Factors Limiting Adenovirus-mediated Gene Transfer into Human Lung and Pancreatic Cancer Cell Lines[1]

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ABSTRACT

Adenoviral vectors are a widely used means of gene transfer. However, transgene expression after adenoviral administration varies among different carcinoma cell lines. We hypothesized that this variation is attributable, in part, to the presence of cell surface molecules involved in adenoviral infection. To test this, we first assessed adenovirus-mediated transgene expression in four human lung carcinoma cell lines and four human pancreatic carcinoma cell lines in terms of luciferase activities and found it to vary from 4.8×10^4 to 6.1×10^7 relative light units/μg of protein. Then, to determine whether the molecules involved in the entry of adenovirus into host cells were responsible for this variation, we evaluated the expression of αvβ5, αv, β3, α5, and β1 integrins and that of coxsackievirus and adenovirus receptor (CAR) in these cell lines. Statistical analysis revealed that the levels of β3 were associated with the levels of transgene expression. Blocking analysis showed that adenovirus-mediated gene transfer could be blocked by antibodies against these six molecules but not by the antibodies against α2 or α3 integrins, thus suggesting that the integrins αvβ5, αv, β3, α5, and β1 and CAR molecules could limit adenovirus-mediated gene transfer when their levels fell below a certain threshold. Furthermore, cells expressing low levels of β3 and resistant to conventional adenoviral vectors were susceptible to a vector containing the heparin-binding domain in its fiber, thus suggesting that redirecting vectors to receptors other than CAR may bypass the integrin pathway. These findings may have implications for improving the efficiency of adenovirus-mediated gene transfer and developing novel adenoviral vectors.

INTRODUCTION

Adenoviral vectors are widely used for both *in vitro* and *in vivo* gene delivery, largely because of their relatively high transduction efficiency in a variety of tissues and cell types (1, 2). However, the transduction efficiencies of such vectors vary greatly among different cell types and established cell lines. For example, hematopoietic cells (3, 4), vascular smooth muscle (5), and stem cells (4, 6, 7) are relatively resistant to adenovirus-mediated gene transfer.

Efforts have been made to characterize the cellular receptors of the adenovirus and their role in adenovirus infection. Studies on viral entry into host cells have revealed that two cell surface events, attachment and internalization, are required for a virus to enter a cell (8). The viral fiber protein will first attach to the CAR[3] on the surface of a host cell (9). The virion then gains entrance into the cell through the interaction of its penton base with the αvβ3 and αvβ5 integrins on the host cell surface (8). Several recent investigations into the levels of these cell surface markers and their correlation with the efficiency of adenovirus-mediated gene transfer have revealed that the integrins αvβ3 (3, 10), αvβ5 (11, 12), and α5β1 (13) and CAR (14, 15) are important for efficient gene transfer and thus potential predictors of transfer efficiency. However, there are, to our knowledge, no reports of any investigations of these markers in the same cell lines that would indicate whether the levels of one or more of these molecules are the limiting factors in adenovirus-mediated gene transfer.

In previous studies of adenovirus-mediated cancer gene therapy, we observed that some cancer cell lines are quite refractory to adenoviral infection. Therefore, to determine whether those molecules known to be involved in attachment and internalization of the adenovirus are also responsible for low transduction efficiencies in these cell lines, we examined adenovirus-mediated transgene expression and the expression of integrins αvβ5, αv, β3, α5, and β1 and CAR in four human lung carcinoma cell lines and four human pancreatic carcinoma cell lines. We then analyzed the correlation of the levels of these six cell surface markers with the levels of transgene expression. Our results suggest that levels of β3 are directly related to the

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[3] The abbreviations used are: CAR, coxsackievirus and adenovirus receptor; Luc, luciferase; RSV, Rous sarcoma virus; CMV, cytomegalovirus; MOI, multiplicity of infection; pfu, plaque-forming unit(s); X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; FACS, fluorescence-activated cell sorting; RLU, relative light unit.

levels of transgene expression in the cell lines examined. Although the levels of CAR, αvβ5, α5, αv, and β1 were not directly related to the levels of transgene expression, antibody blockade of these factors significantly reduced transgene expression. Thus, in the cell lines examined, expression of all six of these molecules appeared to be necessary for efficient adenovirus-mediated gene transfer.

MATERIALS AND METHODS

Adenoviral Vectors. Adenoviral vectors expressing a firefly luciferase gene driven by the RSV long terminal repeat (Ad/RSV-Luc) or a bacterial *LacZ* gene driven by the CMV early promoter (Ad/CMV-LacZ) were used to evaluate the transduction efficiencies of adenoviral vectors in various cancer cell lines. An adenoviral vector containing a heparin-binding domain and expressing a bacterial *LacZ* gene (AdPK; Ref. 16) was obtained from GenVec, Inc. (Rockville, MD). The vectors were expanded in 293 cells and twice purified by ultracentrifugation on a cesium chloride gradient. Virus titers were determined by both optical absorbance at A_{260} (one A_{260} unit = 10^{12} particles/ml) and by plaque assay as described previously (17). All viral preparations were free of $E1^+$ adenovirus as determined by PCR (17).

Cell Lines and *in Vitro* Adenovirus-mediated Gene Transfer. Four established human lung carcinoma cell lines (H1299, H226b, H226br, and H460) and four established pancreatic carcinoma cell lines (MiaPaca, Panc-1, Capan-1, and Panc-28) were used in this study. Cells were cultured in either RPMI 1640 or DMEM supplemented with 5–10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. For *in vitro* adenovirus-mediated gene transfer, cells were seeded onto six-well plates at a density of 5×10^5 cells/well. The cells were then infected with adenoviral vectors at a MOI of 10 pfu and grown at 37°C. Cells were harvested 48 h after infection and frozen at −20°C until used for protein and Luc assays. For blocking analysis, cells were seeded onto 96-well plates at a density of 2.5×10^4/well. After incubation of cells with various antibodies at 4°C for 30 min, 200 pfu of Ad/CMV-LacZ were added to each well. After incubation of cells at 37°C for 30 min, the uninfected vectors were removed. Cells were then washed once with PBS and replaced with fresh medium and incubated for 24 h. Transduced cells were then visualized by X-gal staining.

Biochemical Analysis. Cultured cells were lysed in Luc assay buffer. Cell debris was then removed by microcentrifugation. Protein concentrations were determined using a BCA protein assay kit from Pierce (Rockford, IL) according to the manufacturer's instructions. Luciferase activities were determined using a luminometer and a luciferase assay system according to the manufacturer's instructions (Promega Corp., Madison, WI). X-gal staining was performed as described previously (18). The β-galactosidase enzyme assay was performed by using a Galacto-Light Chemiluminescent Assay kit from Tropix, Inc. (Bedford, MA) according to the manufacturer's instructions.

FACS Analysis. Antibody for CAR was provided by Dr. Finberg (Dana-Farber Cancer Institute, Boston, MA). Antibodies for αvβ5, αv, α2, α3, α5, β1, and β3 integrins were obtained from Life Technologies, Inc. (Gaithersburg, MD), Zymed Laboratories, Inc. (South San Francisco, CA), or BioSource International (Camarillo, CA). Antibody for human IgG was obtained from Zymed Laboratories, Inc. All of these antibodies are monoclonal except for anti-CAR, which is polyclonal antibody obtained from rabbit. For FACS analysis, cells were harvested by trypsinization and washed twice with HBSS with calcium and magnesium (Life Technologies, Inc.) by spinning the cells at $2500 \times g$ for 5 min. Then, 1×10^6 cells suspended in 100 μl were aliquoted into 1.5-ml Eppendorf tubes. Two μl of each antibody were then added to the cell suspension, and the cells were incubated at 4°C for 1 h. After washing twice with HBSS, the cells were suspended in 100 μl of HBSS. One μl of FITC-labeled goat antimouse immunoglobulin (PharMingen, San Diego, CA) was then added to each sample, and the cells were incubated in the dark at 4°C for 30 min. After washing twice with HBSS, the cells were suspended in 1% formaldehyde in PBS. The FACS analysis was performed within 1 week.

Statistical Analyses. Differences among the cell lines in transgene expression and in cell surface marker expression were assessed by ANOVA using Statistical software (StatSoft, Tulsa, OK) and Kruskal-Wallis tests using Statview (SAS Institute, Cary, NC). $P \leq 0.05$ was considered significant. Associations between transgene expression and the expression levels of various cell surface markers were assessed using a linear model. The dependent variable was the logarithm of RLUs; cell surface markers were the independent variables. Replicated measures of both dependent and independent variables were assumed to be subject to normal errors. The mean of the dependent variable was assumed to be equal to the mean of a linear combination of the means of the dependent variables plus a normal error. The variances of the errors were permitted to differ between cell lines and between different variables. Parameter estimation was performed by maximum likelihood estimation using the Mathematica software package (Wolfram Research, Inc., Champaign, IL). Significance tests were performed using the likelihood ratio test.

RESULTS

Transgene Expression in Human Lung and Pancreatic Cancer Lines. To assess the transduction efficiencies of adenoviral vectors in various cancer lines, a pilot study was performed by infecting cells with Ad/CMV-LacZ at different MOIs. Cells were then stained with X-gal, and transduction efficiencies were determined in terms of the percentage of blue cells. The results showed that some cell lines were susceptible to adenoviral infection, whereas others were resistant. At MOI 10, about 80–90% of H1299 and H226b cells were transduced *versus* <10% of H460 and H226br cells (data not shown). To further characterize the adenovirus-mediated gene transduction efficiencies in cancer lines, four human lung carcinoma cell lines (H1299, H226b, H226br, and H460) and four human pancreatic carcinoma cell lines (MiaPaca, Panc-1, Capan-1, and Panc-28) were infected with Ad/RSV-Luc at MOI 10. Cells were harvested 2 days after the infection, and the Luc activities were determined. The relative luciferase activities, given in terms of RLUs, varied from 4.8×10^4/μg protein in H460 cells to 6.1×10^7/μg protein in H226b cells (Fig. 1). Thus, the variation in transgene expression among these cell lines ranged

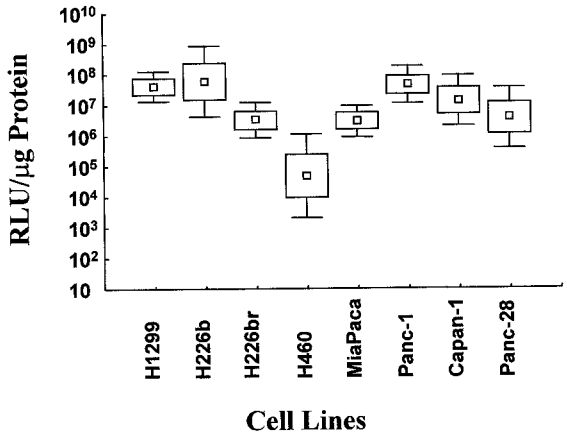

Fig. 1 Transgene expression in human lung and pancreatic cancer lines as indicated by Luc activities. The Luc activities are presented as RLUs/μg cellular protein after adenovirus-mediated gene transfer. Each value represents the mean (*small box*) ± SD (*large box*) and the mean ± 1.96 SD (*bar*) of five repeated analyses for each cell line.

across three orders of magnitude. Statistical analysis showed that the differences among cell lines were significant ($P = 0.0001$). The RLU value for the H460 line was significantly different from the values for all other cell lines, whereas the RLUs for H226b, H226br, and MiaPaca were significantly different from the values for five of the seven other cell lines. The RLU value for H1299, Panc-1, Capan-1, and Panc-28 were significantly different from the values for four of the seven other cell lines.

Expression of Integrins and CAR on Human Lung and Pancreatic Cell Lines. To determine which cell surface molecule or molecules were responsible for the difference in adenovirus-mediated transgene expression among the cell lines, we evaluated the levels of six cell surface molecules known to be involved in the entry-related events of adenoviral infection: integrins αvβ5, αv, α5, β1, and β3 and CAR. To determine the levels of these molecules, cells were harvested by trypsinization and incubated with primary antibodies against one of each these molecules. Cells were then incubated with FITC-labeled secondary antibodies that could specifically recognize the primary antibodies. After washing with PBS, the cells were analyzed by FACS to determine the percentage of fluorescent cells and the mean intensity of the fluorescence for the cell population. Five assays were performed on each cell line, and antibodies against human IgG were used as controls for primary antibodies. The levels detected by this control antibody were used as a basal background for each antibody tested. Although levels of FITC-positive cells detected by this control antibody were comparable with those of the PBS control (data not shown), various levels of FITC-positive cells were detected in each cell lines when a primary antibody against a specific cell surface molecule was used (Fig. 2). Because the efficiency of these antibodies in detecting their target molecules may vary, a direct comparison of the levels of these molecules within the same cell line may not be meaningful. Nevertheless, the levels of the molecules detected by the same antibody across the eight cell lines were compared because detection of the target molecule should occur with the same efficiency. Statistical analysis showed that the levels of these molecules were all significantly different among cell lines ($P \leq 0.0002$). The mean fluorescent intensities usually correlated with the percentage of FITC-positive cells, *i.e.*, a cell line with a high percentage of cells FITC-positive for a particular molecule usually had a high mean fluorescent intensity for that molecule. Analysis of the two parameters together generated results similar to those generated by analysis of percentage alone (data not shown).

Correlation of Transgene Expression with Cell Surface Molecules. To assess which molecule or molecules would limit adenovirus-mediated gene transfer, we analyzed the significance of the association between transgene expression and cell surface molecule expression in each cell line by using the linear model described above. In a univariate analysis, the β3 level was positively associated with the levels of transgene expression (RLUs) among the eight cell lines examined (regression coefficient, 0.066; $P = 0.04$). When a multivariate analysis was performed using forward variable selection, only β3 was selected.

Transgene Expression after Blocking of Cell Surface Molecules. Upon statistical analysis of the correlation of transgene expression and level of cell surface molecules, we observed that CAR (9, 11, 12, 14, 15, 19) and αvβ5 (11, 12), previously reported to be important in the entry of adenovirus into host cells, did not correlate with transgene expression. Therefore, to further test the role of these molecules in adenovirus-mediated gene transfer, we performed blocking analysis with antibodies to the six cell surface molecules. For this purpose, preliminary experiments were performed to test the ability of commercially available antibodies against these molecules in blocking adenoviral infection at various concentrations (dilution range, 1:10 to 1:1000). The majority of the antibodies we tested could block adenovirus infection after 1:10–1:50 dilution (data not shown). The properly diluted antibodies (1:20 for all commercially available antibodies, including controls, and 1:50 for anti-CAR) were then added to H1299 cells seeded in 96-well plates and incubated at 4°C for 30 min. Then, 200 pfu of Ad/CMV-LacZ were added to the cells and incubated at 37°C for 1 h. Uninfected vectors were then removed, and cells were cultured for 2 days before X-gal staining to assess transgene expression. The total number of blue cells was counted and compared with the total number in controls preincubated with PBS or control antibody. The results showed that preincubation of the cells with antibodies against αv, α5, β1, β3, αvβ5, and CAR reduced transgene expression to 26–60% of that in the control (Fig. 3). Preincubation of the cells with antibodies against either α2 or α3 integrins, however, did not change the level of transgene expression (Fig. 3). Thus, the blocking analysis showed that the adenoviral infection could be blocked by antibodies to all six of the target molecules, all of which have been reported to be involved in adenoviral infection.

Transgene Expression of AdPK in Refractory Cells. As shown in Fig. 1, transgene expression from Ad/RSV-Luc differed significantly among H1299, H226br, and H460 cells. Moreover, our study of cell surface molecules showed that H226br and H460 cells, which had low levels of transgene expression, expressed less β3 and/or α5 than did H1299 cells (Fig. 2). All other molecules tested on these two cell lines were

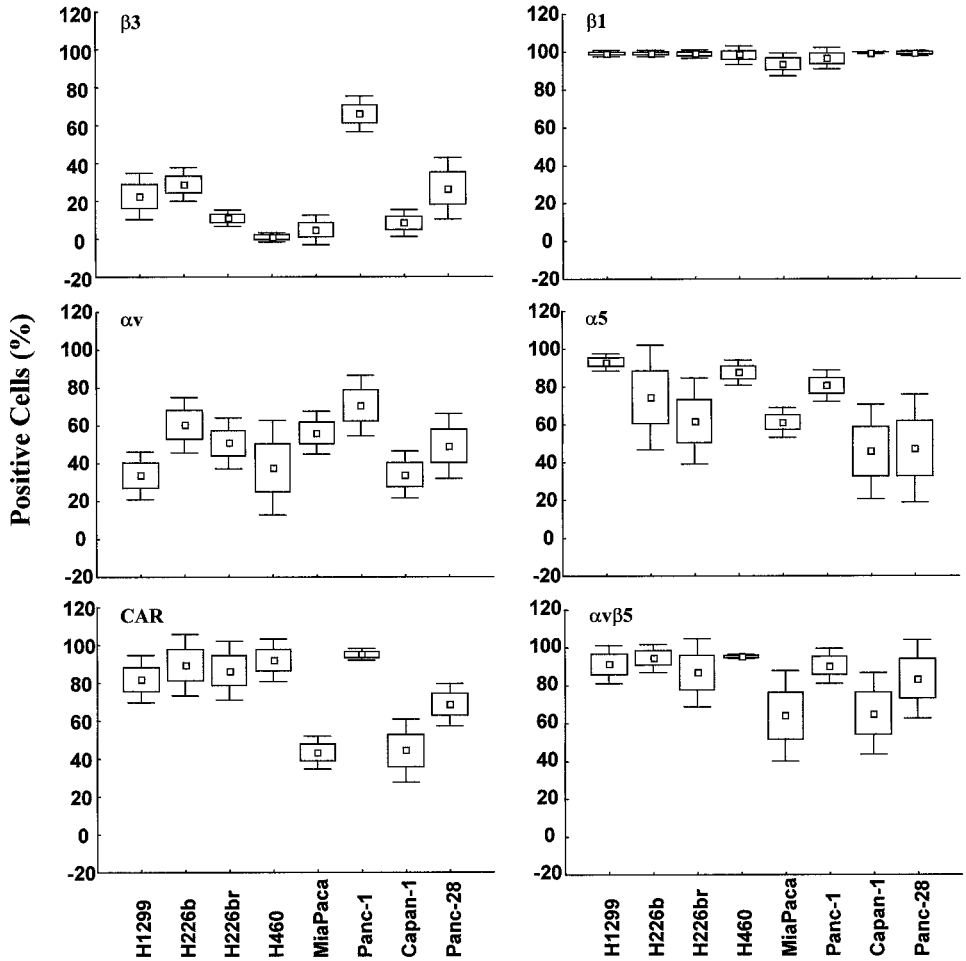

Fig. 2 Levels of six cell surface molecules in each of eight human cancer lines. Each value represents the mean (*small box*) ± SD (*large box*) and the mean ± 1.96 SD (*bar*) of five repeated analyses for each cell line.

expressed at either equivalent or higher levels than in H1299 cells. To test whether the transgene expression in H226br and H460 cells would be increased by using an adenoviral vector with altered receptor tropism, we compared bacterial β-galactosidase activities in H1299, H266br, and H460 cells after infection with Ad/CMV-LacZ, AdPK, or control vector at MOI 10. The cells were then harvested 24 h after infection and assayed for β-galactosidase activities. Although infection with Ad/CMV-LacZ resulted in 30-fold higher transgene activities in H1299 cells than in H226br and H460 cells, infection with AdPK resulted in equivalent β-galactosidase activities in all three cell lines (Fig. 4). The transgene expression was significantly higher when these cell lines were infected with AdPK *versus* Ad/CMV-LacZ ($P = 0.001$). X-gal staining analysis showed an increased percentage of blue cells when cells were infected with AdPK, which correlated with the increase in β-galactosidase activities (data not shown). Because the *LacZ* genes in Ad/CMV-LacZ and AdPK were driven by CMV and RSV promoters, respectively, the difference in the transgene activities within the same cell line may also have reflected a difference in promoter activities. Nevertheless, the differential transgene expression observed after infection with Ad/RSV-Luc was not observed after infection with AdPK. Moreover, a previous study of promoter activities had shown that CMV was more active than RSV in various cell lines *in vitro* as well as *in vivo* (20). Thus, the change in the transgene expression observed in response to AdPK was likely attributable to the increased infectivity of the modified vector.

DISCUSSION

Expression of a transgene delivered adenovirally involves several steps of vector-host cell interaction. This includes attachment of the vector to the host cells, internalization of the vector by the host cells, entry of the vector into the nucleus, transcription of the transgene, translation and modification of the transgene product, and that product's translocation to a proper subcellular compartment, as well as stability of the transgene products once inside target cells. Each step can be affected by a variety of factors that limit the levels of transgene expression. In addition, the limiting step for different vector systems may vary substantially. For example, the entry of conventional retroviral vectors into the nucleus is limited in nondividing cells, but this problem can be overcome by using lentiviral vectors (21, 22). In the case of adeno-associated virus-mediated gene transfer, the limiting step is the conversion of a

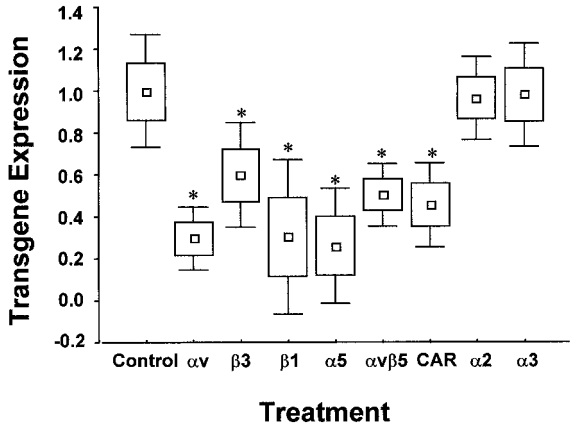

Fig. 3 Blocking analysis. The levels of blockage by the indicated antibodies were determined by comparing the amount of blue cells in each treatment group with that in the control, which was arbitrarily set at 1. Each value represents the mean (*small box*) ± SD (*large box*) and the mean ± 1.96 SD (*bar*) of triplicate assays in H1299 cells. ∗, treatment group that significantly differed from the control ($P < 0.05$).

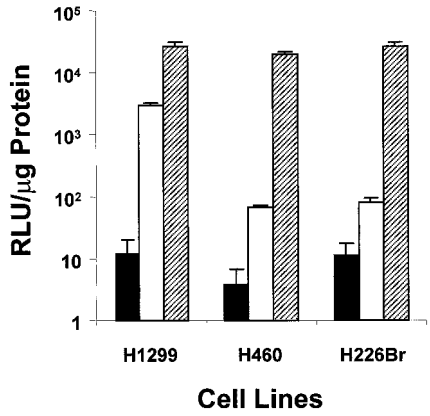

Fig. 4 Comparison of transgene expression after Ad/CMV-LacZ and AdPK infection. The bacterial β-galactosidase activities after vector infection are presented as RLUs/μg cellular protein/5 s. Each value represents the mean ± SD of quadruplicate assays. ■, control vector Ad/E1⁻; □, Ad/CMV-LacZ; ▨, AdPK. The difference between transgene expression after infection with Ad/CMV-LacZ and AdPK within the same cell lines, as expressed in RLUs, was significant ($P \leq 0.01$).

single-stranded vector DNA into a double-stranded DNA so that the transgene will be recognized by the host cell's transcriptional machinery. This can be overcome by the presence of a helper function such as adenoviral E4 and a DNA-damaging reagent (23, 24). The limiting step in adenovirus-mediated gene transfer, however, is not well defined. Several pieces of evidence indicate that the entry of the vector into the host cell may be one such step. For example, Wickham *et al.* (16, 25) have shown that modification of the viral fiber to change its tropism can greatly enhance transgene expression in refractory cells. In addition, other authors have demonstrated that increasing the levels of integrins and CAR in target cells by gene transfer concurrently increases adenovirus-mediated transgene expression (3, 9). Furthermore, various studies have shown that levels of integrins and CAR in target cells seem to correlate with the transduction efficiencies of adenoviral vectors in those cells (10, 11, 14, 15). However, characterization of the expression patterns of the molecules that are known to be associated with the entry of viruses into host cells, as well as statistical analysis to document the correlation of the levels of these molecules with transgene expression, is needed to further define the role of these molecules in adenovirus-mediated gene transfer.

In the study reported here, we studied the levels of six cell surface molecules and transgene expression in eight different cancer cell lines. We observed a positive association between the levels of β3 integrin with adenovirus-mediated gene transfer. Blocking analysis showed, however, that all six molecules we evaluated were required for efficient transgene expression. This may indicate that although CAR, αvβ5, αv, and β1 are all involved and required for the entrance of adenoviral vectors into host cells, they may no longer be limiting once a minimum level is present. Whether this finding can be generalized to other cell lines or cell types is yet to be determined. In addition, the results of the blocking analysis indicated that any one of the six cell surface molecules could be a limiting factor in adenovirus-mediated gene transfer once its expression fell to a certain threshold. This may also explain why these molecules have been reported previously to be the important factors in various other studies (10, 11, 13–15). Furthermore, *in vivo* gene delivery may be limited by other factors, such as a vector's access to target cells through local dissemination or through penetration of vessel walls.

The results of our study also suggest several reasons why transcription or promoter activity may not be the limiting factor when a ubiquitous promoter such as CMV or RSV is used: (*a*) similar patterns of transgene expression were observed among the different cell lines we examined, regardless of whether Ad/RSV-Luc or Ad/CMV-LacZ was used; and (*b*) the cells refractory to transduction by Ad/CMV-LacZ were quite susceptible to AdPK. Moreover, the differential transgene expression seen in H1299, H460, and H266br cells infected with Ad/RSV-Luc was not observed after infection with AdPK, although the two vectors shared the same RSV promoter.

Thus, we have been able to show by correlative studies and blocking analysis that cell surface molecules involved in adenovirus entry are important for transgene expression. Yet, this will still not likely explain all of the differences in the transgene levels observed among various cell types. Other factors, such as the activity of promoters, stability of transgene products, the behavior of the viral genome in host cells, and the sensitivity in measuring transgene products, will certainly contribute to differences in transgene levels among various target cells. In fact, some of these other factors may even explain why transgene expression was no different between H226Br and H460 cells when LacZ was used as a reporter but 79-fold higher in H226Br cells than in H460 cells when Luc was used as a reporter. However, the underlying mechanisms for this phenomenon are yet unclear.

Our observations of enhanced transgene expression in both sensitive and resistant cell lines after infection with adenoviral vectors containing a polylysine sequence in the fiber protein are consistent with observations by others (16). Although the fiber

protein is known to interact with CAR, we have now shown that modification of the fiber protein significantly improved the transgene expression in cells in which such expression was limited by the levels of integrins. This suggests that redirecting a vector to heparin or some other receptor may allow it to bypass the integrin pathway. In addition, the enhancement of transgene expression that we induced by infecting otherwise refractory cells with the vector AdPK suggests that fiber gene-modified vectors may be useful for further studies of gene delivery to cancer cells.

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