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Advances in Brief

In Vivo Gene Therapy with *p53* or *p21* Adenovirus for Prostate Cancer[1]

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Abstract

We introduced the gene for wild-type human *p53* or *p21*, a critical downstream mediator of *p53*-induced growth suppression, into a *p53*-deficient mouse prostate cancer cell line using a recombinant adenoviral vector (Ad5CMV-p53 or Ad5CMV-p21). Elevated levels of endogenous mouse *p21* mRNA provided evidence for the functional activity of virally transduced *p53*. Functional activity of viral-transduced *p21* was demonstrated through immunoprecipitation of cellular protein extracts, which showed that the viral-transduced p21 associates with cyclin-dependent kinase 2 and was sufficient to down-regulate the activity of the cyclin-dependent kinase by approximately 65%. *In vitro* growth assays revealed significantly higher growth suppression after Ad5CMV-p21 infection compared to Ad5CMV-p53. *In vivo* studies in syngeneic male mice with established s.c. prostate tumors demonstrated that the rate of growth and final tumor volume were reduced to a much greater extent in mice that received intratumor injection of Ad5CMV-p21 compared to Ad5CMV-p53. In addition, the survival of host animals bearing tumors that were infected with Ad5CMV-p21, but not Ad5CMV-p53, was significantly extended. These data suggest that Ad5CMV-p21 may be effective as a therapeutic agent for prostate cancer.

Introduction

p53 is one of the most commonly mutated genes in human cancer (1, 2) and is thought to function in two critical pathways: (*a*) the G_1 checkpoint activated in response to DNA damage; and (*b*) radiation-dependent apoptosis (reviewed in Refs. 3 and 4). Cells from *p53*-null mice have defects in both the G_1 checkpoint activated by DNA damage (5, 6) and radiation-induced apoptosis of lymphoid cells (6, 7), and display markedly increased genomic instability (8, 9). In addition, *p53* mutations in prostate cancer have been associated with metastatic disease (10). *p53*-dependent cell cycle arrest may function through the action of the CKI[3] *p21* (Refs. 11–13; reviewed in Ref. 14). *p21* expression is induced by *p53*, either by overexpression of *p53* (11) or after DNA damage (13, 15). Cells overexpressing *p21* accumulate in G_1 (16), and mice lacking *p21* have defects in the G_1 checkpoint induced by DNA damage (17). Together, these data support the idea that *p21* participates in the G_1 checkpoint mediated by *p53*.

Whereas previous studies have demonstrated that introduction of *p53* by viral-mediated delivery can suppress growth in a number of human cancer cell lines *in vitro* and *in vivo* (18–23), the utility of *p21* as an antitumor agent has not been evaluated. In the present study, we evaluated the relative antitumor activities of *p53* or *p21 in vivo* using adenovirus-mediated gene transfer in conjunction with a mouse model for prostate cancer. The results indicate that administration of adenovirus *p21* can significantly extend mouse survival and decrease tumor volume. In addition, *p21* was more effective than *p53* in suppressing tumor cell growth, suggesting that CKIs may prove beneficial in the treatment of prostate and perhaps other cancers.

Materials and Methods

Cell Lines and Recombinant Adenoviruses. The mouse prostate cancer cell line 148-1PA was derived from a primary site tumor using the metastatic mouse prostate reconstitution model system (24). In this model system, urogenital sinus tissue is derived from a transgenic knock-out mouse strain (129/Sv) in which the normal *p53* gene was inactivated (25). Cell line 148-1PA is *p53* null as confirmed by PCR and Southern blotting (23). These cells were grown in DMEM with 10% FCS, 10 mM HEPES, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained with routine media changes, and cultures were passaged by trypsinization with 0.025% trypsin approximately every 7 days. All chemicals for cell culture were obtained from GIBCO-BRL (Gaithersburg, MD).

The recombinant *p53* or *p21* adenovirus, Ad5CMV-p53 (19) or Ad5CMV-p21, contains the CMV promoter, wild-type human *p53* or human *p21* cDNA, and the SV40 polyadenylation signal in a minigene cassette inserted into the E1-deletion region of modified Ad5. The human p21 cDNA (12) was inserted as a 684-bp *Hind*III to *Xho*I fragment into pXCMVpA (20) to generate plasmid pCMVp21, which was cotransfected with pJM17 into 293 cells to generate the Ad5CMV-p21 virus. The control viral vector contains the CMV promoter and the SV40 polyadenylation signal without a cDNA insert (Ad5CMV-vector). Viral stocks were expanded in the 293 transformed tumor kidney cell line. Cells were harvested 36–40 h after infection, pelleted, resuspended in PBS, and lysed. Cell debris was removed by double cesium chloride gradient ultracentrifugation. Virus titer was determined by plaque forming activity in 293 cells. Concentrated virus was dialyzed, aliquoted, and stored at −80°C.

Northern Blot Analysis. Total RNA was isolated using the guanidinium-isothiocyanate method (24). Northern analyses were performed on total RNA (20 μg), which was electrophoresed on a 1% agarose-formaldehyde gel and transferred to a Hybond N membrane (Amersham Corp.) and hybridized with a human *p53* cDNA probe as described previously (24). The membrane was stripped and reprobed with a mouse *p21* cDNA, followed by restripping and probing with mouse actin cDNA for RNA loading control. A similarly prepared blot was also probed with human *p21* cDNA, followed by restripping and probing with actin cDNA for RNA loading control.

Western Blot Analysis and Kinase Assays. Cells from one 10-cm dish infected with the indicated adenovirus and MOI were washed with PBS and lysed in 350 μl of extraction buffers containing 0.4% NP40, as described (17). Protein samples (30 μg) were electrophoresed through 12.5% gels, transferred to nitrocellulose, and blots were probed with a monoclonal antibody against human *p21* (DF10) from Oncogene Science. Detection was accomplished using enhanced chemiluminescence (Amersham Corp.). This antibody does not cross-react with mouse *p21*. Bacterial expressed p21 protein used for positive Western blotting controls was purified as described (17).

For immunoprecipitations, 320 μl of the indicated cell extract were incubated with 600 ng of anti-Cdk2 IgG (Santa Cruz Biochemicals) and 10 μl of Protein A-Sepharose at 4°C for 1 h. Immune complexes were washed three times with 1 ml of NP40 buffer and once with kinase buffer [20 mM Tris-HCl

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[3] The abbreviations used are: CKI, cyclin-dependent kinase inhibitor; Cdk, cyclin-dependent kinase; CMV, cytomegalovirus; MOI, multiplicity of infection.

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(pH 7.5)-15 mM $MgCl_2$), and 10% of the immune complexes was removed for histone H1 kinase assays as described (12). Immune complexes were subjected to immunoblotting using DF10 anti-p21 antibodies as described above.

***In Vitro* Cell Growth Assay.** Cells were plated in triplicate at a density of 2.5×10^4 cells/cm^2 in 24-well tissue culture plates. Twenty-four h later, infection was carried out with Ad5CMV-p53, Ad5CMV-p21, or Ad5CMV-vector in serum-free media. Cells were harvested every 2 days and counted after viability was determined by trypan blue staining.

***In Vivo* Experiments.** All experiments were performed in syngeneic adult male mice (129/Sv). s.c. tumors were generated by injection of 2×10^6 148–1PA cells in 200 μl HBSS into the flank of the 21 host animals. After 3 days of growth, a microliter syringe fitted with a 27-gauge needle was used to transcutaneously inject a 50-μl solution containing 5×10^9 plaque-forming units of Ad5CMV-vector, Ad5CMV-p53, or Ad5CMV-p21. The s.c. prostate tumors were measured with calipers immediately before injection of the adenovirus and every other day until the animals were sacrificed when they exhibited signs of distress or when the tumor was approximately 10% of the weight of the animal. Survival was determined from the time of the cell line injection to the time of sacrifice. All mice were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care, and all animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals. Nonparametric ANOVA was used to test the significance of the differences between the samples using StatView 4.0 Survival Tools (Abacus Concepts Inc.).

Results

Functional Activity of Adenovirus-transduced *p53* and *p21*. Before assessing the effects of adenovirus *p53* and *p21 in vivo*, we characterized the activities of these viruses *in vitro* using mouse prostate cancer cell line 148-1PA derived from a *p53*-null primary tumor (24). This same cell line was used in the tumorigenesis studies described below. Cell monolayers were infected at a MOI of 100 with either Ad5CMV-vector, Ad5CMV-p53, or Ad5CMV-p21. Twenty-four h after infection, total RNA was isolated, and Northern blot analysis was performed. Virus-expressed *p53* mRNA (1.9 kb) was present in the cells infected with Ad5CMV-p53 (Fig. 1*A*) but was not detected in cells infected with control or *p21* adenoviruses (Fig. 1*A*). When this blot was stripped and probed with mouse *p21* cDNA, endogenous *p21* mRNA (2.1 kb) was observed at low levels in control and *p21* adenovirus-infected cells (Fig. 1*B*), whereas the exogenous human *p21* mRNA (at ~0.8 kb) was readily detected only in Ad5CMV-p21-infected cells (Fig. 1, *B* and *D*). Consistent with the ability of *p53* to activate transcription of *p21*, endogenous *p21* mRNA was substantially induced in cells infected with Ad5CMV-p53 (Fig. 1*B*). Thus, Ad5CMV-p53 infected cells have functional p53 protein. Expression of p21 protein in Ad5CMV-p21-infected 148-1PA cells was verified by immunoblotting using a monoclonal antibody toward human p21 (Fig. 2*A*). To evaluate the functional consequences of the expression of *p21*, whole cell lysates were subjected to immunoprecipitation with anti-Cdk2 antibodies and immune complexes used for histone H1 kinases and immunoblotting. p21 was found associated with Cdk2 in Ad5CMV-p21-infected cells but not in control virus-infected cells. In addition, kinase activity was reduced by 65%. Taken together, these data indicate that p21 produced through adenovirus infection is biochemically active. Immunohistochemical analysis of cells infected with Ad5CMV-p53 or Ad5CMV-p21 showed characteristic nuclear staining of p53 or p21 protein, respectively, whereas control cells lacked detectable staining (data not shown).

***In Vitro* Effect of Adenovirus-transduced *p53* or *p21* on Cell Growth.** Cell monolayers of 148-1PA were infected with increasing concentrations of Ad5CMV-vector, Ad5CMV-p53, or Ad5CMV-p21 (Fig. 3). The control Ad5CMV-vector had no effect on cell growth at any MOI tested. In contrast, both *p53* and *p21* reduced cell growth rates, although *p21* was significantly more effective in this assay.

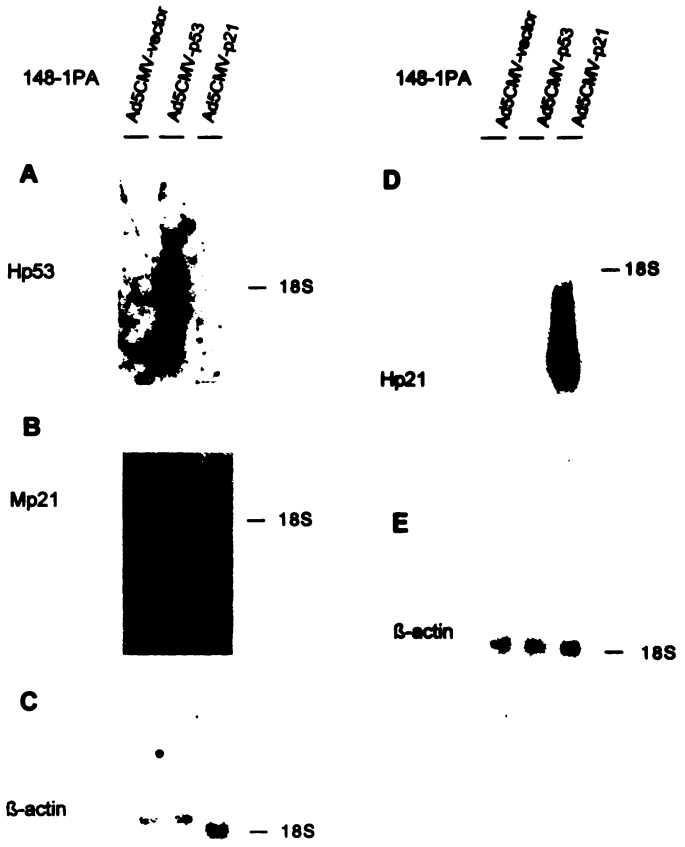

Fig. 1. Adenovirus expression of *p53* and *p21* detected by Northern blotting. RNA was isolated from 148-1PA cells (*Lane 1*), 148-1PA cells infected at a MOI of 100 with Ad5CMV-p53 (*Lane 2*), or 148-1PA cells infected at a MOI of 100 with Ad5CMV-p21 (*Lane 3*). The blots were probed with a human *p53* cDNA probe and exposed for 4 days (*A*); a mouse *p21* cDNA probe, exposed for 4 days (*B*); a human *p21* probe, exposed for 12 h (*D*); or a mouse β-actin probe, exposed for 3 days (*C*) or 12 h (*E*). Equivalent RNA was loaded as determined by ethidium bromide staining of the agarose gel before transfer (*C*). *p53* expression from the Ad5CMV-p53 virus is apparent, and the presence of this virus leads to elevated expression of endogenous *p21* RNA. Expression of the viral encoded *p21* RNA was also evident in Ad5CMV-p21-infected cells.

With a MOI of 100, in cells infected with Ad5CMV-p53, growth rates were reduced by about 30%, whereas with a MOI of 200, growth rates were reduced by about 60%. With *p21*, 50% reduction in growth rates were observed at a MOI of 50 at early times after infection, but with MOIs of 100 or 200, cell growth was almost completely suppressed. Previous studies indicate that *p21* overexpression arrests cells in G_1 (16). Our biochemical data, coupled with *in vitro* tissue culture assays, indicate that *p21* produced by the Ad5CMV-p21 virus actively suppresses growth.

***In Vivo* Effect of Adenovirus-transduced *p53* or *p21* on Tumor Growth and Survival.** s.c. prostate tumors initiated with 148-1 PA cells were given injections of 5×10^9 plaque-forming units of Ad5CMV-vector, Ad5CMV-p53, or Ad5CMV-p21. Tumor volumes were 25–40 mm^3 at the time of virus injection. Tumor volume was measured every other day (Fig. 4*A*), and on day 13 there was a significant ($P < 0.01$) inhibition of tumor growth in animals treated with Ad5CMV-p21 (959 ± 167 mm^3), compared to either Ad5CMV-vector (3040 ± 241 mm^3)or Ad5CMV-p53 (2392 ± 271 mm^3). There was a significant ($P < 0.005$) prolongation of survival in animals treated with Ad5CMV-p21 (17.5 ± 1.0 days), compared to animals treated with Ad5CMV-p53 (13.5 ± 1.0 days) or Ad5CMV-vector (13.5 ± 0.5 days; see Fig. 4*B*). No significant difference in survival

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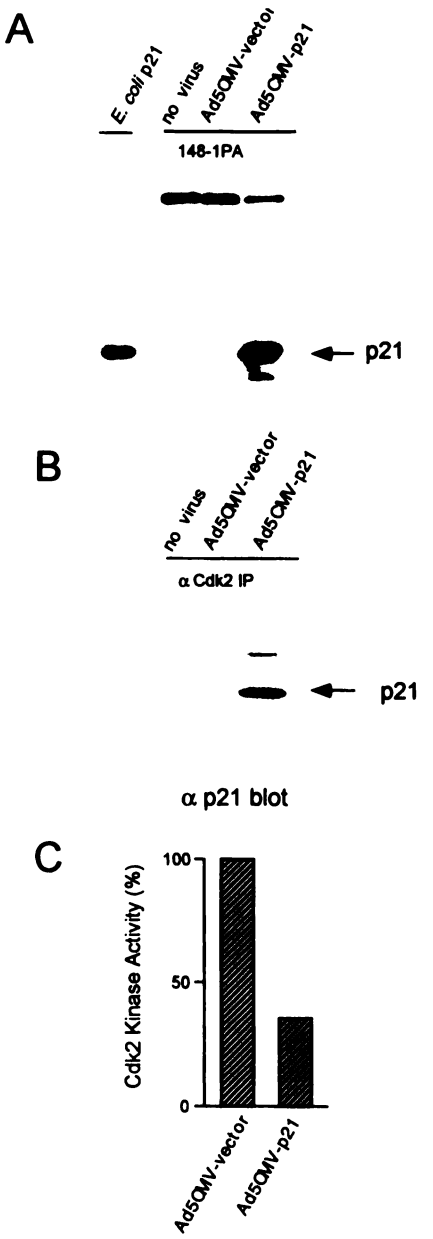

Fig. 2. Expression of p21 protein detected by Western blotting and kinase activity. The 148-1PA cells were infected with Ad5CMV-p21 or Ad5CMV-vector at a MOI of 100, and after 48 h, protein lysates were isolated (20). *A*, immunoblot using a human p21-specific antibody on separated proteins. *E. coli*, *Escherichia coli*. *B*, immunoblotting as in *A* of protein extracts immunoprecipitated with anti-Cdk2 (*α Cdk2 IP*), showing that the viral expressed p21 associates with Cdk2. *C*, histone H1 kinase assay with immunoprecipitated Cdk2, indicating partial inhibition.

was detected between the Ad5CMV-p53-treated animals *versus* those treated with the control Ad5CMV-vector.

Discussion

Cancer is believed to be the end result of an acquisition of genetic alterations in key regulatory molecules, resulting in unregulated cell growth. *p53* is a tumor suppressor gene that is mutated in a wide variety of human malignancies (reviewed in Refs. 1 and 2). *p53* encodes a transcription factor, the targets of which include genes that regulate cellular responses to DNA damage, cell cycle progression, and genomic stability (reviewed in Refs. 3 and 4). Loss of *p53* results in an inability of some cell types to undergo apoptosis in response to DNA damage, and this may be a primary mechanism that gives rise to tumors in cells lacking *p53* (6, 7). In addition, *p53*, when overexpressed, can block the cell cycle in G_1, and this is thought to mimic the stabilization of *p53* after DNA damage. *p53*-dependent cell cycle arrest can function through the action of the Cdk inhibitor p21 (Ref. 17 and references therein). Both of these genes can arrest the cell cycle in G_1 when overexpressed. Viral-mediated gene therapy approaches have been used to deliver *p53* to a number of malignancies with the goal of suppressing tumor growth *in vivo* (18–23). Liu *et al.* (19) showed that after infection of a human head and neck squamous cell carcinoma cell line with the same adenoviral construct as was used in our studies (Ad5CMV-p53), growth arrest occurred. Moreover, injection of Ad5CMV-p53 into s.c. squamous cell carcinoma nodules significantly reduced further tumor development (23).

In the present study, we examined the ability of *p53* and *p21* adenoviruses to block proliferation of a *p53*-null mouse prostate

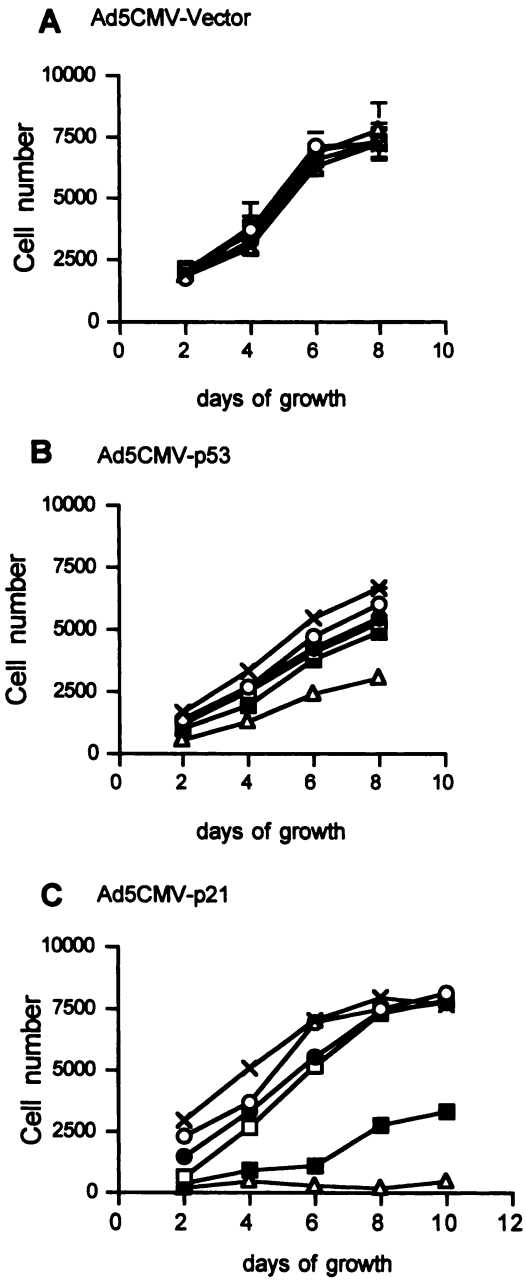

Fig. 3. *In vitro* effect of *p53* or *p21* on cell growth. The growth of 148-1PA cells was determined by counting cell numbers at various time points after infection of cells at a MOI of 0 (*X*), 10 (○), 25 (●), 50 (□), 100 (■), or 200 (△).

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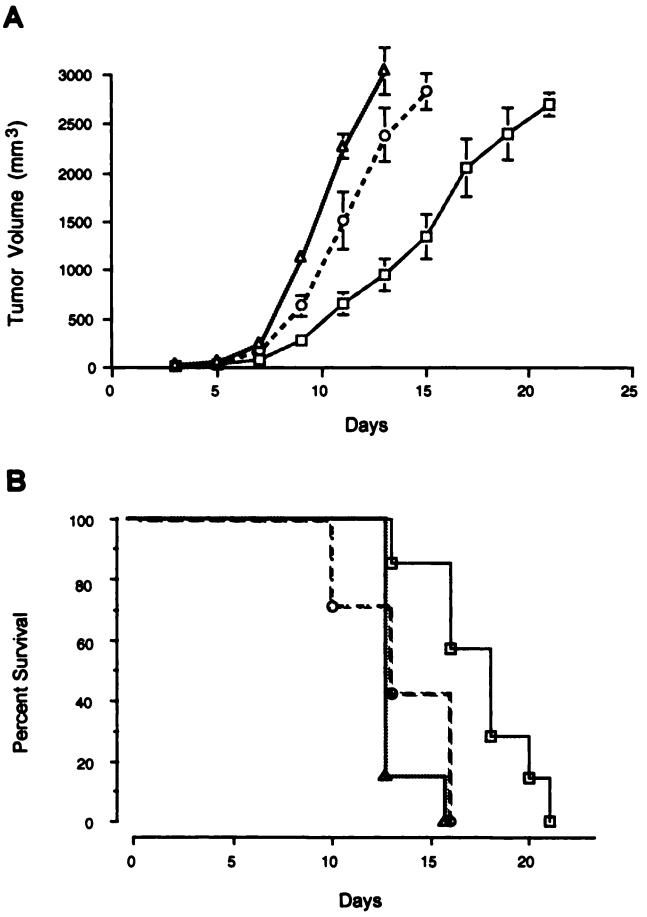

Fig. 4. *In vivo* tumor suppression with Ad5CMV-p21. s.c. prostate tumors were initiated with 2×10^6 148-1PA cells and given injections of 50 μl of adenovirus (5.0×10^9 plaque-forming units) on day 3. Tumor volume was measured every other day, and significant growth suppression was observed in the Ad5CMV-p21-injected tumors (*A*). The effect was less dramatic in the Ad5CMV-p53-injected tumors. *Points*, mean; *bars*, SEM. *B*, Kaplan-Meier cumulative survival plot of animals with s.c. tumors were treated with each adenoviral vector. Animals were sacrificed when they developed cachexia or if tumor burden approached 10% of body weight. Mice treated with Ad5CMV-p21 had significantly better survival. △, Ad5CMV-vector; ○, Ad5CMV-p53; □, Ad5CMV-p21.

cancer cell line *in vitro* and s.c. tumors derived from these cells *in vivo*. In contrast with other cell types that have been examined (18–23), we found that the mouse prostate tumor cell lines derived from *p53*-null animals are only marginally growth suppressed by the action of exogenous *p53* delivered from Ad5CMV-p53 both *in vitro* and *in vivo*, although Northern analysis of cell lines (Fig. 1*A*) and immunohistochemical staining of cell cultures and *in vivo* treated tumors (not shown) showed expression of the viral-encoded *p53*. In addition, induction of endogenous mouse *p21*, presumably by virally delivered *p53*, was evident on Northern blots, indicating that the exogenous *p53* was functionally active. Despite *p53* expression, the growth rates of established s.c. prostate tumor in syngeneic male mice were only slightly reduced by Ad5CMV-p53, as compared with control-treated tumors, and there was no significant increase in survival. The *in vivo* data parallel that found *in vitro* where Ad5CMV-p53 treated cells displayed only about a 2-fold decrease in growth rates.

In contrast with *p53*, Ad5CMV-p21 treatment produced significant growth inhibition both *in vivo* and *in vitro*. *In vivo*, Ad5CMV-p21 treatment reduced the rate of tumor growth by ~60% and increased survival significantly (by ~5 days) at the MOI tested. Moreover, cell growth in culture was essentially abolished with MOIs of 100 or 200. Growth suppression by *p21* correlated with decreased Cdk2 kinase activity measured in infected cells *in vitro*.

The differential activities of *p53* and *p21* in this system suggest that these two growth suppressor do not necessarily behave identically in every tumor cell type, and that *p21* may be a more potent a growth inhibitor of some cell types or under some experimental conditions. Whereas it is clear that *p21* transcription is induced in *p53*-infected cells, the level of *p21* produced via *p53*-mediated transcription may not be as high as that obtainable by adenovirus-mediated transduction because of differences in promoter strength or translational efficiencies of endogenous and exogenous *p21* mRNAs. Previously, we and others (Ref. 16; reviewed in Ref. 14) showed that *p21* in normal diploid fibroblasts is associated with active Cdk complexes, and it is currently thought that multiple *p21* molecules (most likely 2) are required to inhibit a single Cdk. Therefore the growth-suppressive function of *p21* depends critically on the relative levels of *p21* and G_1 Cdks. It is possible that in the prostate tumor cell line used, amplified levels of G_1 cyclins are present, which would increase the level of *p21* required for full cell cycle arrest. It should be noted that cyclin overexpression is a common feature of various tumors (reviewed in Ref. 14) and should be taken into consideration when designing antitumor therapies. Whereas *p21* may have more growth suppression activity in some systems, there may be advantages to the use of *p53* in gene therapy protocols in certain situations. For example, angiogenic activity can be markedly suppressed by increased levels of *p53* via regulation of thrombospondin 1 (26) or fibroblast growth factor (27) expression. In addition, the presence of *p53* may allow for appropriate induction of apoptosis or G_1 arrest in response to DNA damage in certain circumstances.

Overall, our results suggest that treatment of localized prostate cancer may eventually be achievable using adenovirus-mediated delivery of CKIs. The prostate is the most common site of internal malignancy in men, and although there are several treatment options for prostate cancer, few significantly increase long-term survival. At present, it is not clear whether *p21* or one of several other known CKIs would be more advantageous in this regard, but the data presented here indicate that direct administration of *p21* can have a substantial effect on tumor growth *in vivo*.

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In Vivo Gene Therapy with *p53* or *p21* Adenovirus for Prostate Cancer

James A. Eastham, Simon J. Hall, Inder Sehgal, et al.

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