Efficacy of Adenovirus-mediated CD/5-FC and HSV-1 Thymidine Kinase/Ganciclovir Suicide Gene Therapies Concomitant with p53 Gene Therapy

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ABSTRACT

Recent evidence has suggested that tumor cells having a wild-type p53 status are more sensitive to chemotherapeutic agents and radiation than cells that lack functional p53. The heightened sensitivity of wild-type p53 cells is thought to be attributable to their propensity to undergo p53-mediated apoptosis after insult. Given that suicide gene therapy is essentially tumor-targeted chemotherapy, we examined the hypothesis that coexpression of wild-type p53 could enhance the efficacy of adenovirus-mediated suicide gene therapy. Human Hep3B and SK-OV-3 cells, which are null for p53, were infected with a pair of replication-deficient adenoviruses that expressed a cytosine deaminase/herpes simplex virus thymidine kinase (CD/HSV-1 TK) fusion gene without (fusion gene nonreplicative adenovirus, FGNR) or with (FGNRp53) the wild-type human p53 gene. The sensitivity of cells to the CD/5-fluorocytosine (CD/5-FC) and HSV-1 TK/ganciclovir (GCV) enzyme/prodrug systems was determined in vitro and in vivo. Coexpression of p53 did not enhance the cytotoxicity of either the CD/5-FC or HSV-1 TK/GCV system in vitro. The failure to observe an effect of p53 could not be explained on the basis of insufficient or transient p53 expression, because FGNRp53-infected cells growth arrested in G1, induced Bax, and underwent apoptosis at an increased rate after prodrug treatment, particularly when the adenovirus E1A protein was present. Intratumoral injection of FGNRp53 concomitant with single or double prodrug therapy resulted in a tumor growth delay that was equal to or less than that observed with the FGNR virus. Our results indicate that coexpression of p53 may not necessarily improve the efficacy of adenovirus-mediated CD/5-FC and HSV-1 TK/GCV suicide gene therapies in vivo.

INTRODUCTION

Cancer chemotherapy works because it destroys malignant cells more efficiently than normal cells. It was long thought that the basis for this differential effect was attributable to the fact that, in general, tumors contain a higher fraction of cycling cells relative to the surrounding normal tissue. Although this concept may adequately explain the differential sensitivity between malignant and normal tissue, it is insufficient to account for the wide range of chemosensitivities displayed by human tumors because tumor growth fraction is often a poor predictor of the response to chemotherapy (1). Such observations suggest that the effectiveness of a chemotherapeutic agent may not be directly related to its ability to kill actively dividing cells.

Recent evidence suggests that many chemotherapeutic agents exert their cytotoxic effect, at least in part, through the induction of apoptosis. Although apoptosis is a complex process involving many genes (2), one gene that plays a pivotal role in the apoptotic cascade is the tumor suppressor p53. Previous studies have indicated that tumor cells having a wt p53 status are more sensitive to chemotherapeutic drugs, such as 5-FU, and radiation than their isogenic counterparts lacking functional p53 (3, 4). The heightened sensitivity of tumor cells with normal p53 is thought to be attributable to their increased propensity to undergo p53-mediated apoptosis after injury. Such results are buttressed by observations made in the clinic in which for some cancers (mostly leukemia and lymphoma), an altered tumor p53 status has been correlated with a poor response to chemotherapy and radiation therapy and a poorer prognosis (5–8). Although this association is somewhat controversial and may not be applicable to all cancers (9–12), at least for some cancers, the p53 status may be a strong determinant in the response of tumors to chemotherapy or radiation therapy and, therefore, may be a critical factor that determines the outcome of many treatment protocols.

Suicide gene therapy is a new experimental form of cancer chemotherapy that is currently being evaluated in human trials (13). This approach involves intratumoral delivery of genes encoding enzymes that convert nontoxic prodrugs into toxic antimetabolites. Two suicide genes that are being evaluated in the clinic are the Escherichia coli CD and HSV-1 TK genes, which confer sensitivity to 5-FC and GCV, respectively (14–
16). The rationale behind the suicide gene therapy approach is that after “targeted” transfer of these genes to the tumor, only tumor and neighboring cells will be rendered sensitive to their cytotoxic action. Because suicide gene therapy is essentially tumor-targeted chemotherapy, the systemic toxicity commonly associated with, and a major limitation of, conventional chemotherapy is avoided. Although originally conceived as a new form of cancer therapy that could be used independently, we have pioneered the concept of using suicide gene therapy as a neoadjuvant to radiation therapy (17–23). Using a variety of tumor models, we have demonstrated that both the CD/5-FC and HSV-1 TK/GCV systems can potentiate the efficacy of radiation therapy, resulting in significantly better tumor control and tumor cure (18, 22, 23).

Because suicide gene therapy is a form of cancer chemotherapy, we examined the hypothesis that coexpression of wt p53 could potentiate the efficacy of the suicide gene therapy approach. Using a pair of adeno viral vectors that express a CD/HSV-1 TK fusion gene without or with the wt human p53 gene, we found that coexpression of p53 did not enhance the cytotoxicity of CD/5-FC or HSV-1 TK/GCV suicide gene therapies in vitro or in vivo. We present evidence that may explain why previous studies observed an association between tumor p53 status and the sensitivity of tumors to chemotherapy and radiation therapy.

MATERIALS AND METHODS

Generation of FGNR and FGNRp53 Adenoviruses. All plasmids containing adenoviral sequences used in the generation of the FGNR and FGNRp53 viruses were obtained from Microbix (Toronto, Ontario, Canada). Construction and characterization of the CD/HSV-1 TK fusion gene has been described previously (17). The 2.5-kb CD/HSV-1 TK fusion gene was removed from pWZLneoCDglyTK (17) by a partial BamHI-EcoRI digestion and cloned between the BamHI and EcoRI sites of pCA14 (Microbix), generating pCA14-CDglyTK. pCA14 (24) is a left-end shuttle vector that contains Ad5 sequences from base 22 to base 5790 (minus bases 342 to 3523 in the E1 region), the human CMV promoter, a multiple cloning site for insertion of genes, and SV40 polyadenylation sequences. The resulting expression cassette consists of the human CMV promoter, CD/HSV-1 TK fusion gene, and SV40 polyadenylation elements. To construct pCA14-CDglyTK-wtp53, the 1.2-kb wt human p53 gene was generated by PCR using a 1.8-kb BamHI fragment from pCMV-neo-Bam (25) as template. The 5’ primer was 5’-GCGGGGATCCCGATGATAATACCATGGAGGAG-3’, which places the proper spacing and sequences for efficient translation between the EMCV IRES and the authentic human p53 translation start codon (underlined); and the 3’-primer was 5’-GCGGGGATCCGAATTCAAGATCTCGAGTCAAGGC-3’, which places EcoRI and BamHI sites downstream of the authentic p53 stop codon (complement underlined). The resulting 1.2-kb PCR product was digested with BamHI and cloned into the sole BamHI site of pLNS2 (26), a retroviral vector that contains the 500-bp EMVC IRES cloned between the EcoRI and BamHI sites of pLNSN (27). The fused EMVC IRES and wt human p53 gene (1.7 kb) were removed by EcoRI digestion and cloned into the sole EcoRI site of pCA14-CDglyTK, generating pCA14-CDglyTK-wtp53. The resulting expression cassette consists of the human CMV promoter, CD/HSV-1 TK fusion, and p53 genes, separated by the EMVC IRES, and SV40 polyadenylation elements (Fig. 1).

To generate FGNR and FGNRp53 viruses, pCA14-CDglyTK and pCA14-CDglyTK-wtp53 (10 μg) were linearized by Poul digestion and cotransfected with Clal-linearized pBH10 (30 μg) into human embryonic kidney (HEK) 293 cells (Microbix) using the CaPO4-DNA precipitation method. pBH10 (24) is a plasmid that contains the entire Ad5 genome minus bases 188 to 1,339 in the E1 region and bases 28,133 to 30,818 in the E3 region. Isolated plaques were harvested 10 days later and plaque-purified a second time on HEK 293 cells. Virus from twice purified plaques was used to infect 293 cells to generate crude viral lysates and CsCl gradient-purified virus. Crude viral lysates (107–109 pfu/ml) and CsCl gradient-purified virus (1010–1011 pfu/ml) were titered by the plaque assay using HEK 293 cells.
Cell Lines and Adenovirus Infection in Vitro. SK-OV-3, a human ovarian carcinoma cell line, was maintained in MEM supplemented with 10% fetal bovine serum (MF-10) and nonessential amino acids. Hep3B, a human hepatocellular carcinoma cell line, was maintained in the same medium with sodium pyruvate. Both cell lines are null for p53 and were obtained from the American Type Culture Collection. Cells were plated 1 day prior to infection. Cells were infected with virus at the desired MOI for 1 h at 37°C, followed by the addition of growth media.

Western Blotting and Immunofluorescent Staining. Cells (1 × 10^6, 60-mm diameter dish) were infected with virus at the desired MOI. Forty-eight h after infection, cells were harvested, counted, and lysed in Laemmli sample buffer. Extracts representing an equal number of cells were applied to a SDS-10% polyacrylamide gel and transferred to nitrocellulose using standard procedures. Blots were probed with a rabbit polyclonal antibody to CD (provided by C. Richards, Glaxo-Wellcome), a monoclonal antibody to HSV-1 TK (provided by W. Summers, Yale University, New Haven, CT), a monoclonal antibody (DO-1) to human p53 (Santa Cruz Biotechnology), or a rabbit polyclonal antibody (N-20) to Bax (Santa Cruz Biotechnology). Hybridization was visualized using the ECL chemiluminescence detection system (Amersham). For immunofluorescent staining, cells were infected at the desired MOI in four-well chamber slides. Forty-eight h later, cells were fixed in formalin, permeabilized with methanol, and incubated with anti-CD and/or anti-p53 antibodies, followed by a Texas-Red conjugated donkey anti-rabbit IgG and/or FITC-conjugated donkey anti-mouse IgG, respectively. Samples were counterstained with 4,6-diamidino-2-phenylindole and photographed using an Olympus BX-40 fluorescent microscope.

Clonogenic Assays. Cells were seeded into 24-well culture plates (5 × 10^3 cells/well) and infected with virus at an MOI of 100 for 1 h. Forty-eight h after infection, cells were incubated in growth medium containing varying concentrations of 5-FC (Sigma) or GCV (Syntex) for 24 h. Cells were detached by trypsinization and replated in triplicate at low density (10^3 cells/60-mm diameter dish) in drug-free medium. Colonies were fixed, stained with crystal violet, and counted 10–14 days later.

Flow Cytometric Analysis. Actively growing cells (1 × 10^6 cells/T-75 flask) were either mock-infected or infected with virus at an MOI of 100. Forty-eight h later, cells were harvested by trypsinization, washed two times with PBS, and fixed in 70% ethanol overnight at ~20°C. Cells were resuspended in 1 ml of PBS containing 5 μg/ml propidium iodide and 50 μg/ml RNase A and analyzed using a Coulter Epics flow cytometer.

Human Tumor Xenograft Models and Prodrug Therapy. Female athymic nude mice (CD-1 nu/nu; Charles River Laboratories) and male severe combined immunodeficient mice (CB17, SC-M homozygous; Taconic) were used for SK-OV-3 and Hep3B studies, respectively. Tumor cells were resuspended at a concentration of 4 × 10^7 cells/ml in 0.9% NaCl and kept on ice prior to use. Two million (2 × 10^7) cells were injected s.c. into the abdomen. Injection of virus commenced when the tumor volume reached 100 mm^3 (day 0). Prior to treatment, animals were placed randomly into the various groups such that each group contained five (PBS) to eight (treatment groups) mice. Beginning on day 0, 10^8 pfu of CsCl gradient-purified virus (50 μl) was injected intratumorally for 5 consecutive days (days 0–4). Daily i.p. injections of 5-FC (500 mg/kg) and GCV (30 mg/kg) were administered from day 0 to day 27. Tumors were measured every other day until the average tumor volume of the group reached 500 mm^3. Animals were observed for 90 days or until their tumors reached 2 cm^3. In vivo studies were performed twice.

Apoptosis Assays. Apoptosis was measured using the ApoTag in situ apoptosis detection kit (Oncor) according to the protocol provided by the supplier. Apoptotic cells were scored randomly by examination (×500) under a light microscope. At least 600 cells were counted. Where indicated, both attached and detached cells were pooled after prodrug treatment, and cyto- 

Generation of E1A-expressing Hep3B Cell Lines and DNA Transfections. The E. coli β-gal and Ad5 E1A genes were generated by PCR and cloned into the pWZLneo retroviral vector (17). Virus was generated by transient transfection of CAK packaging cells. Hep3B cells (5 × 10^5 cells/60-mm diameter dish) were infected with virus, and 2 days later, replated in growth medium containing 400 μg/ml G418 (Life Technologies, Inc.). Isolated colonies were picked 3 weeks later, grown in mass, and screened for expression of β-gal by histochemical staining and E1A by Western blotting. Hep3B β-gal and E1A clonal lines (4 × 10^5 cells) were transfected with pCA14-CDglyTK or pCA14-CDglyTKp53 using the lipofection method (Lipofectamine; Life Technologies, Inc.). Forty-eight h later, cells were treated with varying concentrations of 5-FC or GCV for 24 h. Cells were scored for apoptosis immediately after prodrug treatment. The DNA transfection efficiency was determined by CD immunofluorescence (21).

RESULTS

Coexpression of CDglyTK Fusion and Human p53 Proteins. To examine the hypothesis that coexpression of wt p53 could potentiate the efficacy of suicide gene therapy, we generated a pair of replication-deficient adenoviral vectors that expressed a CD/HSV-1 TK fusion gene without (FGNR) or with (FGNRp53) the wt human p53 gene (Fig. 1). In FGNRp53, both the CDglyTK fusion and p53 proteins are expressed from the same bicistronic mRNA, thereby increasing the likelihood that they would be coexpressed in the same cell. To demonstrate this point, Hep3B and SK-OV-3 cells were infected with the FGNR and FGNRp53 viruses and examined for expression of the CDglyTK and p53 proteins by Western blotting and immunofluorescence. Hep3B and SK-OV-3 cells were selected for these studies because they are both null for p53 (see below), thereby eliminating any possible interference from endogenous p53 (wt or mutant) proteins. Consistent with our previous observations with retrovirally transduced cells (17), expression of the Cd/HSV-1 TK fusion gene resulted in the production of the M, 90,000 CDglyTK fusion protein that could be detected with antibodies to both CD and HSV-1 TK (Fig. 2A, top). Importantly, the level of CDglyTK fusion protein expression from the FGNR and FGNRp53 viruses was identical. As expected, cells
infected with FGNRp53, but not FGNR, also expressed the human p53 protein. The amount of p53 expressed from the FGNRp53 virus was markedly greater (by 1–2 logs) than, for example, that produced in normal human MRC-5 fibroblasts (Fig. 2A, bottom). Expression of both the CD/HSV-1 TK and p53 transgenes peaked 2–3 days after infection and persisted for at least an additional 4 days (data not shown).

Coexpression of the CDglyTK and p53 proteins was further demonstrated by double immunofluorescence after infection of cells with FGNRp53. At all MOIs examined (1, 5, 10, 50, and 100), the percentage of cells expressing the p53 protein was similar to, but consistently less than, that expressing the CDglyTK protein (not shown). At saturating levels of the virus (MOI, 100), 100% of cells expressed the cytoplasmic CDglyTK protein, whereas 90% expressed p53 in their nucleus (Fig. 2B). Thus, an MOI of 100 was used in all subsequent experiments performed in vitro.

To demonstrate that the FGNRp53 virus produced a functional p53 protein, infected cells were examined by flow cytometry for G1 and G2 growth arrest and for induction of a p53 target, the apoptosis-promoting Bax gene. Whereas FGNR had no effect on the cell cycle distribution of SK-OV-3 cells, infection with FGNRp53 reduced the fraction of cells in S phase by 80%, most of which (76%) arrested in G1 (Fig. 2C). FGNRp53

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**Fig. 2** Coexpression of CDglyTK fusion and p53 proteins. A, Hep3B cells were either mock-infected or infected with the FGNR and FGNRp53 viruses as indicated. Forty-eight h later, whole-cell extracts were prepared and subjected to Western blotting. Triplicate filters were probed with antibodies to E. coli CD, HSV-1 TK, or human p53 as indicated (top). The position of the CDglyTK and human p53 proteins are indicated. The CDglyTK fusion protein is susceptible to proteolysis between the CD and TK moieties and as a result, produces smaller degradation products in Western blots (17). The level of p53 expression in FGNRp53-infected Hep3B cells is compared with human MRC-5 cells (bottom). Extract from an equal number of cells was applied to each lane. B, Hep3B cells were infected with FGNRp53 at an MOI of 100 and analyzed by double immunofluorescence 48 h later. Cells expressing CD fluoresce red (Texas Red) in their cytoplasm, and those expressing p53 fluoresce green (FITC) in their nucleus. Mock-infected cells (not shown) gave a very low background with both fluorescent probes. Similar results were obtained with SK-OV-3 cells. C, SK-OV-3 cells were either mock-infected or infected with the FGNR and FGNRp53 viruses at an MOI of 100. Cells were harvested 48 h later and analyzed by flow cytometry. The percentage of cells in each phase of the cell cycle is indicated. Hep3B cells gave similar results. D, SK-OV-3 cells were infected as described in C and examined 48 h later for Bax expression by Western blotting. The level of Bax induction is compared with human MRC-5 fibroblasts before and after irradiation with 8 Gy.
infection resulted in a 10-fold induction of Bax, which was significantly greater than that (2-fold) observed after FGNR infection and comparable with that (5-fold) observed after irradiation of human fibroblasts (Fig. 2D). Together, the results demonstrate that FGNRp53 coexpresses the CDglyTK and human p53 proteins, the latter of which is expressed at a level sufficient to induce growth arrest and transactivate one of its downstream target genes.

**Effect of p53 Expression on Cytotoxicity of Suicide Gene Systems in Vitro.** To determine whether coexpression of p53 could enhance the cytotoxicity of the CD/5-FC or HSV-1 TK/GCV enzyme/prodrug systems in vitro, Hep3B and SK-OV-3 cells were infected with the FGNR or FGNRp53 viruses, and their sensitivity to 5-FC and GCV prodrug therapy was compared using clonogenic assays. With Hep3B cells, both prodrugs killed in a concentration-dependent manner achieving >2 logs of cell kill at the highest prodrug concentration examined (Fig. 3A). However, after correcting for the effect of p53 itself (see the legend), the 5-FC and GCV survival curves of the FGNR- and FGNRp53-infected cells were essentially identical. The 5-FC + GCV prodrug combination was not examined, because these enzyme/prodrug systems can result in synergistic cell kill, which is often too extensive to quantify using clonogenic assays. SK-OV-3 cells proved to be resistant to the CD/5-FC or HSV-1 TK/GCV enzyme/prodrug systems in vitro; Hep3B and SK-OV-3 cells were mock-infected or infected with FGNR (○) or FGNRp53 (●) at an MOI of 100. Forty-eight h later, cells were incubated with graded concentrations of 5-FC or GCV for 24 h and then replated for clonogenic assays. Colonies were stained and counted 10-14 days later. The results represent the mean of triplicate determinations. Each survival curve (mock, FGNR, and FGNRp53) was normalized to its own no prodrug control (100%) to correct for any effect of p53 itself. In the absence of prodrugs, FGNRp53 yielded an average of 75% and 94% of the number of colonies as FGNR for Hep3B and SK-OV-3 cells, respectively. The clonogenic assays were repeated three times, yielding similar results.

**Effect of Suicide Gene Systems and p53 on Apoptosis.** Recent studies have suggested that many chemotherapeutic agents kill cells by inducing p53-dependent apoptosis (3-5). To determine whether this was true for the CD/5-FC or HSV-1 TK/GCV enzyme/prodrug systems, Hep3B cells, which demonstrated sensitivity to both systems, were infected with the FGNR or FGNRp53 viruses, and the amount of apoptosis was determined after treatment with prodrugs. It is important to note that after prodrug treatment, both attached and detached cells were included in this analysis. In the absence of prodrugs, the amount of apoptosis was not significantly different between FGNR- and FGNRp53-infected cells (Fig. 3A). This observation is consistent with the fact that, typically, p53 expression itself does not result in apoptosis, and the effects of p53 are observed only after insult, for example, with chemotherapeutic agents or radiation. Except for the highest concentration (80 μg/ml) of 5-FC, the amount of apoptosis after infection with FGNRp53 was, on average, 2-fold greater (P < 0.015) than with FGNR. Thus, as expected, p53 is able to stimulate apoptosis after insult with either enzyme/prodrug system. It is important to note, however, that at all prodrug concentrations examined, the amount of average colony size, indicating that p53 expression was inhibiting cell growth. Because it is unlikely that the failure to observe any difference in prodrug sensitivity is attributable to differences in expression of the CDglyTK fusion protein from the FGNR and FGNRp53 viruses (Fig. 2A) or to insufficient or transient expression of the p53 protein (Fig. 2, A and C), we conclude that coexpression of p53 does not enhance the cytotoxicity of either the CD/5-FC or HSV-1 TK/GCV enzyme/prodrug systems in these cell lines in vitro.
apoptosis observed (5-FC, 5%–15%; GCV, 7%–25%) was dramatically less than the cell death (5-FC, 40%–99.8%; GCV, 30%–99.7%) measured in the clonogenic assays (Fig. 3A). Although these differences might be attributable simply to the different assays used (i.e., one measures apoptotic cell death and the other the ability to form a macroscopic colony from a single cell), they, nevertheless, raise the possibility that most of the suicidal effects of the CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems may not be manifested through p53-mediated apoptosis.

Effect of p53 Coexpression on Efficacy of Suicide Gene Therapies in Vivo. Because there are clear examples where results obtained in vitro do not necessarily predict the outcome in vivo (28), the therapeutic effects of the CD/5-FC and/or HSV-1 TK/GCV enzyme/prodrug systems were compared in vivo after intratumoral injection of the FGNR and FGNRp53 viruses. In the Hep3B tumor model, injection of FGNRp53 resulted in a tumor growth delay of 6 days relative to PBS-injected controls (P = 0.0001), whereas FGNR had no effect (Fig. 5; Table 1). This result demonstrates the in vivo biological potency of p53 when expressed from the FGNRp53 virus. Double prodrug therapy (5-FC + GCV) proved to be significantly better (P < 0.001) than either single prodrug therapy (5-FC or GCV) with both viruses, although the combined effects appeared to be additive. When comparing the effects within each prodrug regimen, with one exception, there was no significant difference (P > 0.22) between the FGNR and FGNRp53 viruses. The only exception was double prodrug therapy, in which the FGNRp53 virus proved to be less effective than the FGNR virus (P = 0.003). Similar results were obtained with the SK-OV-3 model. Although FGNRp53 inhibited the growth of SK-OV-3 cells in vitro (Fig. 2C), it failed to result in a tumor growth delay in vivo (Table 1). This may be the result of inefficient infection in vivo. Nevertheless, there was no significant difference in the tumor growth delays when comparing the FGNR and FGNRp53 viruses. Thus, coexpression of p53 did not improve the efficacy of CD/5-FC and/or HSV-1 TK/GCV suicide gene therapies in SK-OV-3 and Hep3B tumor xenografts.

Fig. 4 Determination of apoptosis after prodrug treatment. Hep3B cells were infected with FGNR or FGNRp53 at an MOI of 100. Forty-eight h later, cells were treated with graded concentrations of 5-FC (A) or GCV (B) for 24 h. Three days after prodrug treatment, attached and detached cells were pooled and examined for apoptosis. The results represent the mean of triplicate determinations. The clonogenic assays were repeated three times, yielding similar results. Bars, SD.

Fig. 5 Effect of p53 coexpression on the growth of Hep3B tumors. s.c. tumors (∼100 mm³) were injected with PBS, FGNR (10⁸ pfu), or FGNRp53 (10⁹ pfu) on 5 consecutive days (□, days 0–4). Prodrugs were administered for 28 days (■, days 0–27) to ensure that prodrugs were present throughout the period of transgene expression (14–21 days). Each point represents the average of five (PBS) or eight (all other treatment groups) animals. ——, PBS-injected animals; open symbols, FGNR-injected animals; solid symbols, FGNRp53-injected animals. The dotted line across the graph represents the predetermined end point of five times the initial tumor volume. The in vivo studies were repeated twice, yielding similar results.
Expression of the Adenovirus E1A Protein Sensitizes Cells to p53-mediated Apoptosis after Insult with CD/5-FC and HSV-1 TK/GCV Enzyme/Prodrug Therapy. It was demonstrated previously that E1A-transformed cells having a wt p53 status are dramatically more sensitive to chemotherapeutic agents (5-FU, etoposide, and Adriamycin) and radiation than their isogenic counterparts lacking p53 (3, 4). Such results demonstrated clearly that the cytotoxicity of some anticancer agents can be enhanced by the presence of wt p53, which is somewhat inconsistent with the observations made here. To explore the basis for this apparent inconsistency, we determined whether expression of the Ad5 E1A protein could sensitize Hep3B cells to p53-mediated apoptosis after insult with CD/5-FC or HSV-1 TK/GCV enzyme/prodrug therapies. The FGNR and FGNRp53 adenoviruses could not be used for these studies because the presence of the E1A protein would result in viral replication, leading to massive cell death, particularly in the absence of the adenovirus Mr 19,000 E1B protein (29). Thus, the CD/HSV-1 TK fusion and p53 genes were introduced by lipofection using the left-end shuttle plasmids that were used to generate the FGNR and FGNRp53 viruses (see “Materials and Methods”).

Clonal Hep3B lines stably expressing either β-gal (as a control) or Ad5 E1A were readily generated by retroviral infection (Fig. 6A). Cells were transfected with pCA14-CDglyTK or pCA14-CDglyTKp53, and the extent of apoptosis was determined immediately after a 24-h treatment with 5-FC (10 μg/ml) and GCV (0.2 μg/ml). Because the vast majority of cells were not expected to express the CDglyTK and p53 proteins, results of the apoptosis assays were normalized to the transfection efficiency. When either p53 or E1A (or both) were absent, the extent of apoptosis was low and averaged about 10% of the transfection efficiency (Fig. 6B). As expected, in the absence of p53 (CD/TK), E1A did not sensitize Hep3B cells to apoptosis after insult with either prodrug (compare β-gal versus E1A-expressing cells). By contrast, when p53 was present (CD/Tkp53), expression of E1A increased the extent of apoptosis to a point that approached (5-FC, 75%) or exceeded (GCV, 180%) the transfection efficiency. The latter result might be attributable to the bystander effect of the HSV-1 TK/GCV system, which may induce apoptosis in untransfected cells and be more efficient than that of the CD/5-FC system when examined at this early time point. The results indicate that E1A is able to sensitize a high percentage (75% or more) of transfected Hep3B cells to p53-mediated apoptosis after insult with CD/5-FC or HSV-1 TK/GCV enzyme/prodrug therapy.

DISCUSSION

Using a pair of adenoviral vectors that contain a CD/HSV-1 TK fusion gene without or with the human p53 gene, we found that coexpression of wt p53 did not enhance the effectiveness of the CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems using the SK-OV-3 and Hep3B tumor models. The failure to observe an effect of p53 in vitro cannot be explained on the basis of insufficient or transient p53 expression, because...
the amount of p53 protein produced by the FGNRp53 virus was sufficient to induce G$_1$ growth arrest, induce Bax, and stimulate apoptosis in prodrug-treated cells. Although expression of p53 was transient (~6 days) after FGNRp53 infection, it was highly expressed during, and for several days after, the prodrug treatment period. The in vivo biological potency of p53 when expressed from FGNRp53 was demonstrated by the fact that intratumoral inoculation of the FGNRp53 virus caused a significant delay in the growth of Hep3B tumors. Although our conclusions must be tempered by the fact that only two human carcinomas were examined in this study and the effect of p53 may be cell line dependent, they, nevertheless, raise the possibility that coexpression of p53 may not necessarily improve the efficacy of adenovirus-mediated CD/5-FC and/or HSV-1 TK/GCV suicide gene therapies in vivo. We have made similar observations using isogenic A549 and LNCaP cell lines stably expressing either wt or a dominant-negative mutant (273 his $\rightarrow$ arg) of p53.$^4$

It is interesting that despite the fact that the FGNRp53 virus itself resulted in a significant tumor growth delay with Hep3B tumors, it proved to be equally or less effective than the FGNR virus when used in conjunction with prodrug therapy. These results indicate that the combined effects of p53 and the enzyme/prodrug systems is less than additive in this model, raising the possibility that in some tumors, high expression of p53 may actually antagonize the efficacy of CD/5-FC and HSV-1 TK/GCV suicide gene therapies. Although the basis for this observation is unknown, it is likely that high expression of p53 may reduce the S-phase fraction of Hep3B tumors, thereby rendering them less susceptible to the cytotoxic effects of CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems. This notion is consistent with the fact that both the CD/5-FC (by inhibiting thymidylate synthase) and HSV-1 TK/GCV (by inhibiting DNA chain elongation) enzyme/prodrug systems preferentially kill actively dividing cells.

Using a set of E1A-transformed cell lines that differed only in their p53 status, it was demonstrated previously that cells having a wt p53 status were much more sensitive to chemotherapeutic agents (5-FU, etoposide, and Adriamycin) and radiation than their isogenic counterparts that were null for p53 (3, 4), which is somewhat inconsistent with the observations made here. Although there are many differences between the two studies, including the cell lines, chemotherapeutic drugs, and methods used, we believe the major reason for this apparent inconsistency is the fact that the previous study used cell lines that stably expressed the adenovirus E1A protein. It is well-documented that E1A sensitizes cells to p53-mediated apoptosis (30). By contrast, the cell lines used here were bona fide human tumor lines that lacked E1A. Although Hep3B cells were sensitive to both the CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems, our results suggest that most of the "suicidal effects" of these therapies are not manifested via p53-mediated apoptosis. It is important to note that we did observe a stimulation (2-fold) of p53-mediated apoptosis after prodrug therapy, an effect that was enhanced dramatically in the presence of E1A. Thus, we were able to observe both the stimulation of apoptosis by p53 and the E1A sensitization effect, confirming the previous observations of others (3, 4). Although it is clear that p53 can stimulate apoptosis after insult with chemotherapeutic agents, as demonstrated here with 5-FC and GCV, what is not clear is what proportion of the tumor response observed in vivo after suicide gene therapy is in fact attributable to p53-mediated apoptosis. Our results with SK-OV-3 and Hep3B cells in vitro indicate that p53-mediated apoptosis may account for only a small fraction of the "cell death" that occurs after insult with the CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems. This notion is consistent with the fact that CD/5-FC and HSV-1 TK/GCV suicide gene therapies have demonstrated effectiveness against a variety of tumors that lack functional p53 (e.g., SK-OV-3, Hep3B, 9L, WiDr, U251, DU145, PC-3, C3A3, and many others). Thus, p53 enhancement of CD/5-FC and HSV-1 TK/GCV cytotoxicity in vivo may also require the presence of apoptosis-sensitizing oncogenes that mimic the profound effects of E1A.

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REFERENCES


$^4$ Unpublished data from our laboratory.
Efficacy of Adenovirus-mediated CD/5-FC and HSV-1 Thymidine Kinase/Ganciclovir Suicide Gene Therapies Concomitant with p53 Gene Therapy

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