Wild-Type p53 Induction Mediated by Replication-deficient Adenoviral Vectors

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Abstract

Replication-deficient E1-/E3-deleted adenoviral vectors are commonly used to introduce transgenes into cells in vitro and have been used for certain kinds of gene therapy protocols in vivo. We have demonstrated that transduction of cells using these vectors can induce p53 expression in cells containing a wild-type p53 gene. This response is different from p53 induction observed after DNA damage because the time course of induction is slower and because it occurs in cells with an attenuated DNA damage response. However, this vector-induced p53 is transcriptionally active and, therefore, p53 function is not inactivated by viral proteins. The mechanism of induction appears to be an increased rate of protein translation because immunoprecipitation analyses demonstrated increased levels of 32P-labeled p53 protein, even after a short 15-min labeling time. Induction of p53 by adenoviral vectors may have various effects on transduced cells, including apoptosis and altered chemotherapy chemosensitivity. Therefore, the influence of the vector might confound the impact of any particular gene used in a gene therapy application.

Introduction

The p53 gene encodes a phosphoprotein that is involved in many cellular processes, including inhibition of cell growth, apoptosis, and inhibition of transformation and genetic instability (1). DNA tumor viruses, such as adenovirus, have developed mechanisms to inhibit cellular p53 function (2, 3). Host-induced apoptosis of infected cells prior to virus replication effectively inhibits the spread of infection. Therefore, it is essential for the virus to have a mechanism by which p53 can be inactivated, such that apoptosis is not initiated prior to completion of the viral replication cycle (4). The adenoviral proteins that have been shown to play a role in the inhibition of p53 function are the early genes E1A, E1B, and E4. The E1A gene is primarily responsible for the immortalizing properties of adenoviruses (5). This is mediated in part by the ability of E1A to bind to the product of the retinoblastoma gene, Rb (6) and also by its role in the stabilization of p53 (7). The E1B gene also has transforming properties. It encodes two proteins, E1B M 55,000, which can directly bind to and inactivate p53 (8), and E1B M 19,000, which inhibits initiation of DNA degradation induced by E1A (9). The adenoviral E4 region also encodes a protein, the M 34,000 product of open reading frame 6, which binds to and inhibits the activity of p53 (10, 11).

The adenoviral vector used in these studies is derived from adenovirus of serotype 5 (Ad 5) and has most of the E1 region and all of E3 deleted. For these vectors to replicate E1A and E1B, proteins have to be supplied exogenously, for example, by infecting a complementing cell line such as 293 (12), which expresses these proteins. The E3 region encodes proteins responsible for evading the host immune system (14). Therefore, deletion of this region is a safety feature, ensuring easy detection and elimination by the host. Av1 was developed by Trappnell (12).

A plasmid (pAVS6.DNA, obtained from Genetic Therapy Inc., Gaithersburg, MD) containing an expression cassette consisting of the RSV promoter and SV40 polyadenylation signal but no inserted transgene was homologously recombined with Ad-d327 adenoviral DNA after transfection into 293 cells by standard methodology (15). Plaque purified recombinant virus (Ad-VC) was determined to be free from wild-type virus contamination by PCR analysis for the E1A gene (16). This assay results in a sensitivity of at least 10−9 (16). Ad-VC was originally developed to be used as a control in studies in which results would be comparable with those obtained from similar vectors containing transgenes expressed under the control of the RSV promoter (17). Ad-VC was used at a multiplicity of infection: 1 to 50 plaque-forming units/cell. Initially transduction was for 2 h in small volume of 2% serum containing media to just cover the cells (equivalent to 2 ml in a T75 flask). Complete medium containing 10% serum was then added (equivalent to 12 ml in a T75 flask). The cells were exposed to the adenovirus for a total of 24 h, at which time the medium was replaced. Cells which had been treated in a similar manner without addition of the virus were harvested as “time 0” samples. Any heat inactivation of virus was carried out for 1 h at 56°C. Ad-β-gal (obtained from Genetic Therapy Inc.) was developed by the same methodology as Ad-VC, which allows expression of the β-galactosidase under the control of the RSV promoter after cell transduction.

Antibodies. The p53 monoclonal antibody DO-1, conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA), was used at a concentration of 1:200. DO-1 that was not conjugated to HRP was used for the immunoprecipitation reactions. A p21 polyclonal antibody was obtained from Santa Cruz Biotechnology and used at a dilution of 1:500. A rabbit monoclonal antibody was obtained from Sigma Chemical Co. (St. Louis, MO) and used at a dilution of 1:1000. HRP-conjugated secondary antibodies were used when appropriate, either donkey anti-rabbit IgG or donkey anti-mouse IgG (Amersham, Arlington Heights, IL) at a dilution of 1:1000 and 1:2000, respectively.

Western Analysis. Extracts of soluble cellular protein were prepared by resuspending cell pellets in extract buffer (50 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 1 mM EDTA, 0.5 mM DTT, and 0.1% NP40) containing freshly added

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3 The abbreviations used are: wtp53, wild-type p53; RSV, Rous sarcoma virus; moi, multiplicity of infection; HRP, horseradish peroxidase; PI3K, phosphoinositide-3-OH kinase.

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protease inhibitors; 10 μg/ml each antipain, aprotinin, and leupeptin; 1 mM sodium orthovanadate and 2 mM phenylmethylsulfonyl fluoride. Cells were then frozen and thawed three times in dry ice for 5 min followed by 37°C for 1 min. After microcentrifugation at 14,000 rpm at 4°C for 10 min, the pellets were discarded. Protein concentrations of all extracts were determined using the Bio-Rad protein determination kit (Bio-Rad Laboratories, Richmond, CA). Cell extracts were electrophoresed in 12.5% SDS-polyacrylamide gels, and Western analysis was performed as described previously (18). A tubulin antibody was used as a loading control to confirm that equal amounts of protein had been loaded on to the gel.

**Immunoprecipitation.** Cells plated into T162 flasks were radiolabeled with 120 μCi of [35S]methionine (1000 Ci/mmol; Amersham) in methionine-free medium at a concentration of 12 μCi/ml for 1 h or for 15 min. Cells were harvested immediately after removal of the isotope or 2 h after incubation in methionine-containing medium without isotope. Extracts (500 μg of each) were immunoprecipitated using p53 monoclonal antibody DO-1 (Santa Cruz Biotechnology). After incubation overnight with protein G PLUS-agarose beads (Santa Cruz Biotechnology), the immunoprecipitated p53 was electrophoresed on a 12% SDS-polyacrylamide gel, electroblotted to Immobilon-P membrane (Millipore, Bedford, MA), and exposed to X-ray film (Eastman Kodak Co., Rochester, NY). After a 2-week exposure the film was developed, and the bands were quantitated using Microsoft ImageQuant software. The same membrane was then exposed to p53-DO-1 HRP-conjugated antibody (Santa Cruz Biotechnology) to detect total cellular p53, as described above.

**Results**

Initial experiments used NB-1643, a neuroblastoma cell line that expresses wtP53, which can be induced after exposure to ionizing radiation (Fig. 1A). As expected, DNA damage-induced p53 led to induction of p21, demonstrating transcriptionally active p53. Ad-VC at a moi of 1 was used to transduce these cells for 24 h, after which time fresh medium was applied. Fig. 1A shows Western analysis of a time course of p53 and p21 induction after Ad-VC transduction. Although both ionizing radiation and exposure to Ad-VC induced transcriptionally active p53, the time course of induction was different. Induced p53 could be detected within 4 h postradiation, whereas it took 72 h to detect maximal induction of p53 expression after viral transduction. This difference suggests that these two stimuli, radiation and adenoviral vector transduction, induce p53 by two separate mechanisms or pathways. Exposure to higher doses resulted in a dose-dependent increase in p53 expression (Fig. 2A). A 2–3-fold induction was observed at a moi of 1, which can be increased to a 6-fold induction at a moi of 10 and an 8-fold induction at a moi of 50 (Fig. 2A). A more detailed time course at a moi of 10 revealed that Ad-VC-mediated p53 induction could be observed between 8 and 16 h after exposure to the virus (Fig. 2B). Ad-VC-mediated p53 induction was not observed within 4 h, and therefore, results were still in contrast to p53 induction after radiation exposure, even when cells were exposed to a higher dose of virus (Fig. 1).

Fig. 1B contains data derived from extracts of the normal human fibroblast cell line, IMR90, after exposure to ionizing radiation and Ad-VC. As in the NB-1643 cells, Fig. 1B demonstrates that radiation-mediated p21 induction is evident much sooner than that induced after viral transduction. In this line, Ad-VC induced maximal expression at 24 h after transduction, whereas radiation-induced p21 expression occurs within 4 h of exposure. These data demonstrate that p53 can be induced by adenoviral vectors in nontransformed cells in addition to tumor cell lines.

![Fig. 1.](attachment:image1)

![Fig. 2.](attachment:image2)
exposure to an IC80 dose of ionizing radiation in NB-1691 cells, the activation domain of p53 (19). p53 and p21 cannot be induced after Rh18; Ref. 18). Overexpression of MDM2 protein leads to inactivation of p53 (Fig. 3A). Do-1 cells were transduced with Ad-VC for 24 h. Samples were obtained for immunoprecipitation immediately after a 1 h incubation with [35S]methionine and again after a further 2 h incubation in medium without isotope. The Ad-VC-transduced cells contained 2.3-fold more radiolabeled p53 protein immediately after a 1-h exposure to [35S]methionine than the nontransduced cells. However, after a further 2 h, the amount of [35S]p53 in both samples had reduced to ~50% (Fig. 5, B and C). This suggests that increased p53 expression after Ad-VC transduction is a result of increased p53 protein production and not of increased protein stability. Because the half-life of p53 is only ~30 min (21), the increase in [35S]-labeled p53 protein observed after a 1 h labeling time in Ad-VC-transduced cells could have been due to increased protein stability. Therefore, to minimize [35S]-labeled p53 degradation, an immunoprecipitation was carried out after only a 15-min exposure to [35S]methionine. Results in Fig. 5E demonstrate that the amount of [35S]-

We also examined three cell lines with attenuated endogenous p53 function (either MDM2 amplification or mutant p53) to determine whether these results were dependent upon a wt p53 gene sequence. Because it is the induction of functional wt p53 protein that is of interest and not simply increased protein levels due to stabilization of nonfunctional protein, only the results of p21 expression levels are shown. Fig. 3A shows p21 induction after Ad-VC transduction in two cell lines with not only a wt p53 gene but also an amplified MDM2 gene (a neuroblastoma line NB-1691 and a rhabdomyosarcoma line, Rh18; Ref. 18). Overexpression of MDM2 protein leads to inactivation of p53 function because of the binding of MDM2 to the transactivation domain of p53 (19). p53 and p21 cannot be induced after exposure to an IC80 dose of ionizing radiation in NB-1691 cells, whereas Rh18 cells do still demonstrate a DNA-damage response (Fig. 3B). However, both of these lines can induce p53-dependent p21 expression after Ad-VC transduction in a similar manner as described for NB-1643 and IMR90 cells (Fig. 3A). We have confirmed that Ad-VC transduction does not affect MDM2 binding to p53 (data not shown).

The third line analyzed was Rh30, a rhabdomyosarcoma cell line expressing a mutant p53 gene (18). As expected, neither p53 nor p21 could be induced after exposure to either ionizing radiation (Fig. 3B) or Ad-VC transduction (Fig. 3A), confirming that the induction observed in the other lines was dependent upon a wt p53 gene sequence. We confirmed that Rh30 cells could be transduced by this kind of adenoviral vector by the use of a similar vector containing the wt p53 gene (Ad-p53; Genetic Therapy Inc.). Expression of functional exogenous wt p53 was confirmed by analysis of p21 expression in these cells after Ad-p53 transduction (data not shown).

It has been demonstrated that PI3K is activated after adenovirus receptor binding and that PI3K-activated signal transduction pathways are required for adenovirus internalization (20). We investigated the possibility that adenovirus induced p53 could be mediated by PI3K activation by using wortmannin, an inhibitor of this kinase. Fig. 4 shows Western analysis of p53 and p21 24 h after the addition of Ad-VC (moi of 10) and wortmannin (300 nM), a dose previously shown to inhibit PI3K function (20). Results demonstrated that addition of wortmannin had no effect on p53 or p21 expression after Ad-VC transduction (Fig. 4), and therefore, the PI3K pathway does not appear to be involved in the mechanism of induction. To confirm that wortmannin had no effect on adenovirus transduction, we used an Ad-β-gal vector to transduce NB-1643 cells and confirmed that exogenous β-galactosidase expression was unaffected by addition of wortmannin (data not shown).

We also investigated whether heat-inactivated Ad-VC could induce p53 expression by a receptor-mediated pathway. Fig. 4 shows p53 and p21 expression in NB-1643 cells 24 h after exposure to both heat-inactivated (56°C for 1 h) and non-heat-inactivated virus. Inactivation of the virus was confirmed by a plaque-forming assay (15). Results demonstrated that Ad-VC-mediated p53 induction was inhibited after heat inactivation of Ad-VC.

To further investigate the mechanism by which p53 is induced by adenoviral vector transduction, we carried out Northern analysis to determine whether induction was a result of an enhanced rate of transcription. RNA was isolated from NB-1643 cells prior to and after 24 h exposure to Ad-VC (moi of 10). Results demonstrated no increase in p53 RNA (data not shown), indicating that alterations in transcriptional regulation were not responsible for increasing p53 protein expression levels in the virally transduced cells.

We also investigated whether increased p53 protein stability was responsible for the induction observed after viral transduction in NB-1643 cells. Immunoprecipitation analysis using a p53 antibody (DO-1) with [35S]methionine-labeled cell extracts is shown in Fig. 5. NB-1643 cells were transduced with Ad-VC for 24 h. Samples were obtained for immunoprecipitation immediately after a 1 h incubation with [35S]methionine and again after a further 2 h incubation in medium without isotope. The Ad-VC-transduced cells contained 2.3-fold more radiolabeled p53 protein immediately after a 1-h exposure to [35S]methionine than the nontransduced cells. However, after a further 2 h, the amount of [35S]p53 in both samples had reduced to ~50% (Fig. 5, B and C). This suggests that increased p53 expression after Ad-VC transduction is a result of increased p53 protein production and not of increased protein stability.

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Fig. 4. Western analysis for p53 and p21 expression in NB-1643 cell extracts after exposure to Ad-VC at a moi of 10.
and E4 were obtained.

Discussion

Wild-type adenovirus infection has previously been shown to lead to increased levels of p53 protein in cells. This induction is mediated by the viral E1A protein (7) and inactivation by the E1B protein (8) and inactivation of its activity can have significant effect on the translation of cellular mRNAs. Whereas eIF-4E is likely the target of the translational repression, modulation of its activity can have significant effects on the translation of cellular mRNAs. However, this effect is mostly manifest at late stages of infection and is probably due to the activity of an E1A-encoded protein (29). eIF-4E is responsible for cap-dependent mRNA translation, and modulation of its activity can have significant effects on the translation of cellular mRNAs. Therefore, the overall increase in total cellular p53 after Ad-VC transduction appears to be due to an increased rate of translation and consequently an enhanced rate of p53 protein production.

Fig. 5. 35S-labeled p53 immunoprecipitation of NB-1643 cell extracts with and without 24 h exposure to Ad-VC at a moi of 10. A, total cellular p53 after detection with p53 DO-1 antibody. B, 35S-labeled p53, as detected by autoradiography of the same membrane. C, quantitated amounts of 35S-labeled p53 protein as a histogram. Columns, means of two experiments; bars, span of the two individual points from which the mean was obtained. D, total cellular p53 after detection with p53 DO-1 antibody. E, 35S-labeled p53 generated after only a 15-min exposure to the isotope, as detected by autoradiography of the same membrane.

Labeled p53 increased 2-fold in Ad-VC-transduced cells after this short exposure time. Therefore, the overall increase in total cellular p53 after Ad-VC transduction appears to be due to an increased rate of translation and consequently an enhanced rate of p53 protein production.
initiate a functional p53 response in cells containing a wtp53 gene is important information for anyone using similar vectors for gene therapy. Because the vector itself can mediate changes in gene expression, it is very important that investigators always include appropriate negative controls in their studies, such that results obtained can be attributed to the specific transgene of interest and not to effects of the vector itself. Having said this, vector induction of wtp53 may be beneficial in certain types of gene therapy, particularly for treatment of cancer, because increases in p53 expression have been shown to mediate apoptosis and increase sensitivity to chemotherapeutic agents in model systems (30).

The results presented here are also of importance to those interested in the biology and regulation of p53 expression as they describe identification of a novel mechanism leading to p53 induction which can enhance expression of wtp53, even in cells in which the DNA-damage induction pathway of p53 has been attenuated by the overexpression of MDM2.

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References

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