Adenovirus-mediated Transfer of p53-related Genes Induces Apoptosis of Human Cancer Cells

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Two p53-related genes, p73 and p51, were recently identified as structural homologues of the p53 tumor suppressor gene, suggesting that the roles of these two genes may be similar to those of p53, including growth suppression and induction of apoptosis. Here we show that introduction of p73 or p51 cDNAs into cultured human cancer cells suppressed colony formation in the presence of G418. We then examined the ability of various isoforms of p73 and p51 to activate transcription of a reporter gene. This assay showed that p73β and p51A activated transcription through a consensus p53 binding sequence, while p73α and p51B isoforms minimally transactivated the p53 reporter gene. To characterize further the biological functions of the p53-related genes, we constructed recombinant adenoviruses containing the p73 and p51 cDNAs. Ad-p73β and Ad-p51A induced endogenous p21 gene expression more effectively than Ad-p73α and Ad-p51B, respectively. To evaluate the mode of cell death induced by p53-related genes, Ad-p73β and Ad-p51A or Ad-p51B resulted in DNA fragmentation in a subset of cancer cell lines more efficiently than did infection of Ad-p53. We then examined the combined effect of each p53-related gene and the E1A oncogene in the induction of apoptosis. The E1A oncogene cooperated with p51 as well as p53 to induce apoptosis, while p73 resulted in a weak induction of apoptosis by E1A. Overall, apoptosis induction by p51B and p73α isoforms may be due to mechanisms other than transcriptional activation of p53-target genes. Our results suggest that p53-related genes are both similar to and different from p53 in their pathways leading to growth suppression.

Key words: p73 — p51 — p53 — adenovirus vector — apoptosis

p53 is the most frequently mutated tumor suppressor gene identified in human cancers.1-2 In response to cellular stresses such as DNA damage and oxygen starvation, p53 induces cell-cycle arrest or programmed cell death. It is likely that many of the biological functions of p53 result from transcriptional activation of target genes.3, 4 Two p53-related genes, p73 and p51, were recently identified.5, 6 These p53-related genes possess significant structural homology with the conserved regions of p53 including the DNA-binding, transactivation, and oligomerization domains.5, 6 Furthermore, p73 and p51 can activate p53-responsive promoters and induce apoptosis in tumor cells lacking wild-type p53.5-8 Hence, the idea has arisen that some cellular responses previously assumed to be p53-independent might be attributable to these p53-related genes. Unlike p53, p53-related genes produce several splicing variants corresponding to the various protein isoforms. For example, p73α and p73β, and p51A and p51B are different at their carboxy termini, respectively.5, 6 p51A and p51B have extensive similarity in structure to p73β and p73α, respectively. Different biological functions between p73α and p73β and between p51A and p51B have been identified, but conflicting results have also been reported.5, 7, 11 Furthermore, recent studies demonstrated that viral oncoproteins, adenovirus E1B 55K, SV40 large T, and papillomavirus E6, which bind to and inactivate the p53 protein, do not interact with p73.5, 12 These observations suggest that the functions of p53 and p73 in tumor development may differ. To investigate the distinct biological functions of the two isoforms of p53-related genes, we compared the p73 and p51 isoforms to p53 with respect to apoptosis induction and transcriptional regulation.

MATERIALS AND METHODS

Cell lines and cell culture The following human cell lines were used in this study: SaOS2, a p53-deficient osteosarcoma cell line; NCI-H1299, a p53-deficient lung carcinoma cell line; SW480, a mutant p53 (273His) expressing colon adenocarcinoma cell line; T98G, a mutant p53 (237Ile) expressing glioblastoma cell line. Cells were maintained in Dulbecco’s modified Eagle’s
medium (DMEM) or in RPMI1640 supplemented with 5% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS). SW480 cell line was maintained in Leibovitz’s L-15 supplemented with 5% FBS. p53-deficient mouse fibroblast cell line 10(1) was cultured in DMEM with 5% FBS.

**Plasmid constructions** Human p73 cDNA was cloned using reverse transcriptase-polymerase chain reaction (RT-PCR) from human brain poly(A) RNA (Clontech, Palo Alto, CA). PCR primers were designed corresponding to the nucleotide sequences of human p73 (accession number Y11146). For cloning the entire coding region of p73, the p73 cDNA was divided into two parts. To amplify the first part of the cDNA, the following oligonucleotides were used as primers: S60, 5′-CGGGATCCATGGGGTACACTGATCGGTT-3′; AS875, 5′-CCTACACAGCTGTCTGTTACACATAG-3′; S840, 5′-TATGAGCCACACAGTTG-3′; and AS2060, 5′-CGGGATCCAGGGCCACGCTTTGGGTCTCTG-3′ were used for the second part. A BamHI site was incorporated into the 5′-end of S60 and AS2060. Amplification was performed using KOD DNA polymerase (Toyobo, Osaka) according to manufacturer’s instructions. These PCR products were digested with BamHI and EcoRI and subcloned into pBluescript vector (Strategene, La Jolla, CA). Nucleotide sequencing verified that the two splicing variants corresponded to p73α and p73β. The p51cDNA was cloned using RT-PCR from human skeletal muscle poly(A) RNA (Clontech). The p51 cDNA was divided into two parts for PCR-based cloning. The first part of the cDNA was amplified using the following oligonucleotides: F1, 5′-CGGGATCCAAAGAAAGTTATACCGATCACCACATG-3′; R3, 5′-GGCGACGGCTGGTCTAAAGGTACACTGATCGGTT-3′; F7, 5′-CAGATGTCGGTACCGTCTTATGGGACC-3′; and R7, 5′-CGGGATCCAGGGCCACGCTTTGGGTCTCTG-3′ for p51A; F7 described above, and R9, 5′-CGGGATCTCTCACTCCCCTCTCTTTATGGGACC-3′ for p51B were used for the second part. A BamHI site was incorporated into the 5′-end of F1, R7 and R9. Amplification was performed using KOD DNA polymerase (Toyobo, Osaka). PCR products were digested with BamHI and KpnI, followed by subcloning into the pBluescript vector (Stratagene). All of the cDNA constructs were verified by nucleotide sequencing. Subsequently, the p73α, p73β, p51A and p51B cDNAs were inserted into a mammalian expression vector pcDNA3.1(+). Invitrogen, Carlsbad, CA) and named pcDNA-p73α, pcDNA-p73β, pcDNA-p51A and pcDNA-p51B, respectively. Each of the BamHI cDNA fragments of p73 and p51 was inserted into a Flag-tagged vector, pCMV-Tag2 (Stratagene), in-frame to allow Flag epitope tagging at the N-terminus.

A wild-type (wt) oligonucleotide (5′-TGAGCATCAGGTTCAAGGGAGC-3′) was used to generate a p53-responsive sequence.13,14) A reporter plasmid, pGL3-wt was constructed with three tandem repeats of the wt oligonucleotide sequence inserted into an XhoI site upstream of a basal SV40 promoter of pGL3 plasmid (Promega, Madison, WI). A mutant (mt) oligonucleotide (5′-TGAGAATTCCATGAGGATAC-3′) was used to generate a non-responsive control sequence within the control reporter plasmid, pGL3-mt.

**Western blot analysis** We used ECL western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK) with anti-p21 monoclonal antibody p21(187) (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Flag M2 monoclonal antibody (Sigma, St. Louis, MO).

**Colony formation assay** Cells at 25% confluence in 10 cm dishes were transfected with 5 µg of the indicated expression plasmid and 25 µl of Lipofectin (Gibco BRL, Rockville, MD). At 24 h following transfection, cells were split 1:4 and grown in the presence of GENETICIN (Gibco BRL) (0.4–1.0 mg/ml) for 2 weeks. The cells were fixed and stained with Giemsa, and the number of colonies was scored.

**Luciferase assay** Cultures of SaOS2, NCI-H1299 and SW480 cells at 50% confluence in 6 cm dishes were transfected with a reporter and an effector plasmid using Lipofectin (Gibco BRL). Cells were transfected with an equal amount of plasmid DNAs by supplementing with pUC13 in each experiment. Cells were harvested at 48 h following transfection for measurement of luciferase activity using the Luciferase Assay System (Promega). Cell extract was incubated with luciferin and light emission was measured using a scintillation counter (Beckman LS9000, Beckman, Palo Alto, CA).

**Recombinant adenvirus** The recombinant adenoviral vectors expressing human p73α, p73β, p51A and p51B cDNA were constructed as follows. For example, p73α cDNA was obtained as a HindIII-Xbal fragment from the pcDNA-p73α. The HindIII-Xbal cDNA fragment was subcloned into the HindIII-XbaI site of the pAd-BglII vector15) containing a cytomegalovirus promoter/enhancer and a bovine growth hormone polyadenylation signal which are flanked by Ad5 E1 sequences (nucleotide position 1–356 and 3329–5788) to construct the vector pAd-p73α. Both the pAd-p73α and a plasmid pJM17 containing the genomic sequence of Ad5 (Microbix Biosystems Inc., Toronto, Canada) were cotransfected into 293 cells using Lipofectin reagent (GibcoBRL), and the transfectants were cultured in RPMI1640 medium supplemented with 3% FBS for 3 to 4 weeks to generate a recombinant adenovirus p73α expression vector. Recombinants were purified from single plaques and named Ad-p73α. Culture supernatants of the viral stocks were quantified by a plaque forming assay using 293 cells. To examine the integrity of the cDNA sequence in the recombinant adenovirus, the cDNA fragments were amplified by PCR and their nucleotide sequences were determined. Recombinant adenovirus Ad-LacZ was kindly provided by Dr. M. J.
Imperiale of Michigan University. Ad-p53 and Ad-E1A(12S) are recombinant adenoviruses which express wild-type p53 and Ad E1A 12S, respectively. For expression of lacZ, p53 and the p53-related genes, cells were infected with the corresponding recombinant adenovirus at a multiplicity of infection (m.o.i.) of 20 per cell and cultured for 24–48 h.

Detection of DNA fragmentation Cells were seeded at $4 \times 10^5$ cm dish and cultured for 24 h. The cells were infected with 20 pfu/cell (about 8–20 µl per 6 cm dish) of the recombinant adenovirus, incubated for 1 h and refed in DMEM with 1% FBS. The DNA fragmentation assay was performed as follows. After incubation for 48 h, adherent and floating cells were collected and resuspended in 400 µl of 5 mM Tris-HCl (pH 8.0), 10 mM EDTA and 0.5% Triton X-100. After centrifugation at 16000 $g$ for 20 min, the supernatant was incubated with 100 µg/ml of RNaseA for 1 h at 37°C and then with 200 µg/ml proteinase K and 1.0% sodium dodecyl sulfate (SDS) for 2 h at 50°C. The solution was extracted with phenol followed by precipitation with ethanol. The precipitate was resuspended in TE buffer, electrophoresed in a 1.0% agarose gel and visualized by ethidium bromide staining.

RESULTS

Growth suppression by expression of the p53-related genes Mammalian expression plasmids containing the cDNAs for two isoforms each of p73 and p51, as well as p53 were placed separately under a cytomegalovirus (CMV) promoter, and named pcDNA-p73$\alpha$, pcDNA-p73$\beta$, pcDNA-p51A, pcDNA-p51B and pcDNA-p53, respectively. Expression levels of p73 and p51 proteins were comparable, as detected by immunoblot analysis of cell lysates prepared following transfection with the corresponding Flag-tagged expression plasmids (Fig. 1). The effect of the Flag-tagged p53-related gene expression on growth regulation was also investigated. Introduction of wild-type p53 was previously reported to suppress the growth of a p53-deficient osteosarcoma cell line SaOS2. To determine whether the p53-related genes suppress tumor cell growth, SaOS2 cells were transfected separately with plasmids containing the p53-related gene cDNAs and were grown in the presence of G418. Fig. 2 shows that introduction of the p53-related genes resulted in substantial growth suppression. The growth suppression
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observed in p73β and p51A transfectants was similar to that observed for p53 (Fig. 2). Additionally, we transfected the p53-related genes into a lung cancer cell line (H1299) and a brain tumor cell line (T98G) and observed significant growth suppression (Fig. 2 and Table I). The average size of G418-resistant colonies of H1299 obtained by transfection of p53 or p53-related genes appeared smaller than that of pcDNA transfectants (Fig. 2). Introduction of the p51B and p73α expression plasmids generally resulted in less suppression (greater number of colonies) than that of pcDNA transfectants (Fig. 2). Introduction of the p51B and p73α expression plasmids generally resulted in less suppression (greater number of colonies) than that of pcDNA transfectants (Fig. 2). Introduction of the p51B and p73α expression plasmids generally resulted in less suppression (greater number of colonies) than that of pcDNA transfectants (Fig. 2).

Table I. p53-related Genes Suppress the Growth of Human Cancer Cells

| Transfected DNA      | Number of G418-resistant colonies | SaOS2 | H1299 | T98G |
|----------------------|-----------------------------------|-------|-------|------|
| pcDNA 3.1 (+)        | 689 (1.00)b)                      |       |       |      |
| pcDNA-p53            | 284 (0.41)                        |       |       |      |
| pcDNA-p73α           | 350 (0.51)                        |       |       |      |
| pcDNA-p73β           | 252 (0.37)                        |       |       |      |
| pcDNA-p51A           | 353 (0.51)                        |       |       |      |
| pcDNA-p51B           | 490 (0.71)                        |       |       |      |
| pCMVTag2α            | 745 (1.08)                        |       |       |      |
| pCMVTag2-p73α        | 632 (0.92)                        |       |       |      |
| pCMVTag2-p73β        | 661 (0.96)                        |       |       |      |
| pCMVTag2-p51A        | 644 (0.93)                        |       |       |      |
| pCMVTag2-p51B        | 662 (0.96)                        |       |       |      |
| a) pCMVTag2 is a Flag-tagged expression vector that generates a Flag epitope tagging the N-terminus of the indicated protein. b) The fraction of colonies (ratio) in each dish compared with the control vector transfected cells is indicated.

 Isoforms of the p53-related genes are distinguishable in transcriptional activity through a p53-responsive element We then tested the two isoforms of p73 and p51 for the ability to activate transcription of a luciferase reporter gene from a basal SV40 promoter and a p53 consensus binding sequence, pGL3-wt. A control reporter plasmid (pGL3-mt) was generated by altering the p53 consensus binding sequence. SaOS2 cells were transiently co-transfected with pGL3-wt or pGL3-mt and one of the p53-related gene-expressing plasmids. The ability to stimulate transcription through a p53-responsive element was calculated as the luciferase activity in cells transfected with the non-responsive reporter plasmid (pGL3-mt). As an additional control, we demonstrated that wild-type p53-expressing plasmid pcDNA-p53 had transactivation in a highly sequence-specific manner (Fig. 3). p73β also activated transcription from a reporter containing the p53 consensus binding sequence (Fig. 3). Interestingly, p73α was unable to transactivate the p53-responsive reporter (Fig. 3). This difference in responsiveness was not simply due to a lower expression level of p73α (Fig. 1). The results of the reporter assay with p51 expression plasmids were similar, but not identical, to those with p73 expression plasmids, with the exception of pcDNA-p51B which showed no significant effect on transcriptional activation (Fig. 3). Furthermore, this result was consistent with the luciferase assay using NCI-H1299 cells and a colon cancer cell line SW480 (data not shown), and with a previous report.
showing significant transactivation by p51A and only weak activity by p51B. Interestingly, all p53-related genes suppressed the growth of human cancer cells in culture (Table I), but neither pcDNA-p73α nor pcDNA-p51B produced transcriptional activation of the p53-responsive reporter (Table I, Fig. 3).

p53-related genes can transactivate endogenous p21 To examine whether the p53-related genes could activate transcription of endogenous p53-inducible genes, NCI-H1299 cells were infected with a replication-defective adenovirus containing a cDNA expression cassette, either Ad-p73α, Ad-p73β, Ad-p51A, Ad-p51B, Ad-p53 or Ad-lacZ. To verify the integrity of the cDNA in the recombinant adenovirus, the cDNA cassettes were amplified by PCR and their nucleotide sequences were determined. Western blot analysis revealed a significant induction of endogenous p21 protein in cells infected with Ad-p53 (positive control). Endogenous p21 was induced more effectively following infection with Ad-p73β than with Ad-p73α (Fig. 4). Ad-p51A infection resulted in a similar induction of endogenous p21, while p21 induction by Ad-p51B infection was barely detectable (Fig. 4). This is consistent with the results of transcripational activation of the reporter gene containing a p53-responsive element described above. These results suggest that p53-related genes have the potential to activate p53-target genes.

Growth suppression by p53-related genes involves apoptosis Since the p53-related genes p73 and p51 suppressed tumor cell growth in culture, we tested whether the growth suppression was mediated by induction of apoptotic cell death. To examine the effect of exogenous p53-related gene expression on apoptosis induction, human cancer cell lines were infected with a replication-defective adenovirus containing a cDNA expression cassette, either Ad-p73α, Ad-p73β, Ad-p51A or Ad-p51B. The relative efficiency of adenovirus infection was determined by X-gal staining of cells infected with a control adenovirus vector (Ad-lacZ) containing the bacterial lacZ gene in place of the p53-related genes. In all cell lines infected at an m.o.i. of 20 pfu/cell, more than 80% of the cells expressed β-galactosidase. To measure the apoptotic effect of the p53-related gene expression on several cell lines, the integrity of chromosomal DNA from the infected cells was monitored by agarose gel electrophoresis. p53-deficient NCI-H1299 lung cancer cells were infected at an m.o.i. of 20 pfu/cell with either Ad-p73α, Ad-p73β, Ad-p51A, Ad-p51B, Ad-p53 or Ad-lacZ. DNA from Ad-p53 infected cells showed a nucleosome ladder pattern, presumably as a result of apoptosis. Ad-p73β infection resulted in a similar pattern of DNA fragmentation, but more distinctly than infection with Ad-p73α or Ad-p53 (Fig. 5A). Interestingly, DNA from Ad-p51A and Ad-p51B infected cells showed a strong nucleosome ladder pattern (Fig. 5A). Expression of the p53-related genes in two other cell lines, a breast cancer cell line (ZR-75-1) and a lung cancer cell line (RERF-LC-OK), had an effect similar.
to that observed in NCI-H1299 cells (data not shown). These results suggest that p51, and especially p51A, is more effective than p53 and p73 for induction of apoptosis in several human cancer cells.

**p51 cooperates with E1A to induce apoptosis** To test the role of the p53-related genes on induction of apoptosis mediated by E1A expression, infection of Ad-E1A(12S), encoding adenovirus type 5 E1A 12S protein, together with either Ad-p53, Ad-p73 or Ad-p51 was performed on p53-deficient mouse 10(1) cells. Isolated DNA was analyzed by agarose gel electrophoresis to monitor DNA fragmentation. The Ad-p53/Ad-E1A(12S) coinfection resulted in a significant increase in the level of DNA fragmentation, while only minimal DNA fragmentation was observed in the case of Ad-p73α or Ad-p73β coinfection with Ad-E1A(12S) (Fig. 5B). In contrast to the effect of p73, co-expression of p51A or p51B with Ad-E1A(12S) resulted in a level of DNA fragmentation comparable to that seen in the Ad-p53 coinfection.

**DISCUSSION**

**Transactivation by the p53-related genes through a p53-responsive element** We demonstrated that two isoforms of p73 and p51 have distinct abilities to transactivate a reporter gene containing a p53 consensus binding sequence. p73β and p51A caused significant activation of the p53-responsive reporter gene, while neither p73α nor p51B transactivated the p53-responsive reporter gene (Fig. 3). This result suggests that p73β and p51A have the potential to transactivate p53 target genes. We also demonstrated that transfection of Ad-p73β and Ad-p51A transactivated endogenous p21 more effectively than Ad-p73α or Ad-p51B, respectively (Fig. 4). Yang et al. also reported that p51B failed to activate transcription from a promoter containing a p53-binding sequence (p51A and p51B were referred to as p63γ and p63α, respectively in ref. 10). However, Jost et al. and Laurenzi et al. used reporter assays in SaOS2 cells cotransfected with either of two isoforms of p73 or wild-type p53 and demonstrated transcriptional activation of the reporter genes containing different p53 binding sites. In these previous studies, both isoforms contained an amino-terminal hemagglutinin (HA) epitope tag, which could affect transcriptional activation by the p73 protein. In fact, the corresponding Flag-tagged expression plasmids for p53-related genes exhibited weak cell growth suppression activity compared with the non-Flag-tagged counterparts (Table I). Although the amino acid residues of p53 corresponding to sequence-specific DNA recognition and frequently mutated in human cancers (R175, G245, R248, R249, R273, and R282) are conserved and occupy identical positions in p73 and p51, the degrees of identity of amino acid sequence in the DNA binding region of p53 compared to p73 or p51 are 63% and 60%, respectively. Thus, the binding sequence of p53-related genes may be partly different from that of p53, which may potentially explain some of the similarities and differences between p53 and p53-related genes in the transcriptional activation of specific target genes. It is possible that the carboxy terminus of p73α confers unique structural properties that interfere with the p73 DNA binding domain. Further studies are required to test the function of the C-terminal domains of p73α and p73β. In a recent report, northern blot analysis revealed that most p53 target genes were induced more by p73β than by p73α, and only 14–3–3σ was significantly induced by both of the p73 isoforms. Further studies of the molecular basis of the transactivation by the p53-related genes are required to determine whether the binding sequence and the target genes are identical to those of p53.

**Induction of apoptosis** The p53-related genes p73 and p57 have previously been shown to suppress the growth of SaOS2 and BHK cells in a manner similar to p53, p51A and p51B have the potential to transactivate p53 target genes. We also demonstrated by colony formation assay in the presence of G418 that p73α and p51B were less potent growth suppressors than p73β and p51A, respectively. Adenovirus-mediated transfer of p73β, p51A and p51B induced cell death through apoptosis more extensively than Ad-p53, as assessed in terms of DNA fragmentation in a subset of human cancer cell lines (Fig. 5). Generally, this result was consistent with the tumor growth suppression measured by colony formation assays (Fig. 2). We demonstrated that p51 cooperated with the adenovirus E1A oncogene to induce cell death by apoptosis, while p73 stimulation of E1A-induced apoptosis was minimal (Fig. 5B). Although E1A and E1B 19K were thought to induce or inhibit apoptosis, respectively, the mechanism by which wild-type p53 and p51 stimulate apoptosis by E1A is unclear. As adenovirus-mediated p53-related gene transfer can cause p53-defective tumor cells to undergo apoptosis, the regulation of the p53-related genes might eventually have therapeutic applications. Although further studies are required to evaluate any undesirable effects of adenovirus-mediated p53-related gene transfer to normal tissues in vivo, our results suggest that adenovirus-mediated transfer of the p53-related genes would be a promising approach for the gene therapy of human cancers.

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