An Adenovirus Mutant That Replicates Selectively in p53-Deficient Human Tumor Cells

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The human adenovirus *E1B* gene encodes a 55-kilodalton protein that inactivates the cellular tumor suppressor protein p53. Here it is shown that a mutant adenovirus that does not express this viral protein can replicate in and lyse p53-deficient human tumor cells but not cells with functional p53. Ectopic expression of the 55-kilodalton E1B protein in the latter cells rendered them sensitive to infection with the mutant virus. Injection of the mutant virus into p53-deficient human cervical carcinomas grown in nude mice caused a significant reduction in tumor size and caused complete regression of 60 percent of the tumors. These data raise the possibility that mutant adenoviruses can be used to treat certain human tumors.

Deletion or mutation of the p53 tumor suppressor gene occurs frequently in most types of human cancer (*1*). Tumors lacking functional p53 are, in many cases, refractory to chemotherapy or radiation (*2*). It is therefore critically important to develop therapeutic strategies to treat p53-deficient tumors.

DNA tumor viruses such as adenoviruses infect quiescent cells and induce them into the S phase of the cell cycle so that viral DNA replication can proceed (*3*). The E1A protein of human adenoviruses, which binds pRB, p300, and other related proteins, is largely responsible for this forced entry into the S phase (*4*). The *E1B* region of the viral genome encodes a 55-kD protein (E1B 55K) that binds and inactivates p53 (*5*). This binding is essential to virus replication, possibly because E1A induces p53-dependent apoptosis. We hypothesized that an adenovirus mutant that does not produce E1B 55K should be unable to replicate in normal cells but would be able to replicate in cells lacking functional p53. The dl1520 virus is such a mutant. This human group C adenovirus contains an 827–base pair deletion in the *E1B* region and a point mutation at codon 2022 that generates a stop codon preventing expression of a truncated protein from the deleted gene (*6*). The *E1B 19K* gene, whose protein product suppresses apoptosis, is not affected by this deletion.

We first tested the ability of this virus to grow in cells lacking functional p53. The dl1520 virus grew as efficiently as wild-type adenovirus in C33A cervical carcinoma cells, which express p53 with an inactivating mutation at codon 273 (*7*) (Fig. 1A). Under identical conditions, dl1520 grew poorly in U20S osteocarcinoma cells, which retain wild-type p53 (*8*), producing about 100 times less infectious virus than did wild-type adenovirus. Polymerase chain reaction procedures (*9*) were used to verify that the virus produced by dl1520-infected C33A cells retained the *E1B* deletion and that the infection was not due to wild-type adenovirus contamination (*9*).

We also performed cytopathic effect (CPE) assays on a panel of human tumor cells and normal cells infected with either dl1520 or wild-type adenovirus. The dl1520 virus had no detectable cytopathic effect on normal human diploid fibroblasts or on tumor cells retaining wild-type p53, whereas wild-type adenovirus caused compete lysis under identical conditions (Fig. 1B). In contrast, both dl1520 and wild-type adenovirus killed C33A cells with high efficiency. This analysis was extended to a variety of tumor cell lines of known p53 status (*10*), including four cervical carcinoma cell lines expressing human papillomavirus (HPV) E6, which inactivates p53 through ubiquitin-mediated protein turnover (*7*); four colon carcinoma cell lines containing different mutant forms of p53; U373 glioblastoma cells (codon 273 mutation) (*11*); and HS700T pancreatic adenocarcinoma cells (codon 249 mutation) (*12*). In each case, dl1520 killed cells with an efficiency comparable to that of wild-type adenovirus (*10*).

These results show a clear correlation between p53 status and susceptibility to dl1520 and are consistent with our original hypothesis. However, the dl1520 deletion affects genes other than that encoding E1B 55K; it also deletes a coding sequence from part of the *E3* region (*11*). To test whether the restricted host range of dl1520 is specifically due to loss of E1B 55K, we constructed a U2OS cell line that expressed E1B 55K under the control of the human cytomegalovirus immediate-early promoter (*13*). We predicted that this would render the U20S cells susceptible to dl1520 infection. The E1B 55K protein produced in these transfected cells (designated UFL-A) interacted with endogenous p53 protein, as demonstrated by co-immunoprecipitation of E1B 55K with an antibody against p53, and vice versa (Fig. 2). To test whether these cells had become sensitive to dl1520, we infected them with dl1520 or wild-type adenovirus and monitored them for CPE. Staining of cells with crystal violet 5 days after infection with dl1520 revealed little evidence of CPE in control cells transfected with empty vector (U-vec cells), whereas UFL-A cells were completely lysed by dl1520. Wild-type adenovirus lysed U-vec and UFL-A cells with comparable efficiency. These data confirm that the inability of dl1520 to replicate in and kill $p53^+$ cells is due to lack of E1B 55K expression.

To confirm that the attenuation of dl1520 was due to its inability to inactivate p53, we constructed a U2OS cell line that expressed a mutant E1B 55K protein that was incapable of binding and inactivating p53. The p53-binding domain of the E1B 55K protein [residues 215 to 354 of the 55K protein (*5*)] was deleted at the DNA level, and the modified gene was transfected into U20S cells. A cell line (U4.5-H) expressing the mutant protein was isolated and characterized. The mutant E1B protein was expressed efficiently but did not bind to p53 (Fig. 3), and in transactivation assays with a p53-dependent reporter plasmid, significant p53 activity was retained relative to cells expressing empty vector. In CPE assays, these cells were resistant to dl1520. These data suggest that the inability of dl1520 to produce E1B 55K protein capable of binding p53 is responsible for its inability to replicate efficiently in $p53^+$ cells.

Further evidence to support the hypothesis that the host restriction of dl1520 is due to p53 is presented in Fig. 4. RKO human colon carcinoma cell lines have previously been shown to express functional p53, and a derivative has been made (RKO.p53.13) in which p53 function has been ablated by expression of a dominant negative p53 allele (*14*). RKO cells were not killed by dl1520 at multiplicities of infection (MOIs)

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up to one plaque-forming unit (PFU) per cell, whereas the p53-defective line was killed at MOIs of 0.01 PFU per cell. Thus, p53 inactivation increases sensitivity to dl1520 by a factor of about 100.

We next examined the therapeutic efficacy of dl1520 in vivo by growing $p53^-$ C33A cells and $p53^+$ U87 human glioblastoma multiforme cells as tumor xenografts in athymic mice (U20S cells do not grow as tumors in nude mice). The tumor cells were injected subcutaneously into each flank of each mouse, and after establishment of palpable tumors (mean tumor volume 150 μl), the tumors were directly injected with CsCl-purified wild-type adenovirus, with dl1520, or with ultraviolet (UV)-inactivated wild-type virus as a negative control every other day for three total doses. Tumor growth was then followed for 6 weeks, at which time the mean tumor volume in each group was determined. Treatment of C33A tumors with dl1520 resulted in an 84% reduction in mean tumor volume as compared with wild-type UV-inactivated adenovirus (unpaired two-tailed test: $P = 0.02$; Fig. 5A). One of the tumors treated with dl1520 underwent complete regression. Wild-type adenovirus was slightly more effective in reducing tumor volume (94% inhibition) at an equivalent dose. In contrast, dl1520-injected $p53^+$ U87 tumors were comparable in size to control-injected tumors after 6 weeks, although shrinkage was seen in some cases. Wild-type adenovirus caused significant tumor inhibition (64% inhibition, $P = 0.05$; Fig. 5B). In another experiment, C33A tumor xenografts were injected with dl1520 or buffer control each day for five consecutive days, and the tumor volumes were calculated weekly. The dl1520-treated tumors were significantly inhibited in their growth as compared with buffer controls (Fig. 5C). Of five treated tumors, three showed a complete regression and one showed a partial re-

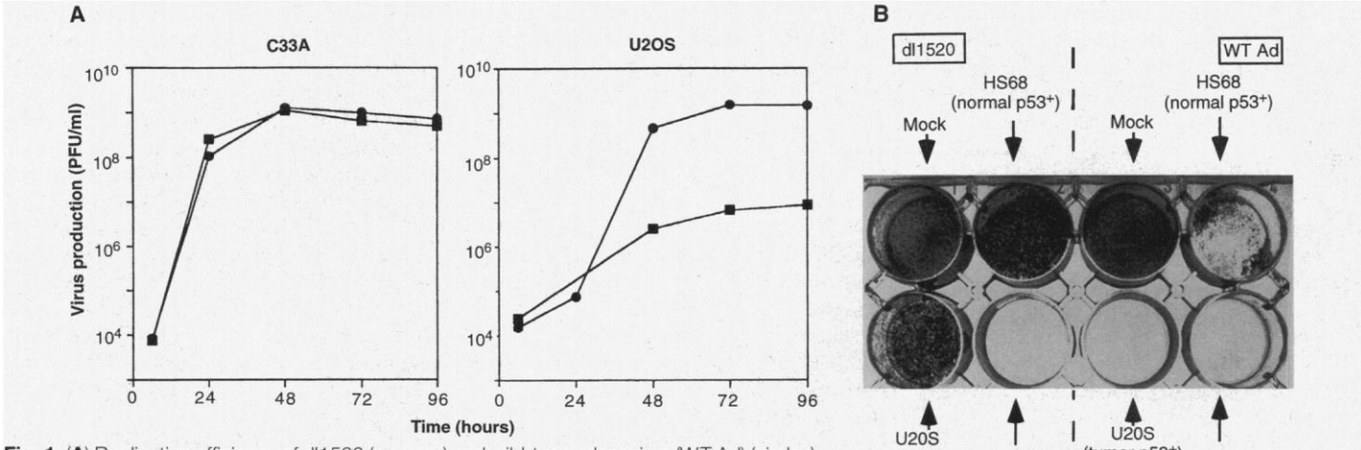

Fig. 1. (**A**) Replication efficiency of dl1520 (squares) and wild-type adenovirus (WT Ad) (circles) in $p53^-$ or $p53^+$ cells. C33A or U2OS cells were infected at a MOI of 1.0 PFU per cell with either WT Ad or dl1520, and virus production was measured by plaque assay (*18*). Data are means of duplicate determinations. (**B**) Selective lysis of $p53^-$ cells by dl1520. Monolayers of C33A cells, HS68 normal diploid fibroblasts, and U2OS cells were infected at a MOI of 10 PFU per cell with either dl1520 (left) or WT Ad (right). After 9 days, viable cells were stained with crystal violet. Uninfected C33A cells were used for the mock infection.

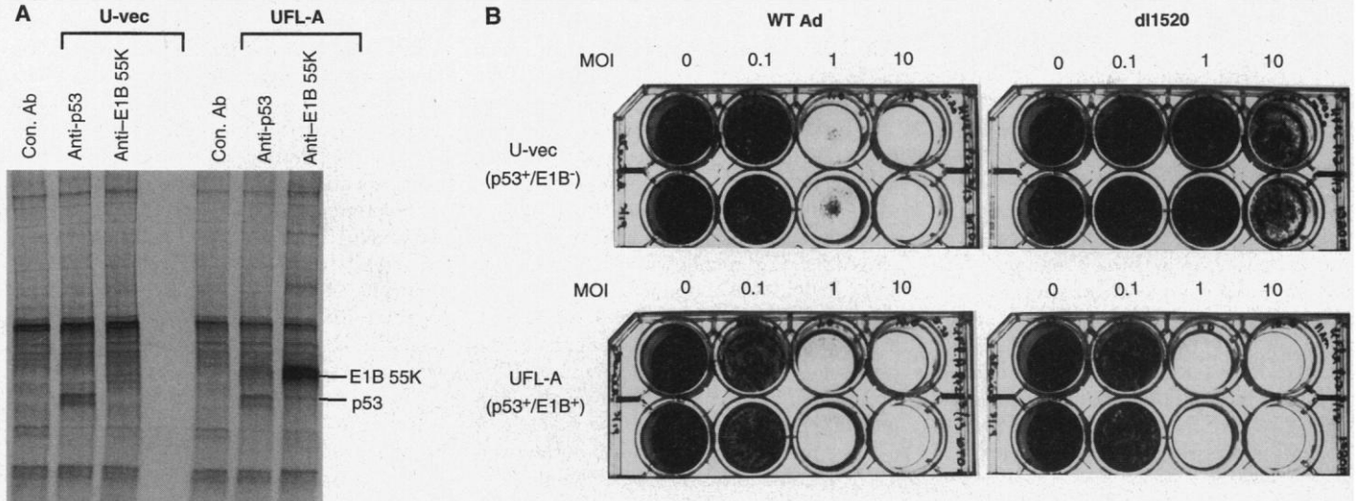

Fig. 2. (**A**) Characterization of a U2OS-derived cell line that expresses E1B 55K. U2OS cells were transfected with either pCMVneo (U-vec) or pCMVFL55 [UFL-A (*13*)]. Cell lysates labeled with [^{35}S]methionine were immunoprecipitated (*19*) with a control antibody (Con. Ab) (lanes 1 and 4); a monoclonal antibody against p53 (Oncogene Sciences) (lanes 2 and 5); or a monoclonal antibody against E1B 55K (Oncogene Sciences) (lanes 3 and 6). (**B**) Expression of E1B 55K renders $p53^+$ U2OS cells sensitive to dl1520. U-vec cells (top plates) and UFL-A cells (bottom plates) were infected with either WT Ad (left) or dl1520 (right) at MOIs of 0, 0.1, 1, or 10. Five days after infection, the plates were stained with crystal violet.

sponse. Tumors responding completely have been followed for over 3 months without evidence of regrowth. In three additional experiments, 12 of 18 C33A tumors injected with 10^8 PFU of dl1520 have shown complete regression (*10*).

To ensure that the reduction of C33A tumor mass was due to dl1520 virus replication, we analyzed sections of the excised tumor for Ad5 capsid proteins. The clear immunohistochemical staining of these sections with antibodies to Ad5 hexon protein (Fig. 6) indicates that dl1520 replicated and disseminated throughout the C33A tumors. No staining was seen in dl1520-treated U87 tumors.

Loss of function of the p53 tumor suppressor gene is the most common genetic defect in human malignancies, affecting more than 50% of all tumors. p53 is thought to monitor the integrity of the

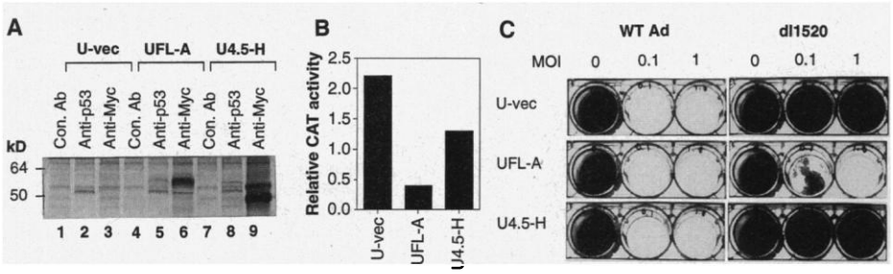

Fig. 3. (**A**) Expression of a mutant E1B 55K protein that does not bind to p53. U-vec, UFL-A, and U4.5-H cells (*13*) were labeled with [^{35}S]methionine and lysates were immunoprecipitated (*19*) with a control antibody (Con. Ab; lanes 1, 4, and 7); a monoclonal antibody against p53 (Oncogene Sciences) (lanes 2, 5, and 8); or a monoclonal antibody against the Myc epitope (lanes 3, 6, and 9). (**B**) The mutant E1B 55K protein produced by U4.5-H cells does not inhibit p53-dependent transactivation of transcription. U-vec, UFL-A, and U4.5-H cells were transfected with 1 μg of pCMVluc and 5 μg of pCOSX1CAT (*20*). CAT activity was normalized to the luciferase activity. (**C**) An E1B 55K mutant incapable of binding p53 does not render p53$^+$ cells sensitive to dl1520. U-vec, UFL-A, and U4.5-H monolayers were infected with either WT Ad (left) or dl1520 (right) at MOIs of 0, 0.1, or 1. Six days after infection, viable cells were stained with crystal violet.

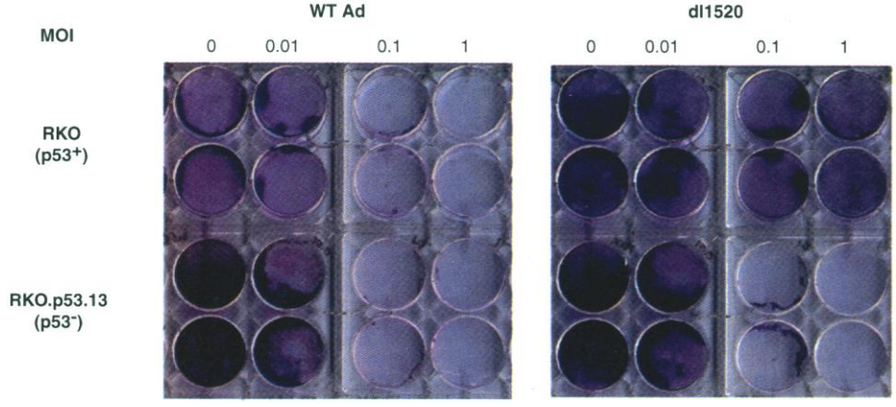

Fig. 4. Effects of dl1520 and WT Ad on RKO cells and RKO.p53.13 cells lacking functional p53. Cells were infected at the MOIs shown and stained for viability 8 days later.

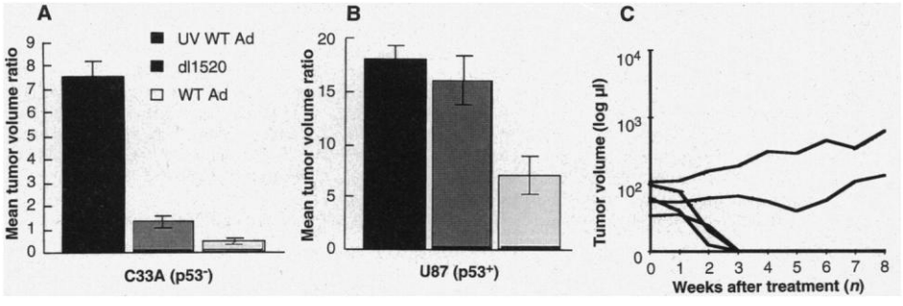

Fig. 5. Effects of UV-inactivated WT Ad, dl1520, or WT Ad on p53$^-$ and p53$^+$ human tumor xenograft growth. (**A**) C33A cells (p53$^-$) or (**B**) U87 human glioblastoma multiforme cells (p53$^+$) were injected subcutaneously into the flanks of *nu/nu* mice. Five weeks later, the tumor volume ratio was calculated (*21*). (**C**) C33A cells were injected subcutaneously as above. Once tumors reached about 80 μl in volume, they were injected with 10^8 PFU of dl1520 (solid lines) or buffer (dashed lines) for 5 days consecutively. The daily dose was divided equally into each tumor quadrant (15 μl per quadrant).

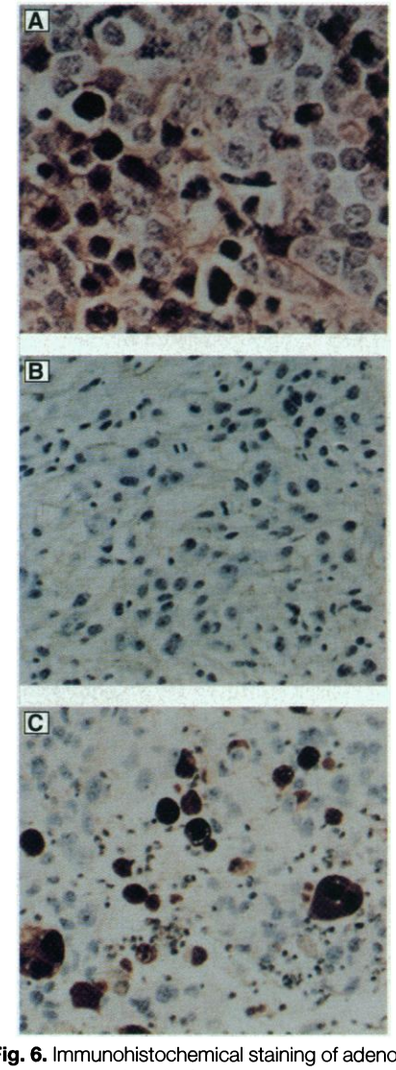

Fig. 6. Immunohistochemical staining of adenovirus hexon protein in human tumor xenografts treated with dl1520 or WT Ad. p53$^-$ C33A tumors and p53$^+$ U87 tumors were excised from nude mice 5 weeks after treatment with dl1520 or WT Ad. Tumors were immunostained with an antibody specific for adenovirus hexon protein (*22*). Cells containing the structural hexon protein, encoded by adenovirus late genes, are stained brown, indicating viral replication. (**A**) p53$^-$ C33A tumor treated with dl1520, showing positive staining in viable tumor cells. (**B**) p53$^+$ U87 tumor after treatment with dl1520, showing absence of hexon protein staining. (**C**) U87 tumor treated with WT Ad, showing numerous tumor cells positively stained for adenovirus hexon protein.

cellular genome and responds to DNA damage by inducing cell cycle arrest or apoptosis. Tumors lacking p53 are unable to mount these responses and therefore respond poorly to radiation or chemotherapy. Thus, a therapeutic strategy that allows selective killing of p53-deficient cells would be of great value.

The strategy presented here takes advantage of the p53 defect in human cancer cells to complement the growth of an adenovirus mutant that has a specific replication deficiency. Our results with cultured cells and tumor-bearing mice show that an adenovirus lacking the E1B 55K gene product replicates in and lyses tumor cells deficient in p53 but replicates 100 times less efficiently in cells expressing functional p53. Recently, it was reported that the orf6 region of adenovirus E4 can participate in p53 inactivation (*15*). The relative contributions of E1B 55K and E4orf6 toward p53 inactivation are currently being investigated.

Our results suggest that adenoviruses with host ranges restricted to tumor cells may be useful in treating human cancers. Several issues remain to be addressed. First is the degree to which the host immune response may affect virus spread and cell killing. Unfortunately, the host range of human adenoviruses is restricted, and no appropriate animal models exist to allow further exploration of this issue. However, the replication of dl1520 is restricted to tumor cells: An immune response directed at late viral antigens expressed on the surface of infected tumor cells might augment tumor killing in an immunocompetent host. In addition, the intratumoral recruitment and stimulation of tumor-specific T lymphocytes theoretically could lead to systemic antitumor immunity. Second, the ability of the virus to spread to distant sites and to infect metastatic tumor cells needs to be addressed because direct intratumoral injection limits the potential benefit of this approach to accessible tumors (primary brain tumors and cancers of the head and neck, for example). However, the selectivity and potency of this virus suggest that this approach should be tested in the clinic. Because of these data as well as data showing that dl1520 is not toxic in mice or cotton rats at doses up to 10^9 PFU (*16*), dl1520 is being tested in Phase I trials in patients with $p53^-$ squamous cell carcinoma of the head and neck (*17*).

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13. A Myc epitope tag was added at the DNA level to the NH_2-terminus of E1B 55K, and the construct was subcloned into pCMVneo. This clone was designated pCMVFL55. Another construct was made in which amino acids 216 through 354 of the Myc-tagged full-length E1B 55K were deleted. This clone, which was expressed in U4.5H cells, was designated pCMV55D4.5. The inserts of pCMVFL55k and pCMV55D4.5 were sequenced to confirm that they were correct (*9*).
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16. Twenty cotton rats (the cotton rat is a permissive host for human adenovirus) were given intraperitoneal injections of up to 10^9 PFU of dl1520 per animal. No evidence of toxicity was seen in these animals, as assayed by animal weight gain, serum chemistries, and organ histopathology (including the liver, lung, kidneys, heart, gastrointestinal tract, and brain).
17. Phase I tests of dl1520 (ONYX-015) began in April 1996 at the University of Texas, San Antonio (by Daniel Von Hoff) and the Beatson Institute, Glasgow, Scotland (by Stanley Kaye).
18. Plates were scraped into 1 ml of media and frozen. Lysates were prepared by three cycles of freezing and thawing, followed by a 30-s pulse in a sonicator water bath. Serial dilutions of the lysates were titered on HEK293 cells (human embryonic kidney cells expressing the *E1* region of Ad2).
19. Cells were harvested after being incubated in media containing 200 μCi of [^{35}S]methionine for 2 hours at 37°C. The cells were lysed in 50 mM Hepes (pH 7.9), 250 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.1 mM Na orthovanadate, and 0.1% Triton X-100. The protein concentration was determined by Bradford analysis. Equal amounts of protein were immunoprecipitated with the appropriate antibody for 1 hour at 4°C, protein G–Sepharose (Sigma) was added, and the samples were incubated for an additional 30 to 60 min at 4°C. The immunocomplexes were washed three times in lysis buffer, resuspended in SDS sample buffer, and resolved on 10% polyacrylamide gels. The gels were fixed, dried, and subjected to autoradiography.
20. Lysates were prepared 36 hours after transfection and chloramphenicol acetyl transferase (CAT) (Boehringer-Mannheim), and luciferase (Promega) assays were performed according to the manufacturer's instructions.
21. C33A human cervical carcinoma and U87 glioblastoma multiforme cells were obtained from the American Type Culture Collection. Female athymic *nu/nu* mice were obtained from the Harlan Sprague-Dawley Company at 4 to 6 weeks of age and were quarantined for at least 2 weeks before the study. Animal experiments were carried out in accordance with both institutional and federal animal care regulations. U87 and C33A cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose and supplemented with 10% fetal bovine serum, nonessential amino acids, L-glutamine, and penicillin and streptomycin until confluence. Cells were harvested through two consecutive trypsinizations, centrifuged at 300*g* for 8 min, washed twice, and resuspended in serum-free DMEM, and the cell density was adjusted to 5×10^7 cells/ml. 1.0×10^7 cells in 0.2 ml were injected subcutaneously into each flank of 7- to 10-week-old female nude mice. Tumor volumes were estimated with the following formula: (maximal length) × (perpendicular width)^2/2. Once tumors reached a mean size of 110 to 150 μl, animals were directly injected with 10^9 PFU of dl1520 (n = 10 U87 tumors and 5 C33A tumors), Ad2 (n = 10 tumors), or UV-inactivated Ad2 (n = 6 tumors) divided equally into four tumor quadrants (15 μl per quadrant) every other day for three total doses. Tumor volumes were recorded weekly until termination of the study. At the time of termination, the tumor volume ratio was calculated as follows: (tumor volume at study termination)/(tumor volume at the time of virus injection). The unpaired t test (two-tailed) was used to compare final tumor volume ratios in various groups.
22. Immunohistochemistry was performed on formalin-fixed paraffin-embedded tumors that had been cut into 4-μm sections, hydrated, and digested with pronase. The primary antibody (MAB805, Chemicon International) is specific for all 41 serotypes of adenovirus hexon protein. Tissues were incubated for 1 hour at 35°C with an antibody dilution of 1:1000. A biotinylated goat secondary antibody to mouse immunoglobulin was then applied, followed by a streptavidin–horseradish peroxidase conjugate. Diaminobenzidine was used as the chromagen, and slides were counterstained with hematoxylin.

23. We thank A. Berk (University of California, Los Angeles) for providing wild-type adenovirus and dl1520; E. White (Rutgers University) for a cDNA encoding full-length E1B 55K polypeptide; M. Kastan (Johns Hopkins University) for RKO cells; J. Hassell (McMaster University) for HEK293 cells; D. Von Moff, G. Mangold, and D. Dexter (San Antonio Cancer Treatment and Research Center) for help with animal models; J. Olesch for technical help; and C. Maack for many stimulating discussions.

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