The Journal of Microbiology, April 2005, p.179-182 Vol. 43, No. 2

Enhancement of Gene Delivery to Cancer Cells by a Retargeted Adenovirus

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(Received February 24, 2005 / Accepted March 31, 2005)

The inefficiency of *in vivo* gene transfer using currently available vectors reflects a major hurdle in cancer gene therapy. Both viral and non-viral approaches that improve gene transfer efficiency have been described, but suffer from a number of limitations. Herein, a fiber-modified adenovirus, carrying the small peptide ligand on the capsid, was tested for the delivery of a transgene to cancer cells. The fiber-modified adenovirus was able to mediate the entry and expression of a β-galactosidase into cancer cells with increased efficiency compared to the unmodified adenovirus. Particularly, the gene transfer efficiency was improved up to 5 times in OVCAR3 cells, an ovarian cancer cell line. Such transduction systems hold promise for delivering genes to transferrin receptor overexpressing cancer cells, and could be used for future cancer gene therapy.

Key words: Adenovirus, cancer cell, ligand-modified fiber, targeting

The ability to target delivery vehicle carrying therapeutics or suicidal genes to cancer cells would be of great use in cancer gene therapy. Adenovirus (Ad) vectors are promising for the gene therapy of cancer, due to their ability to achieve efficient gene transfer upon intratumoral administration, as well as the transient gene expression during limited periods. However, in this context, Ad mediates widespread gene transfer to both tumor and surrounding normal cells, due to the widespread expression of the receptor normally mediating adenovirus entry (the coxsackie adenovirus receptor; CAR) by a variety of cell types (Wickham, 2000). Because of these potential limitations, modified adenovirus vectors, capable of entering cells via specific receptors other than CAR, would be highly useful for delivering genes to cancer cells. As one of several possible strategies for changing the entry tropism of adenoviral vectors for gene therapy (Benihoud *et al.*, 1999; Krasnykh *et al.*, 2000; Wickham, 2000), modification of a viral capsid protein fiber, by adding a short peptide ligand, has been attempted in order to mask the normal CAR-virus interaction and redirect virus particles to specific cell types expressing its cognate receptor (Michael *et al.*, 1995). This particular approach involves the attachment of a ligand to the fiber that mediates normal virus entry through its interaction with CAR.

The human transferrin receptor (hTR) is a type II cell surface receptor that binds to transferrin (Tf), with the complex internalized through clathrin-coated pits. The hTR is ubiquitously expressed, and over-expressed at least 100-fold in oral, liver, pancreatic, prostate and other cancers (Keer *et al.*, 1990; Wen *et al.*, 1993; Miyamoto *et al.*, 1994; Ryschich *et al.*, 2004). This increase in the transferrin receptor in cancers, attributed to the increased metabolism of transformed cells, as well as its endocytic pathway upon binding to Tf, has made hTR a useful target for cancer therapy (Li *et al.*, 2002). Previously, we built an adenovirus vector carrying a small peptide, which binds to the human transferrin receptor (Tf-peptide), onto the C-terminus of fiber protein, through a linker (Joung *et al.*, 2005). The entry of the modified adenoviral vector was shown to be specific for the transferrin receptor. In addition, transferrin, the natural ligand for hTR, does not significantly affect the ability of the virus to deliver a transgene. Here, this adenoviral vector was tested to see if it could provide an effective means for mediating transgenes expression in hTR-positive cancer cells.

The strategy for constructing an adenovirus containing the Tf-peptide on the C-terminus of fiber has been described previously (Joung, *et al.*, 2005). The sequence encoding the *E. coli* β-galactosidase gene was also inserted into the viral genome as a reporter. The CAR-binding site within the fiber gene was retained, because disruption of the fiber's normal structure often cause the instability of virus as the fiber could not form timers, which is prerequisite for viral infection (Hong and Engler, 1996). Such manipulation resulted in a decrease in the

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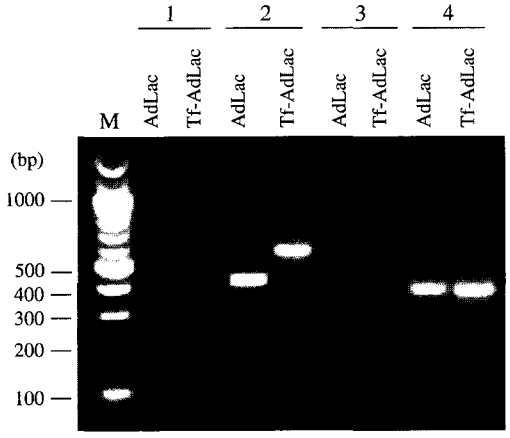

Fig. 1. PCR characterization of a recombinant adenovirus expressing the Tf-peptide on the C-terminus of the fiber. Numbers indicate the primer sets used in the PCR analysis. Pair 1 for the presence of Tf-peptide fused to fiber gene; 5'AGATCTTACTGAAGGCACAGCC 3' (F1, starting at nucleotide 1924 in the Ad5 fiber gene, 332 nucleotides upstream of the Tf-peptide) and 5'TTATCACGGCCACACAGGCGA (Tf-pep), pair 2 for the size of fiber; F1 and 5'GGGTGATCTGTATAAGCTATGT (F2, 114 nucleotides downstream of the stop codon for the fiber gene in the Ad5 sequence); pair 3 for the absence of the Ad early region 1 in order to confirm the replication-deficient adenovirus, and pair 4 for the presence of the β-galactosidase (LacZ) transgene. AdLac contained wild type fiber, while Tf-AdLac had the Tf-peptide attached fiber. Size markers present in the lane marked "M" are the 100 bp ladder.

fiber's trimer formation, with only very low yields of viruses when grown in 293 cells (data not shown). The integrity of the recombinant virus was checked, using the isolated viral DNA, before performing further experiments. The presence of the sequence encoding the Tf-peptide fused to fiber gene was verified by polymerase chain reaction with the primers of corresponding sequences for the Tf-peptide and upstream fiber gene (Fig. 1; panel 1). Adenoviruses with wild type or a modified fiber, named AdLac and Tf-AdLac, respectively lacked detectable early region 1 DNA sequences and contained the *E. coli* β-galactosidase transgene as a reporter (Fig. 1; panels 3 and 4). Viruses used in subsequent experiments were purified by centrifugation in a CsCl gradient, and the infectious units (IU) were determined by a plaque assay on 293 cells.

We first tested whether the recombinant virus carrying the Tf-peptide could redirect recombinant virus to hTR in preference to CAR. To address this question, 293 and HeLa cells were chosen, as both cell lines are widely used for adenovirus infection because of their abundant CAR expression. Immunoblots showed CAR was expressed in both cell lines as expected, with a slightly higher amount in 293 cells, whereas the expression of hTR was expressed to a greater extent in the HeLa cells (Fig. 2A). The greater expression of hTR in the HeLa cells was also verified by ^{125}I-transferrin binding analysis (Fig. 2B). The transduction efficiency of the virus was assayed by infecting 7×10^5 of the 293 cells or 1×10^6 of the HeLa cells in 35 mm plates,

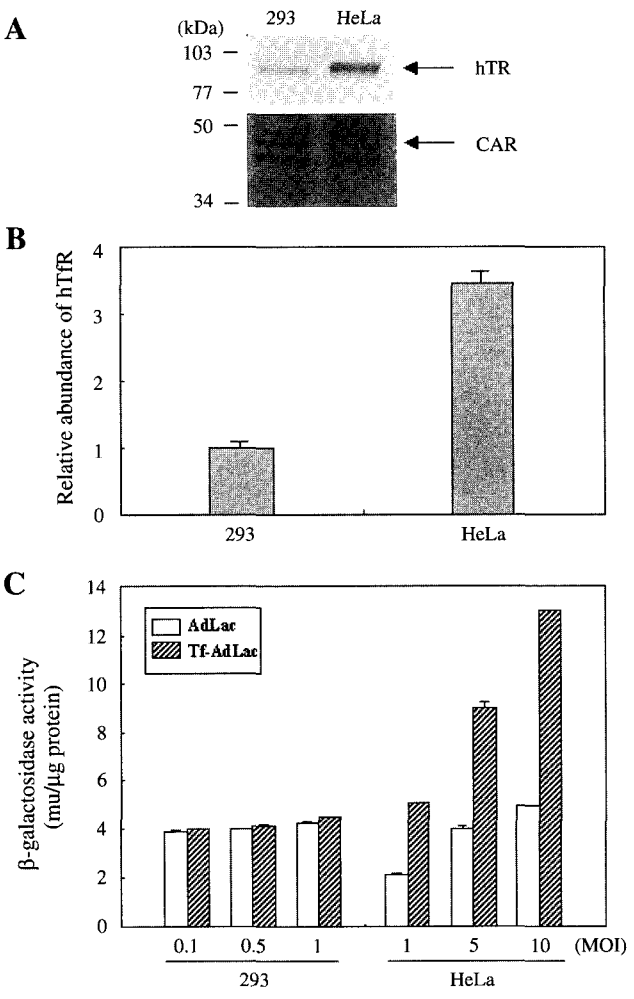

Fig. 2. Virus transduction of β-galactosidase into 293 and HeLa cells. A. Western Blot showing the relative amount of hTR and CAR in the 293 and HeLa cells. For the detections of hTR and CAR, cells were lysed in RIPA buffer (1% Nonidet P-40 (NP-40), 150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors). 30 μg of lysates from each cell type were subjected to 7 or 10% SDS-PAGE, transferred to nitrocellulose paper, and probed with the anti-hTR antibody H68.4 (Biogenex, USA), or the anti-CAR antibody, RmcB (Upstate Biotechnology, USA), respectively. Bound antibodies were detected using the ECL detection kit (GE Healthcare Bioscience, USA). hTR (95 kDa) and CAR (46 kDa) are indicated by arrows. B. The relative abundances of the human transferrin receptor (hTR) in cell lines, as determined by ^{125}I-transferrin binding experiments performed at 4°C. The cpm value for the 293 cells was set to 1, and that for the HeLa cells adjusted accordingly. Data shown represent the average and standard deviation of triplicate samples. C. Virus-mediated transduction of β-galactosidase activity into cell lines, as measured by the enzyme assay (Promega, USA). Data shown represent the average and standard deviation of three independent experiments. Mu = milliunits of activity. The MOI used in this experiment was as indicated.

at the indicated of multiplicity of infection (MOI), in Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum (FBS; Hyclone, USA) at 37°C. After 2 h, the viruses were washed off, the cells incubated

in standard culture media, containing 10% FBS, for 24 h. The β-galactosidase enzyme assay revealed that both the AdLac and Tf-AdLac viruses were able to transduce a functional gene into the 293 cells, with the same efficiency at all MOI (Fig. 2C). However, when tested on the HeLa cells, the Tf-AdLac virus was more efficient at transduction of β-galactosidase than AdLac. The transduction was more pronounced in the HeLa cells, as more viruses were used for infection. The increased transduction efficiency by Tf-AdLac into HeLa cells was correlated with the greater expression of hTR compared to the 293 cells, suggesting that the entry of the fiber-modified virus was mediated through the interaction between Tf-peptide and hTR rather than that between the fiber and CAR.

Next, whether the fiber-modified adenovirus was much more capable of transducing cancer cells other than HeLa, a cervical cancer cell line, was tested. Two different ovarian cancer cell lines, SK-OV-3 and OVCAR3, were infected with either the AdLac or Tf-Adlac viruse at the same MOI, and the β-galactosidase activities measured. Firstly, the cells were tested to see if they express hTR. The OVCAR3 cell line expressed a high level of hTR but the SK-OV-3 cell line showed a lower amount of receptors compared to the other cells when analyzed by Western blot (Fig. 3A). In the β-galactosidase enzyme assay, as high as a 5-fold increase in transduction efficiency was observed in OVCAR3 cells infected with Tf-AdLac. In contrast, the transduction efficiency was not improved in SK-OV-3 cells, which expressed a low amount of hTR (Fig. 3B), which was also confirmed by an X-gal staining experiment. 3×10^5 cells, in a 24-well plate, were infected with each virus and stained with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal), as described previously (Joung *et al.*, 2000). In Tf-AdLac infected OVCAR3 and HeLa cells, higher numbers of dark-stained cells were observed compared to AdLac-infected cells (Fig. 4). In contrast, the number of stained cells was very low when SK-OV-3 cells were infected with either virus (Fig. 4B and F). The relative levels of transduction in different cancer cell lines were approximately as expected based on their expressions of hTR. The correlation between hTR expression and increased transduction efficiency was also detected in other cancer cells, such as DM54, a glioma cell line (data not shown). It should be noted that the increased transduction efficiency was not strictly correlated with hTR expression as observed in the 293 cells. It is possible that virus entry into CAR bearing cells is mediated through CAR as well as hTR since the CAR binding site was not completely knocked out in the fiber protein, due to viral instability. Further study is

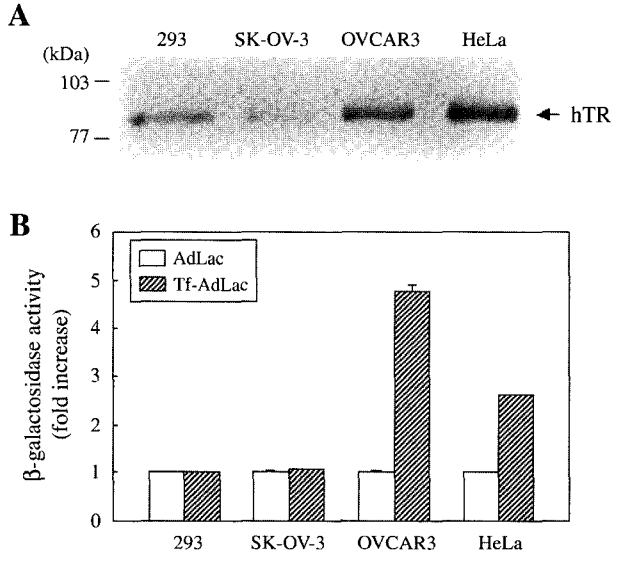

Fig. 3. Tf-AdLac mediated transduction of β-galctosidase in various cancer cells. A. Relative amounts of transferrin receptors as determined by Western Blotting. The same amounts of total protein (30 μg) from each cell were used for the analysis, as described in Fig 2. B. Virus mediated transduction of β-galactosidase activity into cells, as measured by enzyme assays. Enzyme activities in the lysates from AdLac infected cells were set to 1 (open bar) and those detected in Tf-AdLac infected cells are shown as fold increases (hatched bar). The MOI used in these experiments was 10, with the exception of the 293 cells (which was 1).

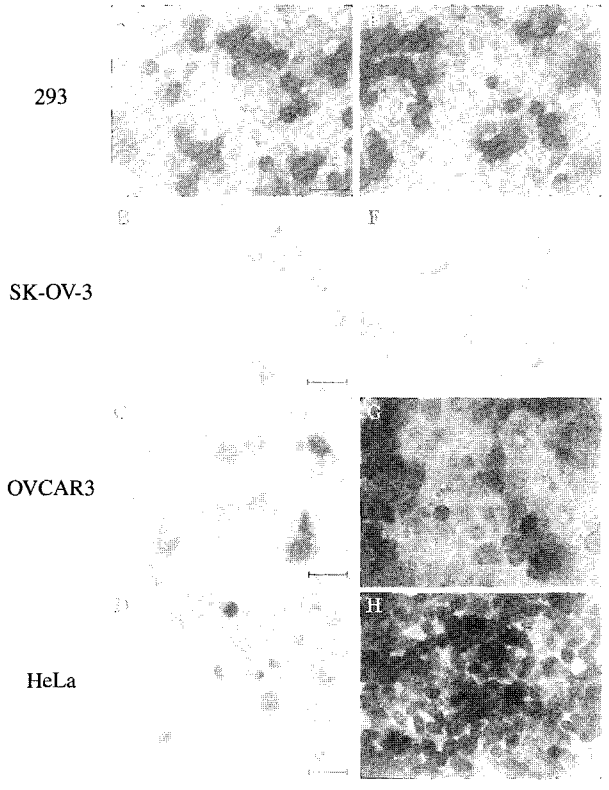

Fig. 4. Virus mediated transduction of β-galctosidase in various cancer cells, as detected by X-gal staining. Cells in a 24 well plate were fixed in 0.25% glutaraldehyde, stained with X-gal and the images captured using an Axioskop microscope (Carl Zeiss, Germany). The expression of β-galctosidase is shown in dark stained cells. Panels A-D, AdLac-infected cells; E-H, Tf-AdLac-infected cells. The MOI used in this experiment was the same as in Fig. 3B. Scale bars: 25 μm.

required for abolishing viral entry through CAR without causing instability of viruses.

The combination between an adenovirus and a Tf complex has been attempted previously. An adenovirus could mediate the transfer of marker genes into several types of tissue culture cells when coupled to Tf-polylysine complexes (Curiel *et al.*, 1991; Wagner *et al.*, 1992). However, most of the carried out at the preclinical studies had problems arising from the interactions with serum protein, which limited blood-stream circulation and restricted access to the target tissue. A cationic immunoliposome system using a lipid-tagged, single-chain antibody Fv fragment (ScFv) against hTR, has also shown an extremely low yield of lipid-tagged ScFv, which further limited downstream development and studies (Xu *et al.*, 2002). The strategy used in this study was easy to manipulate, and produced high yields of viruses. In addition, the gene delivery by fiber-modified viruses would not be affected by serum transferrin since the ligand binds to a different site that of native transferrin. Therefore, such transduction systems hold promise for delivering genes to hTR overexpressing cancer cells, and could be used for future cancer gene therapy.

Acknowledgements

We thank Dr. J. Collawn at UAB and Hak Jae Kim and Seung Ryong Heo at Hanseo University for their technical supports. This work was supported by grants from the Korean Research Foundation KRF 2003-002-C00255 to IJ.

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