Reduced Transduction Efficiency of Adenoviral Vectors Expressing Human p53 Gene by Repeated Transduction into Glioma Cells in Vitro

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ABSTRACT

Purpose: Recombinant adenoviral vectors are widely used in clinical and experimental studies to treat malignant tumors. Recently, host immune responses have been proposed as a major limitation in using adenoviral vectors for repeated gene delivery. We demonstrate another limitation unrelated to host immunity.

Experimental Design: We repeatedly transduced an adenoviral vector expressing the human p53 gene (AxCAhp53) into U373MG, a p53-susceptible cell line, and established the AxCAhp53-resistant cell line U373R. Most U373R cells survived even after AxCAhp53 treatment due to reduced transduction efficiency. Expression levels of adenovirus receptors were estimated to investigate the cause of reduced transduction efficiency. The mutant vector was used to overcome the resistance.

Results: The transduction efficiency of an adenoviral vector possessing the reporter β-galactosidase (LacZ) gene (AxCA2Z-F/wt) for U373R cells was 25.4-fold less than that for parent cells. The expression levels of integrins αvβ3 and αvβ5 were found to be decreased in U373R cells without affecting the expression levels of Coxsackievirus and adenovirus receptor. The mutant vector AxCA2Z-F/K20, with a linker and a stretch of 20 lysine residues at the COOH-terminal of the fiber protein, improved the transduction efficiency of U373R cells to 12.6-fold of that of AxCA2Z-F/wt. A mutant vector carrying the p53 gene, AxCAhp53-F/K20, dramatically induced apoptosis in U373R cells.

Conclusions: Glioma cells expressing low levels of adenovirus receptors might survive and proliferate to recur after repeated adenoviral transduction, even if the adenoviral transduction is effective at first. Changing the tropism of vectors is a potent method to overcome resistance.

INTRODUCTION

Replication-deficient recombinant adenoviruses are most widely used in ongoing or proposed clinical trials of gene therapy because of their large packaging capacity and their infectivity to a wide variety of cells. However, there are some obstacles that limit the use of these vectors for gene therapy. The major limitations of adenoviral vectors are the transient expression of the transgene in vivo and the poor transduction efficiency when readministered. These are caused in part by specific cellular immune responses to recombinant adenoviral vectors and the transgene product, innate immune clearance of adenoviral vectors in vivo, and a neutralizing antiviral humoral response. Host immune responses also limit the use of other viral vectors in vivo when readministered. Controlling host immunity is one of the potential ways to overcome these limitations. However, there is no evidence that tumors become resistant to adenoviral gene transduction by reduced transduction efficiency after repeated adenoviral transductions.

There are great hopes that gene therapy can manage malignant tumors, including gliomas, and various approaches to gene therapy are being tested (for review, see Ref. 3). High-grade gliomas show very malignant biological characteristics. The 5-year actuarial survival rate for glioblastoma is only 7.6%, despite adjuvant radiation and chemotherapy in addition to surgical removal (4). Adenoviral vectors carrying apoptosis-inducing genes are very attractive for the treatment of malignant gliomas. Tumor suppressor protein p53 has been reported to be a promising tool for the induction of apoptosis in tumor cells (5, 6). We have investigated the effects of gene transfer of wild-type p53 on malignant glioma cells. During this investigation, we have found that although U373MG cells are initially susceptible to p53 gene transduction, they become resistant after repeated gene transductions. This finding prompted us to examine the hypothesis that U373MG cells might become resistant due to the reduced transduction efficiency of adenoviral vectors. A fiber mutant vector that improves transduction efficiency on glioma cells was generated and used to examine whether it could overcome the resistance.

MATERIALS AND METHODS

Glioma Cell Lines. The U373MG human glioma cell line was obtained from American Type Culture Collection. The
cell line expresses mutant p53, as shown previously by both genetic and functional assays (7, 8). Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Adenoviral Vectors and Their Transduction Efficiency. The XbaI-XbaI fragment of human wild-type p53 cDNA from pRC/CMV (Invitrogen), provided by Dr. T. Takahashi (9), was inserted into the Nhel-Smal site of pClcC (Promega, Madison, WI; Ref. 10) to generate pCihp53. The human p53 expression cassette from pCihp53 was then ligated to the ClaI site of cosmid pAxcw, resulting in the pAXCI-hp53 cosmid.

The cosmid constructs for AxCAZ2-F/wt (an adenoviral vector possessing the reporter LacZ gene) and AxCAZ2-F/K20 (a mutant vector with a linker and a stretch of 20 lysine residues at the COOH-terminal of the fiber protein) were described in detail by Yoshida et al. (11).

Recombinant adenoviruses were generated by cotransfection of cosmids by the method described by Miyake et al. (12). Titers of infective adenovirus particles were evaluated by the standard plaque-forming assay with 293 cells after the vectors were propagated.

Adenovirus-mediated gene transduction was performed by a method described previously (10), and the transduction efficiency of the adenoviral vectors was evaluated by X-Gal staining using either AxCAZ2-F/wt or AxCAZ2-F/K20 (11, 13), both of which possess the LacZ gene as a reporter. Briefly, glioma cells were seeded in 24-well plates on day 0. The cells were infected with AxCAZ2-F/wt for 2 h at various MOIs and washed twice with PBS, and culture medium was added on day 1. X-Gal staining was performed on day 3, and the number of positive cells per 300 cells in three fields (100 cells/field; magnification, ×400) for each well was counted.

Selection of U373MG Cells Resistant to AxCIp53. A 10-cm-diameter dish of confluent U373MG cells was infected at a MOI of 1000. The adherent cells were fed fresh, complete medium at 4-day intervals. The cells were further infected two times with AxCIp53 (an adenoviral vector expressing the human p53 gene) at a MOI of 1000 after the apparently healthy cell population had grown out. The resistant cell line selected by this method was named U373R.

Flow Cytometry. The expression of integrins α6β1, α6β5, and β1 was analyzed with CELLQuest software using a FACScan (Becton Dickinson, San Jose, CA). Cells in exponential growth were washed three times with PBS and harvested in a single cell suspension using PBS containing 0.5 mM EDTA alone. The harvested cells were centrifuged and washed twice with PBS containing 5% FBS. Then, 5 × 10⁵ cells were incubated with primary antibodies for 30 min at room temperature, further incubated with FITC-labeled secondary antibody for 30 min at room temperature, and washed twice with PBS containing 5% FBS. The cells were then analyzed by flow cytometry. Ten thousand cells were counted in each analysis in this experiment.

Chromatographically purified mouse IgG1 and mouse anti-human integrins α6, β1, and α6, β5 monoclonal antibodies were purchased from Chemicon International, Inc. (Temecula, CA). Mouse anti-human integrin α6 (CD51) and β3 (CD61) monoclonal antibodies were obtained from Immunotech (Marseille, France). FITC-labeled antimouse IgG was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Western Blotting. The protein expression level of integrin β3 was evaluated by Western blotting as described previously (14). Briefly, cultured cells were lysed in lysis buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 20 mM EDTA, 0.5% NP40, 1 mg/ml leupeptin, 1 mg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride] for 30 min on ice and centrifuged at 15,000 rpm for 30 min. Protein concentration was analyzed by a DC protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. A total of 50–200 μg of protein was extracted from 2 × 10⁶ cells. The cell lysates were then frozen quickly and stored at −80°C until use for further analysis. An equal amount of protein (15 μg) from each extract was resolved by electrophoresis on 10% polyacrylamide gels and electroblotted to Immobilon transfer membranes (Millipore, Bedford, MA) at a constant current of 400 mA for 90 min. The membranes were blocked with 5% nonfat dried milk in PBS for 2 h and then incubated with the primary antibodies at a 1:1000 dilution at 4°C overnight. The membranes were washed in PBS, alkaline phosphatase-conjugated secondary antibodies were added at 1:250 dilution, and the membranes were incubated for 2 h at room temperature. The immune signal was detected using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium membrane phosphatase substrate (KPL, Gaithersburg, MD).

Rabbit anti-integrin β3 subunit polyclonal antibody was purchased from Chemicon International, Inc.

Detection of CAR mRNA by RT-PCR. Total cellular RNA was isolated by Isogen (Nippon Gene) according to the manufacturer’s protocol. Two μg of total cellular RNA were reverse transcribed using oligo(dT)₁₂₋₁₈ as primer (Boehringer Mannheim, Indianapolis, IN) and Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim) in a 20-μl reaction. One μl of reverse transcript was used directly for each reaction. In a 50-μl reaction, 25 nmol of forward and reverse primers; 200 μM each of dATP, dCTP, dGTP, and dTTP (Phar-macia); and 47 μl of super Taq premix kit (Sawady Technology, Tokyo, Japan) were used for PCR. The PCR primers used were as follows: (a) CAR sense primer, 5′-CCG-GAA-TTC-CCT-ACC-A₁⁰⁰-TG-GAT-TTC-GCC-AGA-AGT-TTG-AGT-ACT-ACT₄₂₈-3′; (b) CAR antisense primer, 5′-CCG-GAA-TTC-CCT-ACC-A₁⁰⁰-TG-GAT-TTC-GCC-AGA-AGT-TTG-AGT-ACT-ACT₄₂₈-3′; and (c) β-actin sense primer, 5′-GGG-TGG-GAT-AAT-CTT-GTG-AGA-GCC-GAT-CCA-GAT-3′; and (d) β-actin antisense primer, 5′-GGTC-TAA-AAC-GCA-GCT-CAG-TAA-CAG-TCC-GT₄₂₈-3′ (15). EcoRI

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3 The abbreviations used are: X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; CAR, Coxackievirus and adenovirus receptor; MOI, multiplicity of infection; ED₃₀, MOI for transduction of 50% of the population; RT-PCR, reverse transcription-PCR; CMV, cytomegalo virus.
and *NcoI* sites were added to the 5′ end of the CAR sense primer, and a *BamHI* site was added to the 5′ end of the CAR antisense primer. Reactions were incubated in a thermal cycler for 20, 30, and 35 cycles (denaturation, 60 s, 95°C; annealing, 60 s, 60°C; and extension, 90 s, 72°C). The same samples were amplified with β-actin-specific primers to obtain a ratio of CAR:housekeeping gene signal for semiquantitative analysis. The signal intensity was measured by NIH Images version 1.55. Total RNA in 293 cells was used as a positive control for CAR expression.

**Cell Adhesion and Growth.** The ability of cells to adhere to culture plates was estimated by counting the number of cells at 1 and 4 h after seeding 2 × 10⁵ cells in each well of 6-well culture plates (Iwaki Glass). Cells in exponential growth were washed three times with PBS, treated with PBS containing 0.5 mM EDTA alone, harvested in a single cell suspension with culture media containing 10% fetal bovine serum, and deposited in the plates for adhesion. After the cells were cultured for an appropriate time at 37°C, they were trypsinized, harvested, and counted using a cell counter (CDA500; Sysmex, Kobe, Japan). For the growth assay, 1 × 10⁵ cells were seeded on day 0. The numbers of cells were counted on days 2, 4, and 6. The culture medium was changed on days 2 and 4.

**Statistical Analysis.** Data are shown as the mean ± SD. The ED₅₀ was calculated using a logarithmic equation (CA-Cricket Graph III, version 1.5J; Computer Associates International). Data were analyzed by two-sample *t* test, one-way factorial ANOVA, or two-way factorial ANOVA using StatViewJ, version 4.5 (Abacus Concepts, Berkeley, CA). *P* < 0.05 was considered statistically significant.

**RESULTS**

**Effects of AxCIhp53 on U373MG Glioma Cells.** Because malignant tumors, including gliomas, often have mutated p53, the use of wild-type p53 may be a promising tool for gene therapy of malignant tumors. However, transduction of wild-type p53 alone by the adenoviral vector AxCIhp53 was not sufficient to induce apoptosis in glioma cell lines in our preliminary experiments (data not shown). In addition to U373MG, we tested four other established cell lines (A172, U251MG, T98G, and U87MG) and eight primary cell lines. A172, U251MG, and T98G express mutant p53, and U87MG expresses wild-type p53 (7, 8). The p53 statuses of the eight primary cell lines are not examined. Of these cell lines, U373MG and U251MG were susceptible to AxCIhp53. Fig. 1 shows the effects of AxCIhp53 on the growth of U373MG cells. AxCIhp53 strongly inhibited the growth of U373MG cells even at a MOI of 100 (*P* < 0.005 compared with AxCAZ2-F/wt transduced cells). Only 3.6% of the seeded cells survived on day 4 at a MOI of 500. FACScan analysis using the terminal deoxynucleotidyl transferase-mediated nick end labeling method and transmission electron microscopy revealed the induction of apoptosis in U373MG cells by transduction with AxCIhp53 but not AxCAZ2-F/wt on day 2 (data not shown).

**Selection of U373MG Cells Resistant to AxCIhp53.** Repeated transduction of the expanded culture may enrich the population of cells resistant to AxCIhp53. We transduced confluent U373MG cells in 10-cm culture dishes at a MOI of 1000. Most cells underwent apoptosis at this MOI, and only a few cells remained adherent. The adherent cells were fed fresh complete medium at 4-day intervals, and an apparently healthy population of cells grew out in about 1 month. These cells were infected a second time with AxCIhp53 at a MOI of 1000 and then processed exactly as described for the first infection. The number of apoptotic cells was less than that of the first infection, and it took about 2 weeks to recover a healthy cell population. These cells were infected a third time at a MOI of 1000. Most cells survived after three rounds of infection. The cells were trypsinized, replaced at a 1:8 dilution twice a week, and named U373R cells.

**Transduction Efficiency of AxCAZ2-F/wt for U373R Cells.** Fig. 2 shows the effects of AxCIhp53 on the growth of U373R cells at a MOI of 1000. The growth of AxCIhp53-transduced U373R cells was similar to that of AxCAZ2-F/wt-transduced cells. The number of AxCIhp53-transduced U373R cells grew 17.5-fold from the level of seeded cells by day 6, demonstrating the ineffectiveness of AxCIhp53 on U373R cells. To clarify the mechanism by which U373R cells acquired resistance, we first evaluated the transduction efficiency of adenoviral vectors for U373R cells. Fig. 3 shows the results of transduction efficiency of AxCAZ2-F/wt for U373R cells and U373MG cells, as estimated by X-Gal staining. The average ED₅₀ of U373MG cells was 31.24, and 99.42% of cells were positive for X-Gal staining at a MOI of 250. In contrast, the average ED₅₀ of U373R cells was 794, significantly greater than that for U373MG cells (*P* < 0.05). The transduction efficiency of U373R cells was 92.8% even at a MOI of 5000.

**Expression of Integrins and CAR.** Two different receptors are associated with the internalization of group C adenoviral vectors for U373R cells. Fig. 3 shows the results of transduction efficiency of AxCAZ2-F/wt for U373R cells and U373MG cells, as estimated by X-Gal staining. The average ED₅₀ of U373MG cells was 31.24, and 99.42% of cells were positive for X-Gal staining at a MOI of 250. In contrast, the average ED₅₀ of U373R cells was 794, significantly greater than that for U373MG cells (*P* < 0.05). The transduction efficiency of U373R cells was 92.8% even at a MOI of 5000.
Glioma Cells Resistant to Adenovirus Vector with p53

Fig. 2 Effects of AxCbp53 on the growth of U373R cells. Cells were transduced with adenoviral vectors at a MOI of 1000. The numbers of cells were counted on days 2, 4, and 6. The Y axis shows the cell numbers relative to the numbers of U373MG cells on day 0. Transduction with AxCZ2-F/wt, ○; transduction with AxCbp53, ●; no transduction (control), ×. The data shown are representative of two independent experiments. Values are the means ± SD (bars) of duplicate determinations.

Fig. 3 Transduction efficiency of AxCZ2-F/wt into U373MG (dotted line) and U373R (solid line) cell lines. Two days after infection with AxCZ2-F/wt at various MOIs, the cells were stained with X-Gal. More than 300 cells from each of three randomly selected high-power fields (×400) were counted under a light microscope. The X axis shows MOIs in a logarithmic scale, and the Y axis shows the percentage of positively stained cells. ED_{50} was calculated using a logarithmic equation (as described in “Materials and Methods”). The data shown are representative of three independent experiments. Values are the means ± SD (bars) of duplicate determinations.

Characteristics of U373R Cells. The morphology of U373R cells is quite different from that of the parental U373MG cells. The U373MG cells appear fan-shaped and show distinct ruffling around the cytoplasm, whereas U373R cells form short processes and show little ruffling (data not shown).

Fig. 4 shows the abilities of U373MG and U373R cells to adhere to the culture plate. U373MG cells adhere readily to the culture plate. After 1 h of incubation, 86.5% of seeded U373MG cells adhered to the culture plate, and by 4 h, all of the seeded cells had attached themselves to the culture plate. In contrast, U373R cells were less capable of attaching themselves to the culture plate. Only 56.5% and 62.0% of seeded U373R cells adhered to the culture plate after 1 and 4 h of incubation, respectively (P < 0.005 and P < 0.0001, respectively).

The growth rate of U373R cells was slightly but significantly slower than that of U373MG cells (Fig. 5A). The number of U373R cells counted on days 2, 4, and 6 was 80.4%, 73.7%, and 83.4% that of U373MG cells, respectively (P < 0.01, 0.005, and 0.05 on days 2, 4, and 6, respectively).

Transduction Efficiency of AxCZ2-F/K20 and the Effect of AxCbp53-F/K20, a Mutant Vector Carrying the p53 Gene. As reported previously, we generated a fiber mutant adenoviral vector that has a linker and a stretch of 20 lysine residues added to the COOH terminus of the fiber. The vector with a fiber mutant, AxCZ2-F/K20, showed remarkably enhanced efficiency of gene transduction in human glioma cells (11). We transduced AxCZ2-F/K20 into U373R cells to determine whether the vector could improve the transduction efficiency for the U373R cell line as for other glioma cell lines. The ED_{50} in U373MG and U373R cells was 31.24 and 794,
respectively, when the cells were transduced with AxCAZ2-F/wt. AxCAZ2-F/K20 significantly improved the transduction efficiency for both U373MG and U373R cell lines. The ED50 of U373MG and U373R was 7.03 and 63.2, respectively (Fig. 6A).

Fig. 6B shows the results of X-Gal staining of U373R cells transduced with either AxCAZ2-F/wt or AxCAZ2-F/K20 at a MOI of 250. Ninety-three percent of U373R cells treated with AxCAZ2-F/K20 were positive. In contrast, only 22.7% of U373R cells treated with AxCAZ2-F/wt were positive.

The effect of AxCAhp53-F/K20 on U373R cells is shown in Fig. 7. The expression levels of adenovirus receptors in U373MG and U373R cells. A, FACScan analysis of integrin αvβ3, integrin αvβ5, integrin subunit αv, and integrin subunit β3. The top row shows the results in U373MG cells, and the bottom row shows the results in U373R cells. The data shown are representative of three independent experiments. B, Western blot analysis of integrin β3 and cyclin E (control). Lane 1, U373R cells; Lane 2, U373MG cells. The data shown are representative of three independent experiments. C, semiquantitative analysis of CAR mRNA by RT-PCR. The top row shows the expression levels of CAR. The middle row shows the expression levels of β-actin as a control study. The bottom row shows the results of densitometric analyses. Bars represent mean values of three independent determinations, and brackets represent SDs (*, P < 0.005; **, P = 0.08). Reaction mixtures were incubated in a thermal cycler for 20, 30, and 35 cycles. Lane 1, x174 DNA HaeIII digest (New England Biolabs, Inc., Beverly, MA); Lane 2, U373MG cells; Lane 3, U373R cells; Lane 4, 293 cells. The data shown are representative of three independent experiments.
CAhp53-F/K20 possesses a chicken β-actin promoter (CA). To confirm that the apoptosis induced by AxCAhp53-F/K20 is due to improved transduction efficiency rather than to the effect of the CA promoter, we compared the effects of AxCAhp53-F/wt and AxClhp53 on U373R cells (Fig. 6D). As shown in the figure, the effects of AxCAhp53-F/wt were similar to those of AxClhp53, demonstrating that the dramatic induction of apoptosis is due to an improvement in transduction efficiency rather than an effect of the CA promoter.

**DISCUSSION**

Two kinds of receptors, the fiber knob receptor and the penton base receptor, are necessary for the internalization of adenoviral vectors (for review, see Refs. 16 and 17). The expression of both receptors play a crucial role in the transduction efficiency of adenoviral vectors (18–21). Because gliomas, especially high-grade gliomas, express significantly higher amounts of penton base receptors, integrin subunits αv and β3, and integrin αvβ3, than normal brain tissue (22–24), it would be advantageous to use adenoviruses as a vector for gene therapy for gliomas. However, here we provide the first evidence that human tumor cells expressing low levels of adenovirus receptors will survive and proliferate to recur after repeated gene transduction, although the gene transduction is effective at first.

Adenovirus infection starts with the attachment of the viral fiber protein to its cellular receptor, CAR (for review, see Refs. 16 and 17). After fiber-mediated attachment to the cell, the penton base binds via an RGD motif to the αvβ3 and αvβ5 integrin receptors. The cell surface-bound viruses are internalized via receptor-mediated endocytosis. The viruses are then released into the cytosol and bind to the nuclear pore complexes. Next, they release their DNA into the nucleus, and DNA transduction starts (for review, see Ref. 17). The inhibition of any of these steps will cause resistance to infection. In the present study, the ED50 of AxCAZ2-F/wt for U373R was 25.4 times higher than that for U373MG cells, suggesting that reduced transduction efficiency is the major cause of the acquired resistance. FACScan analysis, Western blotting, and RT-PCR revealed that U373R cells express reduced levels of penton base receptors and integrins αvβ3 and αvβ5, without changing the expression levels of the fiber knob receptor CAR. Integrins αvβ3 and αvβ5 are known to be receptors for vimentin, the main adhesive protein in routine cell culture media (25). Cell interaction with vitronectin may induce cell spreading and migration and have positive effects on cell growth (26). U373R cells showed little ruffling, grew more slowly, and were less capable of adherence to the culture plate than parental U373MG cells. All of these characteristics are compatible with the reduced expression levels of integrins, further supporting the notion that resistance is caused by low expression levels of integrins. The decrease in the expression levels of integrin subunits αv and β3 in U373R cells was not so prominent as that of integrins αvβ3 and αvβ5. Integrin subunit αv forms heterodimers not only with integrin subunits β3 and β5, but also with integrin subunits β1, β6, and β8. Similarly, integrin subunit β3 forms a heterodimer with α10 (27). The total amounts of integrin subunits αv and β3 on the cell surface of U373R cells may not decrease as expected.
Recently, other integrin subunits have been suggested to modulate adenovirus-mediated gene transfer (28). We have not examined the expression levels of integrins other than integrins \( \alpha_9 \beta_1 \) and \( \alpha_9 \beta_3 \), and other integrins might also contribute to the transduction efficiency of adenoviral vectors. CAR is indispensable for adenovirus adsorption (18, 19), and the expression level of CAR is reported to be more critical for adenoviral infection than those of integrins (29–31). These reports focused

**Fig. 6** Transduction efficiency and effects on cell growth using AxCAZ2-F/K20 and AxCAhp53-F/K20. **A**, transduction efficiency. U373R (solid lines, circles) and U373MG (dotted lines, squares) cells were transduced with either AxCAZ2-F/K20 (filled symbols) or AxCAZ2-F/wt (open symbols). The X axis shows the MOI in logarithmic scale, and the Y axis shows the percentage of LacZ-positive cells detected by X-Gal staining. The data shown are representative of three independent experiments. Values are the means ± SD (bars) of duplicate determinations. **B**, photomicrographs show the results of X-Gal staining. U373R cells were transduced with either AxCAZ2-F/wt (top panel) or AxCAZ2-F/K20 (bottom panel) at a MOI of 250. Original magnification, \( \times 100 \). **C**, cytotoxic effects of AxCAhp53-F/K20 on the growth of U373R cells. Cells were transduced with fiber mutant adenovirus at MOIs of 100 (squares), 250 (rhombuses), and 500 (circles). The cells were counted on days 2, 4, and 6. The Y axis shows cell numbers relative to the number of cells on day 0. Transduction with AxCAZ2-F/K20, open symbols; transduction with AxCAhp53-F/K20, filled symbols; no transduction (control), \( \times \). The data shown are representative of two independent experiments. Values are the means ± SD (bars) of triplicate determinations (\( *, P < 0.005 \)). **D**, effects of a CMV promoter (CI) and a chicken \( \beta \)-actin promoter (CA) on the growth of U373R cells. The Y axis shows cell numbers relative to the number of cells on day 0. Transduction with AxCIhp53 (○) or AxCAhp53-F/wt (●) is shown at a MOI of 500. No transduction (control), \( \times \). The data shown are representative of two independent experiments. Values are the means ± SD (bars) of triplicate determinations.
mainly on the expression levels of CAR. None or few of the integrins were subjected to evaluation of their expression levels, although multiple integrins have the potential to mediate adenoviral endocytosis into cells (32). In this report, we estimated the expression levels of both CAR and integrins and found that the expression levels of integrins in U373R cells were lower than those in U373MG cells, whereas both cell lines showed similar expression levels of CAR. Our results clearly demonstrate the significance of the integrins on adenoviral infection under similar CAR levels. Pearson et al. (28) also indicated the significance of integrin expression. According to Asaoka et al. (33), wild-type p53 may repress the expression levels of CAR. In our experiments, no significant difference in the expression levels of CAR was detected between U373R and U373MG cells, despite the fact that U373R cells were selected by wild-type p53. First of all, U373MG cells are very susceptible to wild-type p53, and few cells survive after transduction of the p53 gene. Secondly, adenovirally transduced gene expression is transient, and it is unlikely that the transduced gene affects exponentially growing cells. Therefore, adenovirally transduced p53 seems to have little effect on the expression levels of CAR in U373R cells. In our laboratory, the efficacy of adenoviral-delivered wild-type p53 tends to correlate at least with the expression level of CAR, as other laboratories have indicated (30, 31), which would corroborate de novo sensitivity to restored wild-type p53 as a therapy for gliomas. However, there might also be other mechanisms that regulate susceptibility of glioma cells to wild-type p53.

A single round of gene delivery may not be sufficient to eradicate malignant tumors in clinical use, and multiple administration of vectors will be indispensable. However, we have shown the possibility that tumors become resistant to vectors by repeated administration because the population of cells with low expression levels of receptors will survive and proliferate irrespective of the genes transduced. To overcome this resistance, a new device for improving transduction efficiency is indispensable. There are several strategies to enhance the efficiency of adenovirus gene transduction. The simplest approach is to use higher MOIs to get higher transduction. This approach, although simple, is costly and laborious in clinical gene therapy situations. Moreover, dose-limiting vector-related toxicities (e.g., immune reaction, reaction to toxic adenovirus components, and replication-competent adenovirus) may limit the overall therapeutic index achievable with adenoviral vectors. Another approach is to test chemical methods and physical conditions to enhance adenovirus infection efficiency (34). An alternative strategy is to develop adenovirus mutants. Mutations in the fiber knob or penton base will change the tropism of target receptors. The advantage of the F/K20 adenoviral vector on glioma cells was described previously in detail by Yoshida et al. (11). In the present study, the F/K20 adenoviral vector also improved the transduction efficiency into U373R cells, and AxCAhp53-F/K20 strongly induced apoptosis in U373R cells. The results indicate the advantages of using F/K20 adenoviral vector not only for established glioma cell lines but also for cells whose receptors for F/wt adenoviral vectors are significantly reduced.

Wickham et al. have generated either seven lysine residues (35) or variable numbers (36) of lysine residues at the COOH-terminal end of the fiber. The receptor molecules for these fibers with lysine oligomers are supposed to be the negatively charged cell surface molecules such as heparin and heparan sulfate. These heparan sulfate proteoglycans are expressed in large amounts in gliomas, especially in high-grade gliomas (37, 38). There might be a positive correlation between the expression levels of heparan sulfate proteoglycans and the transduction efficiency of F/K20 adenovirus vectors, leading to efficient gene transduction into human glioma cells as demonstrated by Yoshida et al. (11). The expression levels of heparan sulfate proteoglycans may be independent of those of integrins, and heparan sulfate proteoglycans may remain abundant in U373R cells as they do in U373MG cells. Consequently, F/K20 adenovirus could improve the transduction efficiency of U373R cells and thereby induce apoptosis by p53 protein. Further study is necessary to elucidate the mechanism.

This report suggests the disadvantages of repeated administration of the same vector and further advantages of F/K20 adenovirus. In general, tumor cells are heterogeneous in terms of levels of integrins. Therefore, some cells intrinsically resistant to adenoviral infection because of low expression of adenovirus receptors will survive and proliferate to recur, which is very critical in clinical use. This problem may not be limited to gliomas but could also be applicable to other malignant tumors. It may also be said that this is not only a problem with adenoviral vectors with wild-type fiber knob but also in other vectors that are integrated through specific receptors. Changing the tropism of vectors is a potent method to overcome resistance. In addition to low expression levels of vector receptors, the Achilles’ heel of this and all p53-based gene transfer approaches is that it is simply not possible to deliver the transgene into all cells. Therefore, these approaches will always have a population of tumor cells that will regrow. Multiple gene therapy strategies (e.g., suicide or prodrug strategies, immunogene therapy, and antiangiogenesis) should also be applied together with this replacement/therapeutic gene transfer.

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