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Ectopic Expression of the Coxsackievirus and Adenovirus Receptor Increases Susceptibility to Adenoviral Infection in the Human Cervical Cancer Cell Line, SiHa

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The expression level of the coxsackievirus and adenovirus receptor (CAR) gene in human cervical cancer cell lines (Hela, Caski, HT-3, and SiHa) appears to be correlated with their susceptibility to adenoviral vector-based gene transfer. Hela, Caski, and HT-3 cells, which express the CAR molecule on the cell surface, showed a higher susceptibility to infection of AdCMVGFP than SiHa cells with no detectable level of CAR expression. Transient expression of the CAR gene in SiHa cells dramatically enhanced the susceptibility to adenoviral infection. Furthermore, SiHa-CAR, a stable transfectant which expresses the CAR gene showed a highly increased susceptibility to adenoviral infection in contrast to SiHa. These results demonstrate that the low susceptibility of SiHa to adenoviral infection is closely related to its loss of the CAR gene expression. In addition, the low infection efficacy can be overcome by the ectopic expression of the CAR gene. These results also give insight into a possible application of the CAR gene to adenovirus-mediated gene delivery.

Key Words: **coxsackievirus and adenovirus receptor; adenovirus vector; infection efficacy; cervical cancer cell lines.**

The adenoviral vector is a useful tool for gene delivery and gene therapy (1, 2). Even though a recombinant adenovirus derived from subgroup C has a wide host range, infection efficacy is very low in some cell types and established cell lines (3–5). The mechanism of adenovirus entry to target cells is only partly known. Briefly, the adenovirus attaches to the primary receptor, the coxsackievirus and adenovirus receptor (CAR), on the host cells (6) and its internalization is facilitated by the interaction between the virus and αv integrins on the cell surface (7).

The CAR is a 46-kDa transmembrane protein that is composed of an extracellular domain, a hydrophobic transmembrane domain, and a cytoplasmic domain (3). Even though the accurate cellular function of the CAR has not been elucidated, it has been shown that the CAR functions as a high-affinity receptor for both subgroup C adenovirus and coxsackie B virus and that its cytoplasmic and transmembrane domains are not essential for coxsackievirus and adenoviral infection (8).

Recently, several groups reported that the difference in the expression levels of the CAR (9, 10) and integrins (11–14) is associated with their sensitivity to viral infection in various cells. However, there has been no direct evidence of any relationship of the expression level of the CAR and susceptibility to adenovirus infection in cervical cancer cell lines.

In this report, we demonstrate that the loss of the CAR due to its down-regulation at the transcription level is directly correlated with low infection efficacy of adenovirus in the cervical cancer cell line, SiHa. Transient expression of the CAR in SiHa increases its sensitivity to adenovirus-mediated gene transfer. In addition, the SiHa-CAR cell line that stably expresses the CAR gene shows an enhanced susceptibility to adenovirus-mediated gene transfer. These results also indicate that the low susceptibility of SiHa to adenoviral infection can be overcome by the ectopic expression of the CAR gene and suggest that the CAR gene may be applicable as a useful tool to boost infection efficacy of adenovirus.

MATERIALS AND METHODS

Cell lines. The cervical cancer cell lines HeLa, Caski, HT-3, and SiHa were maintained in Dulbecco's modified Eagle medium

Abbreviations used: CAR, coxsackievirus and adenovirus receptor; SiHa-CAR, SiHa cell which stably expresses CAR gene; GFP, green fluorescent protein; AdCMVGFP, a recombinant adenovirus which expresses GFP; AdCMVp53, a recombinant adenovirus which expresses p53; AdRSVβgal, a recombinant adenovirus which expresses β-galactosidase; m.o.i., multiplicity of infection.

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(DMEM) supplemented with 10% fetal bovine serum. The human embryonic kidney cell line 293 was maintained in Eagle's minimal Essential medium (EMEM) supplemented with 10% fetal bovine serum. All cell lines were obtained from ATCC (American Type Culture Collection, Rockville, MD).

Adenoviral vectors and infection. The recombinant adenovirus, AdCMVp53, was generated as described previously (15). AdCMVGFP was also generated by the same method, except that the 0.8 kb cDNA of green fluorescent protein (GFP) was inserted at the *Hpa*I site in the multiple cloning region of pΔACMVpA to give rise to AdCMVGFP. One of the replication-deficient AdCMVGFP clones was selected and propagated. Another replication deficient adenovirus, AdRSVβgal, a β-galactosidase-expressing adenovirus, was provided by Wei Wei Zhang (GenStar Therapeutics Corporation, San Diego, CA). Virus titers were determined by both plaque assay and optical observance at A_{260} (one A_{260} unit = 10^{12} particles/ml).

Flow cytometry analysis. To measure infection efficiency of AdCMVGFP in four cervical cancer cell lines, cells were infected with PBS or 10 multiplicity of infection (m.o.i.) of AdCMVGFP. After infection for 24 h, cells were collected with trypsinization and washed twice with PBS. The expression of the GFP transgene product was assayed by flow cytometry. For CAR gene expression analysis, HeLa, Caski, HT-3, and SiHa cells were washed twice with PBS and then incubated for 30 min at 4°C with RmcB monoclonal antibody (1:1000), obtained from Dr. Jeffrey M. Bergelson (Children's Hospital of Philadelphia, Philadelphia PA). Cells were washed with PBS and incubated for 30 min at 4°C with a secondary FITC-conjugated goat anti-mouse antibody (1:50). For negative control, only secondary FITC-conjugated goat anti-mouse antibody (Zymed, South San Francisco, CA) was treated.

RT-PCR analysis. Total cellular RNA was extracted from each cell line using TRIzol (GIBCO-BRL, Gaithersburg, MD). Two micrograms of total RNA was reverse transcribed and total cDNA was subjected to a 50 μl-PCR using a CAR gene primer set (5′-ATCAGAAGGTGGATCAAGTG-3′ and 5′-TTACTGCCGATGTAGCTTCT-3′) or a GAPDH primer set (5′-CGTGGAAGGACTCATGACC-3′ and 5′-TCCACCACCCTG-TTGCTGTA-3′) by a RT-PCR kit (Promega, Madison, WI). The final PCR products were electrophoresed in 1.2% agarose gel and photographed under UV light.

Transient transfection and construction of a stable cell line, SiHa-CAR. CAR cDNA was provided by Dr. Jhin-Gook Kim (Samsung Medical Center, Seoul, Korea). CAR cDNA was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) for mammalian expression with sense (pcDNA3.1-sCAR) or antisense direction (pcDNA3.1-asCAR). For transient transfection, approximately 2×10^5 cells of SiHa were seeded in s 60-mm dish 24 h before transfection. Cells were transfected with 5 μg of empty vector, pcDNA3.1-sCAR or pcDNA3.1-asCAR using FuGene6 reagent (Roche, Mannheim, Germany). For the construction of a stable cell line, transfected cells with pcDNA3.1-sCAR were screened with geneticin (400 μg/ml, GIBCO-BRL, Gaithersburg, MD). A single clone isolated from this screening was propagated in a 100-mm dish for further study.

Western blot analysis and β-galactosidase assay. For Western blot analysis, cells were lysed in RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP40, 0.1% SDS, and 0.5% sodium-deoxycholate]. Equivalent amounts of whole cell extracts (10 μg) were separated in 12% polyacrylamide gel and transferred to Hybond-ECL nitrocellulose filter paper (Amersham, Buckinghamshire, UK). Filters were blocked in 25 mM Tris (pH 8.0) containing 125 mM NaCl, 0.1% Tween 20, and 5% skim milk. Protein bands were probed with anti-p53 antibody (Novocastra, Newcastle on Tyne, UK) followed by labeling with horseradish peroxidase-conjugated anti-mouse antibody (Amersham, Buckinghamshire, UK). Bands were visualized using an ECL kit (Amersham, Buckinghamshire, UK) according to the manufacturer's protocol. β-galactosidase assay was performed by using a β-gal assay kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Gene therapy is a new approach to treat diseases including cancer, genetic disorders, and various incurable diseases. The basic concept of gene therapy is to replace a malfunctional mutated gene with a normal wild-type gene or to express a therapeutic gene in target cells. It is generally accepted that a critical prerequisite for gene therapy is a delivery system of the gene into target cells with high efficiency and accuracy. A recombinant adenovirus derived from subgroup C adenovirus is highly infectious and is capable of delivering therapeutic genes to many different cell types. However, it has been shown that the susceptibility to an adenoviral vector is very low in some cancer cells and some cell types (3–5), indicating that the susceptibility of target cells to an adenoviral vector limits a broad-range application of adenovirus-mediated gene therapy. Although it has not been completely elucidated, a possible mechanism responsible for the entry of an adenoviral vector into target cells centers around the role of the coxsackievirus and adenovirus receptor (CAR) that binds adenovirus as the primary receptor (9, 10) as well as two integrins, αvβ3 and αvβ5, that facilitate the internalization of adenovirus as a secondary receptor (11–14). These previous results suggest that the infection efficacy of adenovirus may be mainly correlated with the expression level of the CAR and integrins.

Recenlty, we investigated the infection efficacy of adenovirus in four human cervical cancer cell lines (HeLa, Caski, HT-3, and SiHa) in an attempt to examine whether adenoviral vector-mediated gene therapy is applicable to the treatment of human cervical cancer. Monolayers of individual cervical cancer cell lines were infected with 10 m.o.i. of a recombinant adenovirus possessing GFP gene, AdCMVGFP. The infection with AdCMVGFP appeared to have no significant effect on cell growth at the concentration tested. To measure the infection efficacy of AdCMVGFP, the cells were collected by trypsinization after 24 h of infection, and 10^5 cells were analyzed by flow cytometer. As shown in Fig. 1, a cervical cancer cell line SiHa appears to be refractory to the infection of AdCMVGFP. This is different from the other three cervical cancer cell lines (HeLa, Caski, and HT-3), which were all highly susceptible to the infection of AdCMVGFP. This lower susceptibility of SiHa cells to adenoviral infection compared with that of HeLa, Caski, and HT-3 cells was consistently observed when these cervical cancer cell lines were infected with another recombinant adenovirus, AdRSVβgal, which expresses β-galactosidase gene (data not shown).

Since the CAR is known to be a primary molecule for the attachment of adenovirus to target cells and thus is believed to play an important role in adenovirus entry into host cells (7, 9, 10), it seems likely that the lower

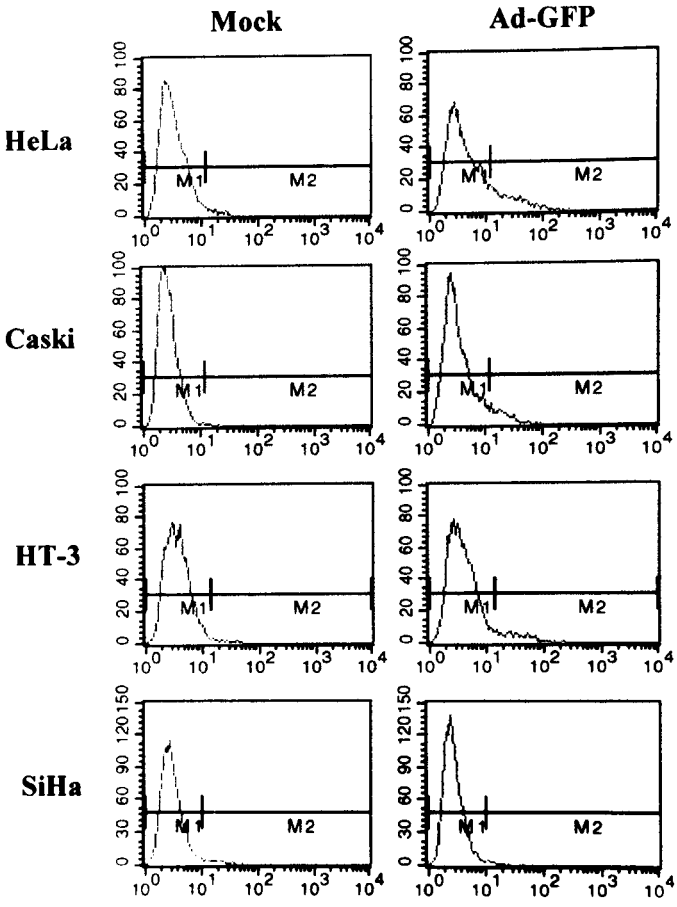

FIG. 1. Infection efficacy of AdCMVGFP in cervical cancer cell lines. Four cervical cancer cell lines were treated with PBS (mock) or 10 m.o.i. of AdCMVGFP (Ad-GFP). After 24 h of infection, cells were collected and green fluorescent protein (GFP) transgene product was assayed by flow cytometry.

susceptibility of SiHa cells to adenoviral infection might be related to the difference in the expression level of the CAR. To test this prediction, we decided to examine the correlation between the low susceptibility to AdCMVGFP and the expression level of the CAR in SiHa cells. To quantify the expression level of CAR-specific mRNA in individual cervical cancer cell lines (HeLa, Caski, HT-3, and SiHa), RT-PCR was performed using total RNA extracted from these cell lines. As shown in Fig. 2, the PCR product from CAR-specific mRNA was easily detectable in HeLa, Caski, and HT-3 cells but was undetectable in SiHa cells. Under the same conditions, there was no difference in the level of the PCR product from GAPDH-specific mRNA. To confirm the results of the RT-PCR analysis, the expression level of the CAR molecule on the cell surface was also investigated by immunofluorescent assay using anti-CAR monoclonal antibody, RmcB. In accordance with the results of the RT-PCR analysis which revealed the expression level of CAR mRNA, the expression of the CAR molecule on the cell surface was undetectable only in SiHa cells among four cervical cancer cells tested (Fig. 3). These results indicate that the low susceptibility of SiHa cells to the recombinant adenoviral infection is possibly due to their failure to express the CAR gene at the transcription level.

To obtain direct evidence that the low susceptibility of SiHa to adenoviral infection is due to its loss of the CAR expression, we examined whether the low susceptibility of SiHa cells to the adenoviral infection can be restored by introducing the CAR gene into SiHa cells by transient transfection. In this regard, we transiently transfected the CAR gene into SiHa cells to induce the expression of the CAR on the cell surface prior to AdRSVβgal infection. After SiHa cells were transfected with pcDNA3.1-sCAR for 24 h, the cells were infected with 50 m.o.i. of AdRSVβgal. Sequentially, the cells were collected at 48 h after AdRSVβgal infection and then β-gal assay was performed. As shown in Fig. 4, the susceptibility of SiHa to AdRSVβgal appeared to significantly increase when SiHa regained CAR expression following transfection with pcDNA3.1-sCAR. However, under the same conditions, the cells transfected with either pcDNA3.1 vector only or pcDNA3.1-asCAR did not show any increase in their susceptibility to adenoviral infection (Fig. 4). These results indicate that the loss of CAR expression in SiHa is a main cause in lowering the susceptibility to adenoviral infection. Thus a transient introduction of the CAR gene into SiHa is sufficient to boost its susceptibility to adenoviral infection.

To further demonstrate that the direct involvement of the CAR in determining the susceptibility to adenoviral infection, we tried to construct a stable transfectant, SiHa-CAR, which constitutively expresses the CAR. The results of the RT-PCR analysis using total RNA from SiHa-CAR showed that SiHa-CAR cell line expresses an easily detectable level of CAR-specific

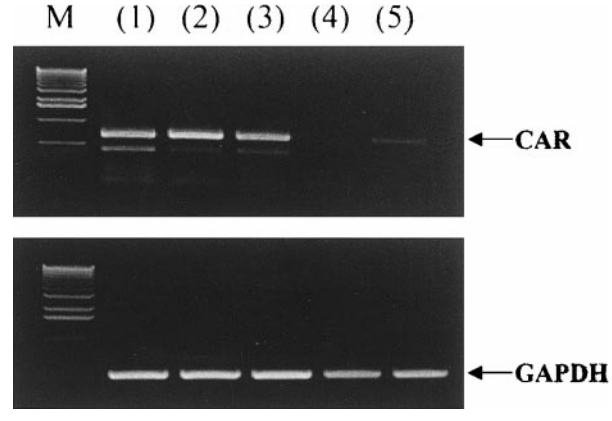

FIG. 2. CAR expression in four cervical cancer cell lines. Total RNA was extracted from four cervical cancer cell lines and a stable cell line, SiHa-CAR. CAR expression of each cell line was analyzed by RT-PCR using a CAR primer and GAPDH primer set. (1) HeLa, (2) Caski, (3) HT-3, (4) SiHa, and (5) SiHa-CAR.

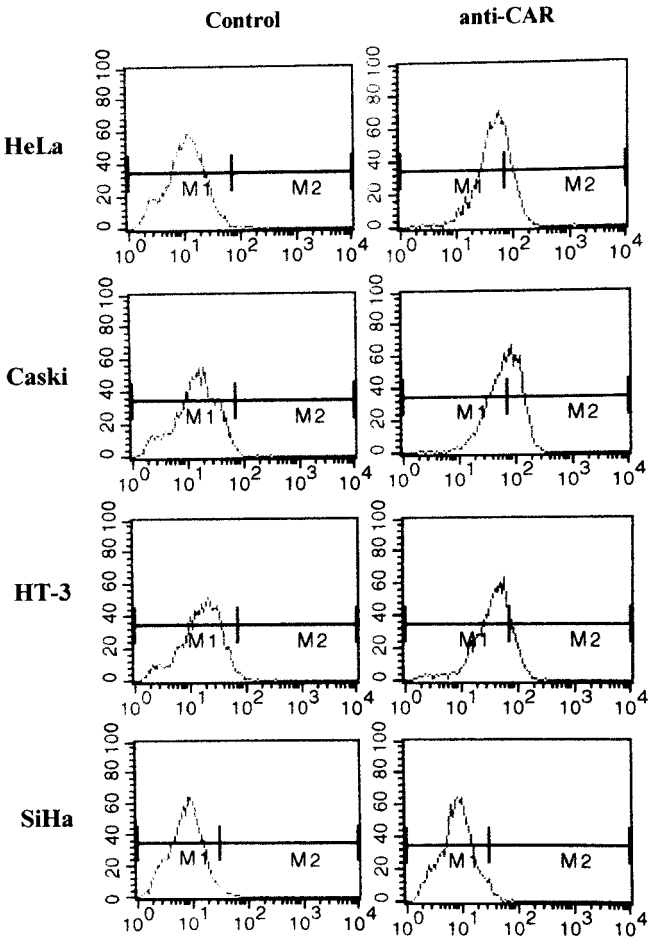

FIG. 3. Expression of surface CAR on four cervical cancer cell lines. Individual cancer cells were analyzed by flow cytometry after the cells were treated with anti-RmcB antibody (1:1000 dilution) and then FITC-labeled secondary antibody (1:50 dilution).

mRNA (Fig. 2A, lane 5). When SiHa and SiHa-CAR cell line were infected with 50 m.o.i. of AdRSV-βgal for 24 h and were subjected to β-gal assay, the stable transfectant SiHa-CAR retained a significantly higher susceptibility to adenoviral infection in contrast to SiHa cells (Fig. 5A). Sequentially, we employed another recombinant adenovirus encoding p53, AdCMVp53, to investigate the relationship between the ectopic expression of the CAR and adenoviral infection efficacy. When SiHa and SiHa-CAR were infected with 25 or 50 m.o.i. of AdCMV-p53, and the expression level of p53 protein was analyzed by Western blot analysis, the level of p53 protein appeared to be significantly higher in SiHa-CAR than in SiHa cells at the tested m.o.i. of AdCMV-p53 (Fig. 5B). Particularly, although SiHa-CAR was able to express an easily detectable level of p53 protein following transfection with 25 m.o.i. of AdCMV-p53, SiHa expressed a hardly detectable level of p53 protein under the same conditions. These results demonstrate that the CAR gene plays a critical role in determining adenoviral infection efficacy in cervical cancer cell line SiHa. It also demonstrates that introducing the CAR gene into SiHa by transfection is enough to enhance the susceptibility to adenovirus-mediated gene delivery. It has recently been shown that a recombinant adenoviral vector encoding CAR (Ad-CAR) improves adenovirus-mediated gene delivery to skeletal muscle (16). In these experiments, mouse myoblast cell line C2C12, which was infected with Ad-CAR at high m.o.i. prior to Ad-GFP infection, successfully expressed GFP at a low dose of Ad-GFP infection. These previous results and our results raise the possibility that coexpression of the CAR and a therapeutic gene by adenovirus vector may be employed as a novel strategy to gene therapy.

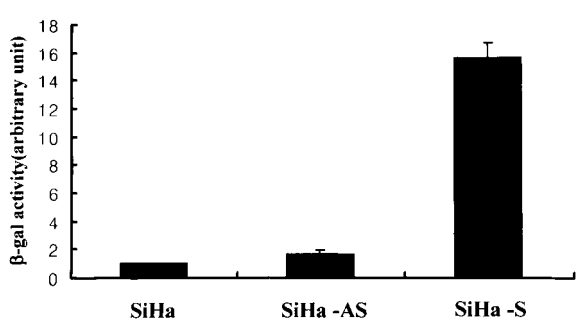

FIG. 4. Enhanced βgal gene expression after transient CAR transfection in SiHa cell line. SiHa cell line was transfected with an empty vector, pcDNA3.1-sCAR or pcDNA3.1-asCAR vector before AdRSVβgal infection. After infection for 24 h, cells were collected and β-gal activity was determined by β-gal assay.

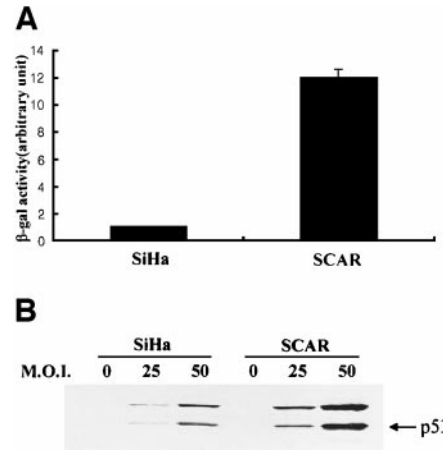

FIG. 5. Increase of adenoviral infectivity in SiHa-CAR cell line. (A) SiHa and SiHa-CAR cell line was infected with 50 m.o.i. of AdRSVβgal. After infection for 24 h, cells were collected and β-gal activity was determined by β-gal assay. (B) SiHa and SiHa-CAR cell line were infected with 25 or 50 m.o.i. of AdCMV-p53. After infection for 48 h, the expression of p53 protein was analyzed by Western blot analysis.

In summary, we demonstrated that a failure to express the CAR gene at the transcription level was directly correlated with low adenovirus susceptibility

in the cervical cancer cell line SiHa, and that the low susceptibility of SiHa cells to adenoviral infection can be restored by introducing the CAR gene into SiHa. In addition, we suggest that the CAR can be applicable as a useful tool to extend the efficiency of adenoviral vector-mediated gene therapy.

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REFERENCES

1. Kovesdi, I., Brough, D. E., Bruder, J. T., and Wickham, T. J. (1997) Adenoviral vectors for gene transfer. *Curr. Opin. Biotechnol.* **8,** 583–589.
2. Zhang, W. W. (1999) Development and application of adenoviral vectors for gene therapy of cancer. *Cancer Gene Ther.* **6,** 113–138.
3. Feldman, E., Ahmed, T., Lutton, J. D., Farley, T., Tani, K., Freund, M., Asano, S., and Abraham, N. G. (1997) Adenovirus mediated α interferon (INF-α) gene transfer into CD4(+) cells and CML mononuclear cells. *Stem Cells* **15,** 386–395.
4. March, K. L., Madison, J. E., and Trapnell, B. C. (1995) Pharmacokinetics of adenoviral vector-mediated gene delivery to vascular smooth muscle cells: Modulation by poloxamer 407 and implications for cardiovascular gene therapy. *Hum. Gene. Ther.* **6,** 41–53.
5. Watanabe, T., Kelsey, L., Ageitos, A., Kuszynski, C., Ino, K., Heimann, D. G., Varney, M. T., Shepard, H. M., Vaillancourt, M. T., Maneval, D. C., and Talmadge, J. E. (1998) Enhancement of adenovirus-mediated gene transfer to human bone marrow cells. *Leuk. Lymphoma* **29,** 439–451.
6. Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. (1997) Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* **275,** 1320–1323.
7. Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. (1993) Integrins αvβ3 and αvβ5 promote adenovirus internalization but not virus attachment. *Cell* **73,** 309–319.
8. Wang, X., and Bergelson, J. M. (1999) Coxsackievirus and adenovirus receptor cytoplasmic and transmembrane domains are not essential for coxsackievirus and adenovirus infection. *J. Viol* **73,** 2559–2562.
9. Hemmi, S., Geertsen, R., Mezzacasa, A., Peter, I., and Dummer, R. (1998) The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures. *Hum. Gene Ther.* **9,** 2363–2373.
10. Li, Y., Pong, R. C., Bergelson, J. M., Hall, M. C., Sagalowsky, A. I., Tseng, C. P., Wang, Z., and Hsieh, J. T. (1999) Loss adenoviral receptor expression in human bladder cancer cells: A potential impact on the efficacy of gene therapy. *Cancer Res.* **59,** 325–330.
11. Croyle, M. A., Walter, E., Janich, S., Roessler, B. J., and Amidon, G. L. (1998) Role of integrin expression in adenovirus-mediated gene delivery to the intestinal epithelium. *Hum. Gene Ther* **9,** 561–573.
12. Goldman, M. J., and Wilson, J. M. (1995) Expression of αvβ3 integrin is necessary for efficient adenovirus-mediated gene transfer in the human airway. *J. Viol.* **69,** 5951–5958.
13. Takayama, K., Ueno, H., Pei, X. H., Nakanishi, Y., Yatsunami, J., and Hara, N. (1998) The levels of integrin αvβ5 may predict the susceptibility to adenovirus-mediated gene transfer in human lung cancer cells. *Gene Ther.* **5,** 361–368.
14. Davison, E., Diaz, R. M., Hart, I. R., Santis, G., and Marshall, J. F. Integrin α5β1-mediated adenovirus infection is enhanced by the integrin-activating antibody TS2/16. *J. Viol.* **71,** 6204–6207.
15. Hwang, E. S., Kim, J., Kim, J. S., Kao, C., Ko, S.-C., Chung, L., and Lee, J. H. (1998) The effects of the adenovirus-mediated wild-type p53 delivery in human epithelial ovarian cancer cell line *in vitro* and *in vivo. Int. J. Gynecol. Cancer* **8,** 27–36.
16. Kimura, E., Maeda, Y., Arima, T., Nishida, Y., Yamashita, S., Hara, A., Uyama, E., Mita, S., and Uchino, M. (2001) Efficient repetitive gene delivery to skeletal muscle using recombinant adenovirus vector containing the Coxsackievirus and adenovirus receptor cDNA. *Gene Ther.* **8,** 20–27.