Suppression of Cancer Cell Growth by Adenovirus Expressing p21\(^{WAF1/CIP1}\) Deficient in PCNA Interaction\(^1\)

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ABSTRACT

p53 tumor suppression is deficient in the majority of human cancers. Efforts to understand this pathway have identified cyclin-dependent kinase (CDK) inhibitors and suggested a potential for their replacement in human cancer. In the present studies, expression of a C-terminal deletion mutant of the human p21\(^{WAF1/CIP1}\) CDK inhibitor completely suppressed the growth of colon cancer cells, whereas full-length p21 only partially suppressed growth. We prepared a replication-deficient adenoviral recombinant which expresses the p21 C-terminal mutant (Ad-WAF1-341) and compared its tumor suppressive abilities with Ad-p53 and Ad-LacZ. Ad-WAF1-341- and Ad-p53-infected cancer cells, but not Ad-LacZ-infected cancer cells, expressed a nuclear protein recognized by anti-p21 antibody and were deficient in cell cycle progression. The exogenous p21 mutant interacted with CDK2 but not proliferating cell nuclear antigen following infection of p21\(^{--}\) cancer cells. Ad-WAF1-341 was more potent than Ad-p53 in inhibiting DNA synthesis in human papillomavirus 16 E6-expressing cancer cells. Most importantly, the Ad-WAF1-341-infected E6-expressing cells died, whereas most of the Ad-p53-infected cells continued to proliferate. Endonucleolytic cleavage of DNA was observed in Ad-WAF1-341-infected cancer cells. These observations suggest that Ad-WAF1-341 should be evaluated in the treatment of human papillomavirus-associated neoplasia and other neoplasias resistant to p53.

INTRODUCTION

The most common genetic alteration in human cancer involves the p53 tumor suppressor gene (1, 2) and results in defective checkpoint control and cell death following exposure to DNA-damaging agents (3). Loss of p53 function has been associated with increased resistance of thymocytes to apoptosis following \(\gamma\)-irradiation (4), decreased apoptosis following treatment of fibroblasts with chemotherapeutic agents (5), and decreased apoptosis in in vitro models of tumorigenesis (6, 7). The mechanism of p53-mediated apoptosis is not entirely clear but may involve activation of genes such as bax or Fas/APO1 or may involve transcriptional repression (8–11). Another target for transcriptional activation by p53 is the CDK\(^1\) inhibitor p21\(^{WAF1/CIP1}\) (12), a negative regulator of cell cycle transitions (13, 14) following DNA damage (15, 16) or during differentiation (17). The expression of p21\(^{WAF1/CIP1}\) during development (18, 19), in adult tissues (18, 20), in senescence (21), or following treatment of cells with growth factors (22), differentiating agents (23–25), or transforming growth factor \(\beta\) (26–28) appears to be p53 dependent. The cell cycle inhibitory effects of p21\(^{WAF1/CIP1}\) may be attributed to its ability to bind CDKs as well as PCNA (29, 30). Targeted disruption of both p21\(^{WAF1/CIP1}\) alleles was compatible with normal mouse development, i.e., was not required for apparently normal development, but fibroblasts isolated from these mice had a deficiency in their G1 checkpoint following exposure to DNA damage (31). Although p21\(^{WAF1/CIP1}\) induction during p53-mediated apoptosis (15), its expression did not appear to be required for p53-mediated apoptosis of mouse thymocytes (31).

Because p21\(^{WAF1/CIP1}\) was growth inhibitory when expressed in tumor cells (12), and because its levels were low in mutant p53-expressing tumor cells (15), we chose to use it as a candidate for gene replacement in cancer. Because in our preliminary experiments we found that transfection of a C-terminal truncation mutant of p21 was more potent than w.t. p21 in tumor growth suppression, we prepared a C-terminal truncation mutant, p21\(^{WAF1/CIP1}\)-expressing, replication-deficient Ad (Ad-WAF1-341) and studied its biochemical and biological effects on tumor cells in vitro compared with Ad-p53 and Ad-LacZ.

We found that infection of mutant p53-expressing tumor cells with either Ad-WAF1-341 or Ad-p53 inhibited cell cycle progression into S-phase and prevented tumour cell proliferation. Of particular interest was our observation that Ad-WAF1-341, but not Ad-p53, infection of HPV16 E6-transfected tumor cells resulted in growth suppression. Endonucleolytic cleavage of DNA was observed in Ad-WAF1-341-infected cancer cells.

\(^1\) The abbreviations used are: CDK, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; Ad, adenovirus; w.t., wild-type; MOI, multiplicity of infection; kDa, kilodalton; HPV, human papillomavirus.
Western blot analysis following infection of either 293 or SW480 cells, and further purified as described previously (12). Adenoviral titers were determined by plaque formation following infection of 293 cells essentially as described previously (12). The MOI was defined as the ratio of total number of plaque-forming units used in a particular infection:total number of cancer cells to be infected. Ad-LacZ and Ad-p53 were prepared as described previously (12).

Transfections and Growth Assays. Approximately 1.5 × 10⁶ cells were seeded in 25-cm² tissue culture flasks 24 h before transfection. Six µg CsCl-banded DNA and 25 µg lipofectin (Life Technologies, Inc., Gaithersburg, MD) were used for transfections as described previously (12). Hygromycin (0.25 mg/ml) selection of growth inhibition experiments was initiated 24 h following transfection and maintained as described previously (12). In each experiment, we used β-galactosidase expression as a marker of transfection efficiency. In some experiments, we monitored the interflask variability in transfection efficiency by including a trace amount (4% of total DNA) of a luciferase reporter driven by a Rous sarcoma virus promoter and found that efficiency varied ±5% between different identical flasks (data not shown).

Immunoprecipitations and Western Blot Analysis. Transfected or infected cells were harvested in 1× SDS-PAGE-loading buffer and boiled for 5 min. A total of 50–100 µg protein/lane was electrophoresed through 15% denaturing polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes as described previously (15). Immunodetection of p21WAF1/CIP1 was performed using the anti-human WAF1 monoclonal antibody EA10 (Oncogene Science). Immunoprecipitations were carried out using either anti-human p21 antibody or anti-human CDK2 antibody, followed by Western blot analysis using anti-human p21, anti-human CDK2, or anti-human PCNA antibodies as described previously (15).

Immunofluorescence. Monolayers of infected tumor cell lines were fixed in Histochoice at room temperature for 5 min and permeabilized by incubation in 0.5% Triton X-100 in PBS at room temperature for 5 min. Permeabilized cells were treated with goat serum and then incubated in primary antibody, the monoclonal anti-human p21WAF1/CIP1 (Oncogene Science) in goat serum at room temperature for 2 h. The secondary antibody was a biotinylated goat antimer IgG (Pierce Chemical Co.). Avidin-conjugated fluorescein was used to develop staining, and cells were visualized under fluorescence microscopy.

Cell Cycle Arrest following Ad-WAF1-341 Infection. At 20 h following adenoviral infection, 5 µCi [³H]thymidine were added to wells containing approximately 20,000 tumor cells/well in 24-well plates. The plates were incubated for 2 h at 37°C, and incorporation of [³H]thymidine into newly synthesized DNA was determined as described previously (15).

Cell Death following Ad-WAF1-341 Infection. Rhodamine 123 and propidium iodide were used to distinguish between live and dead cells by visualizing under fluorescence as described. Analysis of DNA integrity was performed as described elsewhere (33). There may be a role for Ad-WAF1-341 in the treatment of cancers that are resistant to p53-induced cell death.
scribed previously (33), except that high molecular weight genomic DNA was not removed.

RESULTS

Expression of Transfected w.t. and Mutant p21WAF1/CIP1 in Tumor Cells. In transient transfection experiments, we verified that expression of p21 protein was increased following introduction of pC-WAF1-S or pC-p53-w.t., and expression of a truncated p21 (341-mutant) was detected following introduction of either pC-WAF1-341 or pMV60-WAF1-341 expression vectors in human colon adenocarcinoma SW480 cells (Fig. 1). The truncated p21 protein migrates at 12-13 kDa. The exogenous p21 proteins appeared to be overexpressed, because elevated levels were detected with only about 10% transfection efficiency of the SW480 cells. The increase in w.t. p21 protein following pC-p53-w.t. transfection appeared unimpressive due to the relatively low transfection efficiency in the transient transfections; however, this vector was an extremely potent growth suppressor following stable transfection (see below).

Tumor Growth Suppression following Stable Expression of a p21WAF1/CIP1 C-Terminal Deletion Mutant. To evaluate the tumor growth-suppressive properties of the p21WAF1/CIP1 C-terminal deletion mutant, we transfected

Fig. 2  A p21WAF1/CIP1 C-terminal deletion mutant (WAF1-341) is more potent than w.t. p21 in growth suppression. SW480 human colon adenocarcinoma cells were transfected with pCEP4 vector (A), pC-WAF1-S (B), pC-p53-w.t. (C), or pC-WAF1-341 (D) and selected in the presence of hygromycin as described in “Materials and Methods.” Numbers in parentheses, percentages of colonies relative to vector-alone transfection (100% = 600 colonies in this experiment).
SW480 colon cancer cells with w.t. p53 and w.t. or mutant p21<sub>WAF1/CIP1</sub> expression vectors carrying the hygromycin resistance gene and selected for stable colony formation in the presence of hygromycin. Fig. 2 shows, as reported previously (12), that transfection of the vector alone resulted in numerous colonies following hygromycin selection (100%), whereas transfection with w.t. p53 resulted in no stable clones (0%). Transfection of w.t. p21<sub>WAF1/CIP1</sub> resulted in few colonies (10% of the vector alone), as reported previously (12, 36). However, similar to w.t. p53 transfection, expression of the p21<sub>WAF1/CIP1</sub> C-terminal deletion mutant (341-mutant) completely suppressed growth of SW480 cells, i.e., there were no colonies following selection in the presence of hygromycin (0%). A similar result has recently been reported, using a different C-terminal deletion mutant of p21<sub>WAF1/CIP1</sub> (37). In addition to SW480 cells, in which we have reproduced the absence of stable colonies in six independent transfections, we have reproduced this observation in brain and lung cancer cells (data not shown).

**Ad-WAF1-341 Infects p21<sup>-/-</sup> Colon Cancer Cells and Expresses a Truncated p21 Protein That Associates with CDK2 but not PCNA.** Based on its potent tumor growth-suppressive effects, we chose to isolate an adenoviral recombinant expressing the 341 C-terminal deletion mutant of human p21<sub>WAF1/CIP1</sub> to evaluate its potential role in gene replacement in human cancer. The 341-mutant p21 protein lacks the C-terminal PCNA-interacting domain. To show that this truncated protein, produced following adenoviral infection, was still capable of interacting with CDK2 but was not capable of associating with PCNA, we examined the cyclin-CDK-p21 and PCNA-p21 complexes in p21<sup>+/+</sup> and p21<sup>-/-</sup> colon cancer cells (Fig. 3). Following infection of p21<sup>+/+</sup> cells by either Ad-LacZ or Ad-p53, we found evidence for association of w.t. p21 with CDK2 (Fig. 3A). Whole-cell lysates immunoprecipitated using anti-human CDK2 antibodies contained w.t. p21 protein (Fig. 3A, Lanes 1 and 2). The C-terminal truncation mutant of p21 also associated with CDK2 following infection of either p21<sup>+/+</sup> or p21<sup>-/-</sup> cells with Ad-WAF1-341 (Fig. 3A, Lanes 3 and 6). As expected, infection of p21<sup>-/-</sup> cells with either Ad-LacZ or Ad-p53 resulted in p21-lacking CDK2 immune complexes (Fig. 3A, Lanes 4 and 5). In contrast, the C-terminal truncation mutant of p21 failed to associate with PCNA in p21<sup>-/-</sup> cells, although PCNA was clearly present in the whole-cell lysates, and the truncated p21 protein was expressed (Fig. 3B, Lane 4). As expected, infection of p21<sup>+/+</sup> cells with either Ad-p53 or Ad-WAF1-341 and immunoprecipitation with anti-human p21 antibody resulted in association of PCNA with w.t. p21 (Fig. 3B, Lanes 1 and 2). Infection of p21<sup>-/-</sup> cells with Ad-p53 produced no p21-PCNA complexes, although PCNA was clearly present in the whole-cell lysates (Fig. 3B, Lane 3).

**Ad-WAF1-341 Targets Mutant p21<sub>WAF1/CIP1</sub> Protein to the Nucleus and Inhibits DNA Replication.** Because the C-terminus of p21<sub>WAF1/CIP1</sub> contains a putative nuclear localization signal (12), we investigated the subcellular localization of p21<sub>WAF1/CIP1</sub> protein following infection of tumor cells with Ad-WAF1-341. Fig. 4 shows that following infection of SW480 colon cancer cells with either Ad-p53 or Ad-WAF1-341, a nuclear protein recognized by anti-human p21<sub>WAF1/CIP1</sub> antibody is overexpressed, compared with either uninfected or Ad-
LacZ-infected cells. Using a MOI of 20 in these adenoviral infections, we observed nuclear p21 expression in >90% of Ad-p53- and Ad-WAF1-341-infected cells. Ad-WAF1-341-infected cells displayed increased cytoplasmic reactivity versus Ad-p53-infected cells, suggesting that loss of the nuclear localization signal affects nuclear transport. However, the majority of the p21\textsuperscript{WAF1/CIP1} deletion mutant gets into the nucleus. We would speculate that it may get into the nucleus by diffusion, because it is a relatively small protein (~12–13 kDa), or it may cross while associated with cyclin-CDK complexes.

Fig. 4 also shows that following infection of SW480 cells with Ad-p53 or Ad-WAF1-341, but not Ad-LacZ, there was a potent inhibition of new DNA synthesis. Thus, expression of the p21\textsuperscript{WAF1/CIP1} C-terminal deletion mutant was associated with inhibition of cell cycle progression despite a lack of association with PCNA.

**Ad-WAF1-341 Is More Potent Than Ad-p53 in Inhibiting Proliferation of HPV16 E6-transfected Cancer Cells.** We next tested the cell cycle effects of varying doses of Ad-WAF1-341 and Ad-p53 following infection of other cell types.
Fig. 5 Cell cycle arrest following infection of human cancer cells by Ad-WAF1-341 or Ad-p53, but not Ad-LacZ. SKBr3 (A), SW480 (B), H460/neo (C), and H460/E6 (D) cancer cells were infected with different MOIs (as indicated under the columns) of Ad-LacZ (●), Ad-p53 (□), or Ad-WAF1-341 (■), and incorporation of thymidine into newly synthesized DNA was measured (Y axes) as described in “Materials and Methods.” Bars, SD.

(Fig. 5). Because expression of HPV type 16 or 18 E6 protein targets the p53 tumor suppressor for degradation, we chose to use E6-expressing cancer cells to evaluate whether expression of the p21 C-terminal deletion mutant might offer a way to bypass the defect and possibly to offer a therapeutic advantage over replacement of wt. p53. We found that by MOIs of 20 or greater, both Ad-p53 and Ad-WAF1-341 (compared with Ad-LacZ or uninfected cells) inhibited cell cycle progression and cell proliferation of the mutant p53-expressing human breast cancer cell line SKBr3 (Fig. 5A) and the mutant p53-expressing colon adenocarcinoma cell line SW480 (Fig. 5B) or the wt. p53-expressing human non-small cell lung cancer cell line H460 (Fig. 5C). However, H460 cells, which were transfected by HPV16 E6 (H460/E6 cells), appeared more sensitive to the cytostatic effects of Ad-WAF1-341 than Ad-p53 (Fig. 5D).

We suspected that the decreased sensitivity of H460/E6 cells might be due to active degradation of exogenous p53 due to the presence of E6 protein. We have previously shown that H460/E6 cells have undetectable levels of p53 and p21, either before or after exposure to DNA-damaging agents, presumably due to degradation of DNA damage-induced, endogenous p53 (33). The absence of p53 and p21 following exposure of H460/E6 cells to DNA-damaging agents such as etoposide was correlated with a defective G1 checkpoint (33). Fig. 6 shows that Ad-p53 infection of H460/neo cells resulted in accumulation by 24 h of endogenous p21 protein in the nuclei of infected cells (Fig. 6a), whereas Ad-p53 infection of H460/E6 cells resulted in lower p21 accumulation, and this was observed in only a minority of the cells (Fig. 6, b and c). In contrast, following Ad-WAF1-341 infection, the exogenous C-terminal p21 truncation mutant was expressed at similar levels in the nuclei of either H460/neo or H460/E6 cells (Fig. 6, d and e). Thus, it appears that the relative resistance of H460/E6 cells to the cytostatic effects of Ad-p53 might be due to degradation of the exogenous p53 protein, accompanied by a failure to arrest the cell cycle. Not unexpectedly, the exogenous C-terminal truncation mutant of p21 appeared to be resistant to E6-mediated degradation.

We next compared the cytotoxic effects of Ad-p53 or Ad-WAF1-341 infection of H460/neo and H460/E6 cells (Fig. 6). At a MOI of 150, most Ad-LacZ-infected H460/neo or H460/E6 cells were proliferating by day 3 (Fig. 6, f and i). Under the same conditions, both Ad-p53-infected (Fig. 6g) or Ad-WAF1-341-infected (Fig. 6h) H460/neo cells underwent massive cell death. In addition to arresting cell growth of the H460/E6 cells (Fig. 5), Ad-WAF1-341-infected H460/E6 cells died by day 3 (Fig. 6i), whereas most of the Ad-p53-infected H460/E6 cells appeared to proliferate (Fig. 6j). Thus, in the presence of HPV16 E6 protein, there was greater cytotoxicity following Ad-WAF1-341 infection than Ad-p53 infection. We have observed similar results at a MOI of 30. We have repeated these cytotoxicity experiments in three independent experiments, each time in duplicate, with similar results. We cannot rule out the possibility that E6 could be affecting the sensitivity of the cells to p21WAF1/CIPI through a p53-independent mechanism. This possibility can be specifically ruled out in the future by creating lines that express HPV16 E6 protein in cells containing mutant p53 protein or that are null for p53 and studying the effect of Ad-WAF1-341 infection. It should be noted, however, that Ad-WAF1-341 inhibited cell cycle progression in human cancer cells in the absence of any E6 (Figs. 4e, 5, A–C, and 6h).

Ad-WAF1-341-infected Cancer Cells Undergo Apoptotic Death. To further investigate the mechanism of cell death in Ad-WAF1-341-infected cells, we isolated genomic DNA and analyzed its integrity. For these experiments, we used HCT/neo and HCT/E6 cells and first confirmed that they were sensitive to Ad-WAF1-341 infection (as the H460/neo and H460/E6 cells shown in Fig. 6; data not shown). Analysis of cellular DNA integrity revealed evidence for endonucleolytic cleavage in the HCT/neo and HCT/E6 cells following Ad-WAF1-341, but not Ad-LacZ, infection (Fig. 7; compare Lanes 3 and 5 with 2 and 4). An identical pattern of DNA fragmentation was observed in H460/neo and H460/E6 cells infected by Ad-WAF1-341 versus Ad-LacZ (data not shown). These results suggested that the truncated p21-expressing Ad was capable of inducing apoptosis following infection of human cancer cells.

DISCUSSION

We showed that a replication-deficient Ad encoding a C-terminal deletion mutant of human p21 (Ad-WAF1-341) was capable of infecting human cancer cells, expressing a ~12–13-kDa truncated p21 nuclear protein that associated with CDK2 but not PCNA. Ad-WAF1-341- or Ad-p53-infected cancer cells underwent cell cycle arrest and eventual cell death. We found that HPV16 E6-transfected human lung cancer cells were more resistant to both the cytostatic and cytotoxic effects of Ad-p53 versus Ad-WAF1-341. Ad-WAF1-341-infected, E6-transfected cells arrested and eventually underwent apoptosis, whereas Ad-p53 infection of the same cells resulted in continued proliferation, presumably due to degradation of the exogenous p53.
It is not clear why the C-terminal truncation mutant of p21 is a more potent tumor growth suppressor compared with w.t. p21. It is possible that drastically altering the distribution of p21 between G1 cyclin-CDK complexes and PCNA might signal an irreversible catastrophe, which may induce programmed cell death. Alternatively, it is possible that in the absence of PCNA interaction, the p21 association with cyclin-CDK complexes is so potent that irreversible growth arrest occurs, and the cells eventually undergo apoptosis. There has been only one report that w.t. p21 was capable of inducing cell death following...
transfection (37). Future studies will investigate the reason(s) why cells are more sensitive to truncated p21 and whether interaction with PCNA by w.t. p21 might offer a protective role, possibly by allowing such cells time to repair, or whether the absence of cyclin-CDK effects at the replication fork might actively induce apoptosis, as observed here following infection with Ad-WAF1-341 (Fig. 7).

The Ad-WAF1-341 reagent described here should be a useful tool for biochemical studies of cell cycle regulation, because Ad infects a wide range of human cell types, and the truncated p21 clearly arrests the cell cycle but does not associate with PCNA. Thus, as has been found by others, the C-terminal PCNA-interacting domain of p21 is not required for growth suppression (38) but because Ad infects a wide range of human cell types, and the truncated p21 clearly arrests the cell cycle but does not associate with PCNA. Thus, as has been found by others, the C-terminal PCNA-interacting domain of p21 is not required for growth suppression (38) but may be required to bring cyclin-CDK complexes to replication centers (39), although the C-terminal PCNA-interacting domain is clearly not required for cell cycle arrest by p21. Finally, Ad-WAF1-341 suppression of HPV16 E6-transfected cancer cells may provide a way to bypass a pathogenic defect in controlling the growth of HPV-associated cancers or other cancers in which w.t. p53 function is disrupted by downstream inhibitors (40). Future studies will explore this possibility further.

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