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


Scott Department of Urology [J. A. E., S. J. H., I. S., W., T. L. T., G. Y., T. C. T.], Verna and Mears McLean Department of Biochemistry [L. C.-C., S. J. E., J. W. H.], Howard Hughes Medical Institute [S. J. E.], and Departments of Human and Molecular Genetics [S. J. E.], Cell Biology [T. C. T.], and Radiology [T. C. T.], Baylor College of Medicine, Houston, Texas 77030, and Gene Therapy Unit, Baxter Healthcare Corporation, Round Lake, Illinois 60073 (W-W. Z]

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 G1 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 G1 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-deficient cell cycle arrest may function through the action of the CKI βp21 (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 G1 (16), and mice lacking p21 have defects in the G1 checkpoint induced by DNA damage (17). Together, these data support the idea that p21 participates in the G1 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, 100 IU/ml penicillin, and 100 μg/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 HindIII to Xhol fragment into pXCMVpA (20) to generate plasmid pCMVp21, which was cotransfected with pJMl7 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 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 Hybridon 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 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-}

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2 To whom requests for reprints should be addressed, at 6560 Fannin, Suite 1004, Houston, TX 77030. Phone: (713) 799—8718; Fax: (713) 799—8712; E-mail: timothyt@bcm.tmc.edu.

3 The abbreviations used are: CKI, cyclin-dependent kinase inhibitor; Cdk, cyclin-dependent kinase; CMV, cytomegalovirus; MOI, multiplicity of infection.
(pH 7.5)-15 mM MgCl₂), 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 × 10⁴ cells/cm² 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 × 10⁶ 148-1PA 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 × 10⁷ 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. 1A) but was not detected in cells infected with control or p21 adenoviruses (Fig. 1A). 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. 1B), 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. 1B). 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. 2A). 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.
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 G1, 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 G1 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 cell line 148-1PA. Ad5CMV-p53 and Ad5CMV-p21 were constructed by subcloning the human p53 or p21 cDNA into the E1-deleted Ad5.5 vector, under the control of a strong early promoter (Ad5CMV). The cell line 148-1PA was infected with Ad5CMV-p53 or Ad5CMV-vector at a MOI of 100, and after 48 h, protein lysates were isolated. Immunoblotting of infected 148-1PA cells showed that expression of p21 protein was present in cells infected with Ad5CMV-p21 but not in cells infected with Ad5CMV-vector. Western blot analysis showed that the viral expressed p21 associates with Cdk2, indicating partial inhibition.

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. 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 (a Cdk2 IP), showing that the viral expressed p21 associates with Cdk2. C, histone H1 kinase assay with immunoprecipitated Cdk2, indicating partial inhibition.

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 (▲).
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 G1 C/dks. It is possible that in the prostate tumor cell line used, amplified levels of G1 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 G1 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.

References


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