Therapy of colon cancer with oncolytic adenovirus is enhanced by the addition of herpes simplex virus-thymidine kniase. *Cancer Res* 1999; **59**: 410–413.
10 Wildner O, Morris JC, Vahanian NN, Ford Jr H, Ramsey WJ, Blaese RM. Adenoviral vectors capable of replication improve the efficacy of HSVtk/GCV suicide gene therapy of cancer. *Gene Therapy* 1999; **6**: 57–62.
11 Liang Y, Yang CT, Jablons DM. ONYX-015 works synergistically with chemotherapy lung cancer cell lines and primary cultures freshly made from lung cancer patients. *Cancer Res* 2000; **60**: 1009–1013.

12 Barker SD, Dmitriev IP, Nettelbeck DM, Liu B, Rivera AA, Alvarez RD *et al*. Combined transcriptional and transductional targeting improves the specificity and efficacy of adenoviral gene delivery to ovarian carcinoma. *Gene Therapy* 2003; **10**: 1198–1204.
13 Barnett BG, Tillman BW, Curiel DT, Douglas JT. Dual targeting of adenoviral vectors at the level of transduction and transcription enhances the specificity of gene expression in cancer cells. *Mol Ther* 2002; **6**: 377–385.
14 Reynolds PN, Nicklin SA, Kaliberova L, Boatman BG, Grizzle WE, Balysnilova IV *et al*. Combined transductional and transcriptional targeting improves the specificity of transgene expression *in vivo*. *Nature Bio* 2001; **19**: 838–842.
15 Chung I, Schwartz PE, Crystal RG, Pizzorno G, Leavitt J, Deisseroth AB. Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cell. *Cancer Gene Ther* 1999; **6**: 99–106.
16 Peng XY, Won JH, Rutherford T, Fujii T, Zelterman D, Pizzorno G *et al*. The use of L-plastin promoter for adenoviral-mediated, tumor-specific gene expression in ovarian and bladder cancer cell lines. *Cancer Res* 2001; **61**: 4405–4413.
17 Akbulut H, Zhang L, Tang Y, Deisseroth A. Cytotoxic effect of replication-competent adenoviral vectors carrying L-plastin promoter regulated E1A and cytosine deaminase genes in cancers of breast, ovary and colon. *Cancer Gene Ther* 2003; **10**: 388–395.
18 Liu YZ, Ye T, Sun DJ, Maynard J, Deisseroth A. Conditionally replication-competent adenoviral vectors with enhanced infectivity for use in the gene therapy of melanoma. *Human Gene Ther* 2004; **15**: 637–647.
19 He TC, Zhou SB, Costal LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci USA* 1998; **95**: 2509–2514.
20 Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promoter adenovirus internalization but not virus attachment. *Cell* 1994; **73**: 309–319.
21 Hallenbeck PL, Chang YN, Hay C, Golightly D, Stewart D, Lin J *et al*. A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. *Human Gene Ther* 1999; **10**: 1721–1733.
22 Lin CS, Chang CH, Huynh T. The murine L-plastin gene promoter: identification and comparison with the human L-plastin gene promoter. *DNA Cell Biol* 1997; **1**: 9–16.
23 Zhang L, Akbulut H, Tang Y, Peng X, Pizzorno G, Sapi E *et al*. Adenovirus vectors with E1A Regulated by Tumor-specific promoters are selectively cytolytic for breast cancer and melanoma. *Mol Ther* 2002; **6**: 386–393.
24 Louis N, Fender P, Barge A, Kitts P, Chroboczek J. Cell-binding domain of adenovirus serotype 2 fiber. *J Virol* 1994; **68**: 4104–4106.
25 Xia D, Henry LJ, Gerard RD, Gerard RD, Deisenhofer J. Crystal structure of the receptor-binding domain of adenovirus 5 fiber protein at 1.7A resolution. *Structure* 1994; **2**: 1259–1270.
26 Dmitriev I, Krasnykh V, Miller CR, Wang M, Kashentseva E, Mikheeva G *et al*. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol* 1998; **72**: 9706–9731.
27 Roelvink PW, Mi LG, Einfeld DA, Kovesdi Z, Wickham TJ. Identification of a conserved receptor-binding site on the fiber protein of CAR-recognizing adenoviridae. *Science* 1999; **286**: 1568–1571.
28 Jakubczak JL, Rollence ML, Stewart DA, Jafari JD, Von Seggern DJ, Nemerow GR *et al*. Adenovirus type 5 viral particles pseudotyped with mutagenized fiber protein show diminished infectivity of coxsackie B-adenovirus receptor-bearing cells. *J Virol* 2001; **75**: 2972–2981.
29 Okegawa T, Pong RC, Li YM, Bergelson JM, Sagalowsky AI, Hsieh JT. The mechanism of the growth-inhibitory effect of coxsackie and adenovirus receptor (CAR) on human bladder cancer: a functional analysis of CAR protein structure. *Cancer Res* 2001; **61**: 6592–6600.