P53-Dependent Cell-Killing by Selective Repression of Thymidine Kinase and Reduced Prodrug Activation

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ABSTRACT

Selective killing of tumor cells is an important goal for cancer therapeutics. The tumor suppressor transcription factor p53 is absent or mutated in more than 50% of human tumors. Thus, determining approaches that use p53 status to regulate therapy may be an important strategy for attaining cancer selectivity. We have shown previously that a designed transcriptional repressor, K2–5F, strongly and selectively reduces the expression of its target gene MDR1. In this study, we exploited p53 status and the strong repressor activity of K2–5F to establish a system for preferential killing of p53-negative cells. In this system, the expression of K2–5F is induced by p53 in normal cells, and the K2–5F repressor then inhibits the expression of herpes simplex virus thymidine kinase (HSV-TK) driven by an MDR1 minipromoter. In p53-deficient cells, little K2–5F is expressed, and thus HSV-TK is expressed, allowing the cells to be killed by ganciclovir (GCV). K2–5F induced by exogenous p53 dramatically reduced the expression of HSV-TK in human embryonic kidney 293 cells, and it subsequently increased cell survival in response to GCV. To further evaluate this approach in a uniform genetic background, we developed Saos-2 cells stably expressing physiological levels of p53 and paired them with wild-type p53-negative Saos-2 cells. Stable expression of moderate levels of p53 in Saos-2 cells was able to induce the expression of K2–5F and reduce HSV-TK expression and resulted in a modest but distinct protection from GCV toxicity. Thus, this system may be suitable for further development as an approach to selective cancer therapy.

Tumor selective cell-killing is one of the ultimate goals of cancer therapy. One approach to this has been to optimize the expression of therapeutic genes in tumor cells and to minimize their expression in normal cells. Several strategies have been tried, including direct gene delivery to tumors (Mohr et al., 2001), retroviral integration into rapidly dividing cancer cells (Tamura et al., 1998), and tumor-specific control of transcription (Ido et al., 2001). Targeting tumor cells via the control of transcription has been tested extensively, and several tumor-selective promoters have been identified, such as the hepatoma-associated α-fetoprotein promoter (Ido et al., 2001), the carcinoembryonic antigen promoter in colorectal and lung cancer cells (Kijima et al., 1999), and the tyrosinase gene promoter in melanomas (Siders et al., 1998). These promoters have been used to drive therapeutic genes to selectively kill tumor cells. The most commonly used therapeutic killing tool is a suicide enzyme/prodrug combination: the enzymes produced from suicide genes convert nontoxic drugs into cytotoxic compounds. For example, herpes simplex virus thymidine kinase (HSV-TK)

and *Escherichia coli* cytosine deaminase convert ganciclovir and 5-fluorocytosine to the toxic products ganciclovir-triphosphate and 5-fluorouracil, respectively (Ichikawa et al., 2000; Loimas et al., 2001). Although the suicide enzyme/prodrug approach is powerful and controllable, cell type-specific promoters are relatively weak and are not applicable to many types of tumors, resulting in limits to the efficiency and specificity of the killing.

The tumor suppressor p53 is absent or mutated in more than 50% of human tumors (Hainaut, 2002; Lane and Lain, 2002), and abnormalities in the regulation of p53 contribute to cancer (Prives, 1998; Thomas et al., 1999). Thus, several therapeutic strategies have formulated by evaluating the function and regulation of p53. In some studies, the wild-type p53 gene was delivered to tumor cells, causing apoptosis of the cells in response to cytotoxic drug treatment (Merritt et al., 2001). Another important study (Bischoff et al., 1996) produced a mutant adenovirus that does not express E1B, a protein that binds and inactivates p53. Thus, this mutant virus could replicate in and lyse p53-deficient human tumor cells but not cells with functional p53 (Heise et al., 1997, 1999a,b).

A powerful approach to the selective regulation of transcription involves the design of novel proteins based on the

Cys2-His2 type of zinc finger (Zif) DNA binding domain. This has allowed the creation of chimeric proteins that have novel DNA sequence binding specificities and strong transcriptional regulatory effects (Beerli et al., 1998; Kim and Pabo, 1998). Novel DNA binding Zifs coupled with transcriptional activator or repressor domains produce strong transcriptional regulatory effects on reporter genes (Kim and Pabo, 1997; Beerli et al., 1998; Kang and Kim, 2000) and on endogenous chromosome-embedded genes (Bartsevich and Juliano, 2000; Beerli et al., 2000; Kang and Kim, 2000). We have reported previously (Bartsevich and Juliano, 2000) the use of a yeast combinatorial library approach to produce a 5Zif DNA binding domain directed against a 15-base sequence in the promoter of the MDR1 gene. This was linked to two KRAB-A repressor domains to form K2–5F, a designed sequence-selective repressor that regulated the expression of reporter genes driven by the MDR1 promoter sequence. Furthermore, we have recently shown that this designed transcriptional regulator strongly and selectively repressed the expression of the MDR1 gene in multidrug-resistant human tumor cells (Xu et al., 2002).

In the current study, we sought to achieve selective killing through the control of the transcription of a suicide gene. Thus, we used K2–5F to repress the expression of the HSV-TK enzyme. The expression of K2–5F itself is driven by a p53-responsive promoter. Therefore, in normal cells with wild-type p53, K2–5F expression should be high, and HSV-TK expression should be repressed. However, in p53-deficient cells there will be little K2–5F, and thus HSV-TK should be expressed at higher levels. HSV-TK converts ganciclovir (GCV) to its monophosphate, which can be converted by cellular kinases to the toxic triphosphate, a terminator of DNA polymerization (Belcourt et al., 1998). Thus, in this system, the concentration of GCV triphosphate in cells should be regulated by their p53 status. Here, we show that in HEK 293 cells and Saos-2 cells, K2–5F induced by exogenous p53 dramatically repressed the expression of HSV-TK, resulting in increased cell survival in response to GCV. A reduction of HSV-TK expression and a consequent protection against GCV toxicity were also observed in p53 stably transfected Saos-2 cells. Thus, this study suggests that coupling a strong and selective transcriptional regulator with p53 status could be a powerful strategy for selective cancer therapeutics.

Materials and Methods

Cell Culture. U-2OS and Saos-2 human osteosarcoma cancer cell lines, as well as HEK 293 cells, were purchased from the Tissue Culture Facility (Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC). The HEK 293T cell line was kindly provided by Dr. Alice Ma (University of North Carolina at Chapel Hill). Saos-2 and U-2OS cells were cultured in McCoy's 5a medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. Both HEK 293T and HEK 293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum.

Plasmid Construction. Two copies of the K2–5F binding sequence in the MDR1 promoter were excised with *Bam*HI from plasmid 2MDR-LUC (Bartsevich and Juliano, 2000) and then cloned into *Bgl*II-digested pRL-TK (Promega, Madison, WI) to create pRL-2MDR-TKp. Full-length cDNA of HSV-TK with *Nhe*I and *Not*I sites at the ends and including a polyhistidine tag at the carboxyl termi-

nus was obtained by 30 rounds of PCR amplification of plasmid LNC-TK (Hoganson et al., 1996), kindly provided by Dr. John Olsen (University of North Carolina at Chapel Hill). The oligonucleotide primers used were 5'-TAGGCTAGCCACCATGGCTTCGTACCCCT-GCCA-3' and 5'-GAAGCGGCCGCTCTAGAATCAATGATGATGAT-GATGATGGTTAGCCTCCCCCATCT-3'. The amplified cDNAs were inserted into *Nhe*I- and *Not*I-digested pRL-2MDR-TKp and pRL-TK, resulting in vectors pRL-2MDR-TKp-TK (producing HSV-TK regulated by K2–5F) and pRL-TKp-TK (producing HSV-TK not regulated by K2–5F), respectively. The recombinant molecules were sequenced to verify that no mutations had been introduced during PCR amplification and cloning.

The p53-responsive vector pFR-2p21-K2–5F is based on the reporter plasmid pFR*-2p21 (Falke et al., 2003), which contains two copies of the p53 binding site of the p21 promoter. The luciferase gene of pFR*-2p21 was excised with *Eco*RI/*Xba*I and replaced by an *Eco*RI/*Spe*I-digested linker containing the restriction sites *Kpn*I, *Nco*I, and *Xba*I. K2–5F sequence with *Nco*I and *Xba*I sites at the ends and including an myc tag at the carboxyl terminus was obtained by PCR amplification of plasmid pcK2–5F (Bartsevich and Juliano, 2000) and inserted into the *Nco*I/*Xba*I-digested pFR*-plasmid, resulting in plasmid pFR-2p21-K2–5F. Primers used to create the linker were 5'- GCGAATTCCAGCTTGGCATTCCGGTACTGTTG-GTACCATGGCGTCTAGAC-3' and 5'-GCACTAGTGTATTACAAT-AGCTAAGAATTTCGTCTAGACGCCATGGTACC-3'. The primers were annealed, and the ends were filled in with T4 DNA polymerase. The primers used to amplify K2–5F were 5'-CCACCATGGCTAGCT-GTTTC-3' and 5' CGTCTAGACTGAATACAGTTACATTTCAATG-ATGATGATGATGAT-3'.

Plasmid pCMV-P53-Myc was constructed from vector pcDNA3.1(−)/Myc-HisA (Stratagene, La Jolla, CA) by inserting a p53 coding sequence from plasmid pCMVP53 (BD Biosciences Clontech, Palo Alto, CA) through *Hin*dIII/*Eco*RI sites.

Transfection. Transfection was carried out using Fugene 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Cells were cotransfected with the indicated vectors. The total amount of DNA was adjusted with empty vectors or salmon sperm DNA.

Immunoprecipitation, Nickel-Bead Purification, and Western Blotting. Forty eight hours after transfection, cells were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 2 mM phenylmethylsulfonyl fluoride, and 0.1% aprotinin), and lysates were centrifuged at 12,000 rpm for 10 min at 4°C. For Western blotting, equal amounts of protein (20 μg) were mixed with 2× SDS sample buffer and boiled for 5 min. For nickel-bead pull-downs, the supernatants were incubated with aprotinin-pretreated nickel beads for 2 h. The resulting beads were washed three times with the modified RIPA buffer and boiled with 1× SDS sample buffer (with the addition of 200 mM imidazole) for 5 min.

For immunoprecipitation, the supernatants were incubated with antibody for 2 h at 4°C followed by the addition of protein G-sepharose and further incubation for 2 h at 4°C. The precipitates were washed three times with modified RIPA buffer and boiled with 1× SDS sample buffer for 5 min. The proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred onto polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). K2–5F was detected using monoclonal anti–c-myc antibody 9E10 (Berkeley Antibody Company, Richmond, CA) at a dilution of 1:2000. HSV-TK was detected using monoclonal anti-polyhistine antibody H-3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:1000. p53 was detected using monoclonal anti-p53 antibody DO-1 (Santa Cruz Biotechnology, Inc.) at a dilution of 1:5000. Secondary antibody was peroxidase-conjugated goat anti-mouse IgG antibody (Calbiochem, San Diego, CA) at a dilution of 1:5000. Immunoprecipitated K2–5F and p53 were detected using biotin-labeled anti–c-myc antibody 9E10 (Berkeley Antibody Company) at a dilution of 1:1000 followed by strepta-

vidin-horseradish peroxidase at a dilution of 1:5000 (Amersham Biosciences Inc., Arlington Heights, IL). Signals were detected by enhanced chemiluminescence (ECL kit, Amersham Biosciences Inc.).

GCV Sensitivity Assays. Cells were seeded in 12-well plates and transfected as described above. The transfection mixture also included luciferase-expressing plasmid pGL3 (Promega) or β-galactosidase–expressing plasmid to mark the transfected cells. Twenty-four hours after transfection, cells in each well were divided equally into six parts and replated into six-well plates. Twenty-four hours later, GCV (Sigma Chemical Co., St. Louis, MO) was administered in varying concentrations (Mavria and Porter, 2001). After 4 days of treatment, cells were lysed, and luciferase activities or β-galactosidase activities were measured according to the manufacturer's protocol (Promega). Cell viability was estimated by luciferase activity or β-galactosidase activity, with results expressed as a percentage of the activity in the absence of GCV. Although the transfection of various plasmids may affect total luciferase or β-galactosidase expression, each concentration-response profile is normalized against its own control at zero concentration of GCV; thus, the relative response is independent of differences in the absolute level of enzymatic activity. Therefore, in this assay, the amount of luciferase or β-galactosidase retained after GCV treatment is an indicator of cell survival.

Stable Cell Line Production. Saos-2 cells were transfected with linearized pCMV-P53-Myc vector or with pcDNA3.1 vector as a control. Forty-eight hours after transfection, cells resistant to neomycin were selected in medium containing 1 mg/ml G418 (Invitrogen). Clones were tested for constitutive p53 expression by Western blot. Eight p53-positive clones (Saos-2/p53+) were selected and maintained in the presence of G418 (0.5 mg/ml), as were several p53-negative control clones (Saos-2/p53−).

Colony-Formation Assay. Cells were transfected with the plasmids indicated, along with a β-galactosidase–expressing plasmid to mark the transfected cells. Twenty-four hours after transfection, 600 cells were replated in 10-cm plates, and after another 24 h, GCV was administered in varying concentrations. After 10 days, surviving blue (β-galactosidase–positive) colonies larger than 50 cells were counted. Survival was expressed as a percentage of colonies formed at 0 GCV.

Results

Strategy and Plasmid Construction. An overview of our strategy is shown in Fig. 1. The goal was to develop a set of vectors that permits effective cell killing in the absence of functional p53, but not in its presence. To attain this we constructed two plasmids. The first, pFR-2p21-K2–5F (Fig. 2), contains two copies of a p53 binding sequence taken from the p21 gene, followed by a TATA box and then the coding sequence of the K2–5F–designed repressor protein. This vector will thus express K2–5F in the presence of functional p53. The second vector, pRL-2MDR-TKp-TK (Fig. 2), contains two copies of a 15-base sequence (taken from the MDR1 promoter) that specifically binds K2–5F; these were placed upstream of a partial HSV-TK promoter, which in turn is upstream from the HSV-TK coding sequence. This vector will constitutively express HSV-TK, but it can be repressed by the expression of K2–5F. Thus, when both vectors are present in a cell, one would expect high levels of HSV-TK expression in the absence of p53 and low levels in its presence.

Repression of HSV-TK by Constitutively Expressed K2–5F. To initially assess the ability of K2–5F to repress the expression of HSV-TK from the pRL-2MDR-TKp-TK vector, we used a vector that constitutively expressed K2–5F driven by a cytomegalovirus promoter. HEK 293T cells were transfected with equal amounts of the plasmids pcK2–5F and pRL-2MDR-TKp-TK or pRL-TKp-TK, or with empty vectors pcDNA3.1A and pRL-2MDR-TKp-TK or pRL-TKp-TK. As seen from the Western blotting result in Fig. 3, constitutively expressed K2–5F dramatically inhibited the expression of HSV-TK from pRL-2MDR-TKp-TK, but not from pRL-TKp-TK, suggesting that the repression can be attributed to the two copies of the K2–5F–binding 15-base sequence from the MDR1 promoter.

Regulation of the Response to GCV by Constitutively Expressed K2–5F. We next evaluated the pharmacological consequences of K2–5F expression. HEK 293T cells were cotransfected with pRL-2MDR-TKp-TK and pcK2–5F or empty vector pcDNA3.1A and with the luciferase vector pGL3 or β-galactosidase–expressing vector. Cells transfected with two empty vectors (pcDNA3.1A and pRL-TK) were used as controls. After 4 days of treatment with GCV, luciferase or β-galactosidase activity was measured as an indication of cell
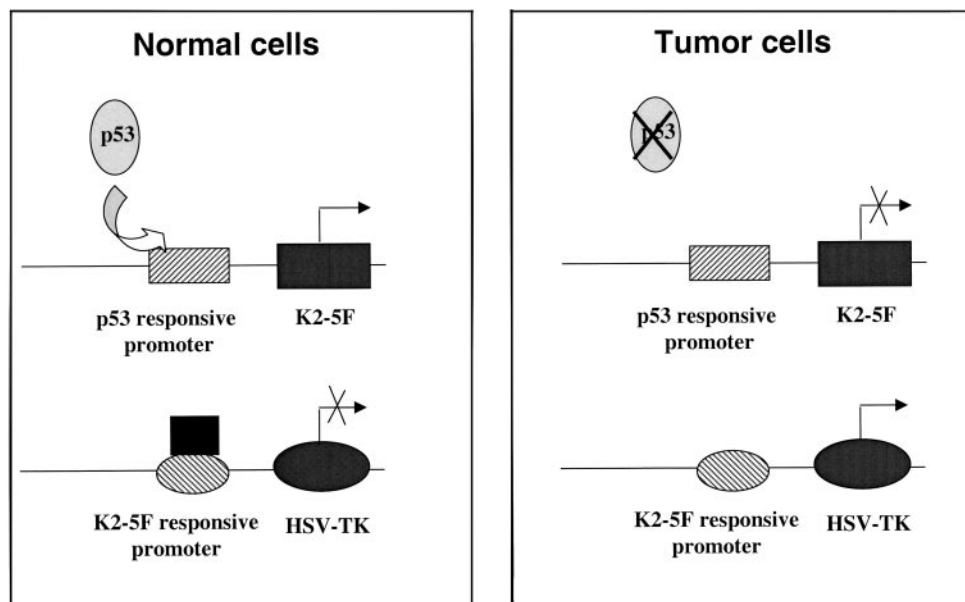

Fig. 1. Strategy to selectively kill p53-negative tumor cells. Constructs of p53-inducible K2–5F and K2–5F–regulated HSV-TK are introduced into cells by transfection. The expression of the transcriptional repressor K2–5F is under the control of a p53-responsive promoter. The modified HSV-TK promoter contains sites that bind the K2–5F repressor. In normal cells (left), wild-type p53 activates the expression of K2–5F, which binds to the TK vector and represses the expression of HSV-TK. In p53-negative cells, there is no K2–5F to inhibit the expression of HSV-TK; thus, these cells make ample enzyme and are susceptible to cell-killing by GCV.

viability (Fig. 4). The repression of HSV-TK expression by K2–5F resulted in a significant right shift of the dose-response profile of cells cotransfected with pRL-2MDR-TKp-TK and pcK2–5F. Thus, the IC_{50} for GCV in the cells transfected with pRL-2MDR-TKp-TK alone was approximately 2×10^{-7} M, whereas that in the cells transfected with both pRL-2MDR-TKp-TK and pcK2–5F was approximately 1×10^{-5} M. Therefore, constitutively expressed K2–5F substantially reduced cell killing by HSV-TK and GCV. The use of two reporter genes, luciferase or β-galactosidase, validates the reliability of this assay. In addition, the use of the β-galactosidase marker allowed an estimate of the transfection efficiency, which was approximately 60%.

Repression of HSV-TK by p53-Induced K2–5F. These results show that the expression of HSV-TK from pRL-2MDR-TKp-TK could be substantially inhibited by constitutively expressed K2–5F. We then questioned whether similar inhibition could be achieved through p53-mediated induction of K2–5F. Because the T-antigen in HEK 293T cells interacts with and inactivates p53, we used HEK 293 cells, which also have high transfection efficiency but lack the T-antigen. Figure 5 shows that K2–5F can be strongly induced by cotransfection with a p53-expressing plasmid. Furthermore, this leads to a dramatic p53-dependent reduction in the level of expression of HSV-TK. The small amount pf K2–5F seen in the absence of cotransfected p53 may be caused by endogenous p53 in the HEK 293 cells or to some degree of "leakiness" in the promoter for K2–5F expression.

Regulation of the Response to GCV by p53-Induced K2–5F. To test the pharmacological consequences of p53-mediated expression of K2–5F, HEK 293 cells were transfected with the luciferase reporter plasmid and with various combinations of TK-, K2–5F-, and p53-expressing plasmids. The cells were then treated with GCV, and cell viability was evaluated by the use of the luciferase assay. As seen in Fig. 6, the inhibition of HSV-TK expression by p53-induced K2–5F resulted in an approximately 1-log right shift of the dose-response profile of cells cotransfected with these three vectors compared with cells transfected with the TK vector only or with the TK vector and the K2–5F vector. Although the rescue of cell viability was not as pronounced as the one caused by constitutively expressed K2–5F, the impact of p53-induced K2–5F was very clear. Because p53 itself is toxic to some degree, only low levels of p53 expression were used.

Repression of HSV-TK by p53-Induced K2–5F in Saos-2 Cells. The ultimate goal of this approach was to
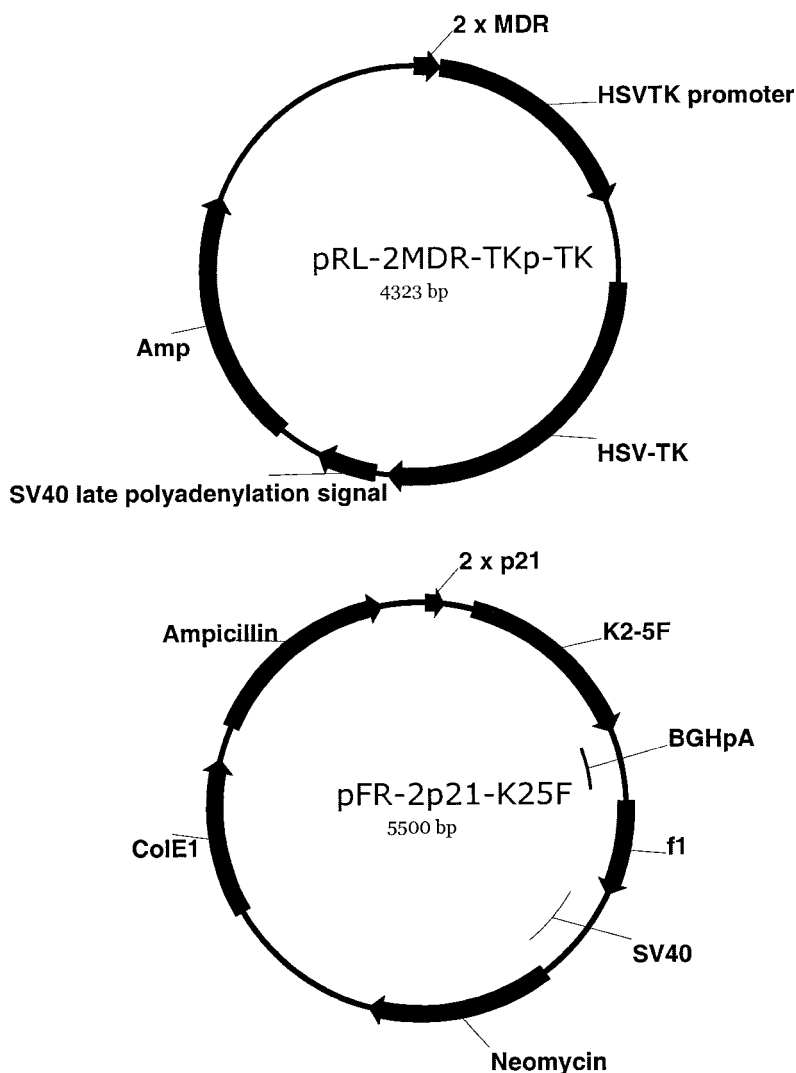

Fig. 2. Constructs of two essential vectors. Top, pRL-2MDR-TKp-TK produces HSV-TK but is regulated by K2–5F. This vector contains two copies of a K2–5F binding sequence from the MDR1 promoter followed by the HSV-TK promoter and the coding sequence for HSV-TK. The vector pRL-TKp-TK is the same as pRL-2MDR-TKp-TK, except it lacks the two K2–5F binding sites. Bottom, pFR-2p21-K2–5F produces p53-inducible K2–5F. This vector contains two copies of a p53-binding sequence from the p21 promoter followed by a TATA box and the K2–5F coding sequence.

selectively kill p53-deficient tumor cells. To test this strategy, we needed a model system using tumor cell lines that differ only in p53 status. We turned to Saos-2 cells, a p53-null osteosarcoma cell line. First, we tested whether TK expression from pRL-2MDR-TKp-TK could be repressed by p53-induced K2–5F in these cells. Cells were cotransfected with
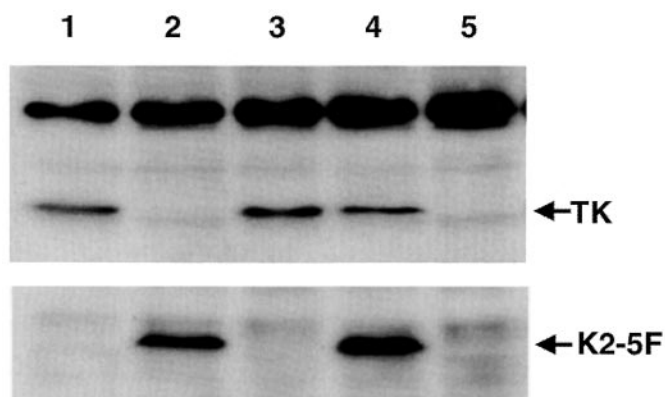

Fig. 3. Repression of TK by pcK2–5F measured by Western blot. Cells (HEK 293T) were cotransfected with equal amounts of the following plasmids: pRL-2MDR-TKp-TK and pcDNA3.1A (lane 1), pRL-2MDR-TKp-TK and pcK2–5F (lane 2), pRL-TKp-TK and pcDNA3.1A (lane 3), pRL-TKp-TK and pcK2–5F (lane 4), or pcDNA3.1A and pRL-TK (lane 5). Expression of HSV-TK and K2–5F was detected by Western blotting using monoclonal anti-His antibody or monoclonal anti–c-myc antibody, respectively.

various combinations of TK-, K2–5F-, and p53-expressing plasmids. Because protein expression is much lower in Saos-2 cells than in HEK 293 cells, hexahistidine and myc-tagged–expressed proteins were enriched by nickel-bead affinity or by immunoprecipitation with anti-myc antibody. Figure 7 shows that p53-induced K2–5F effectively repressed the expression of HSV-TK in SAOS-2 cells.

We then established pairs of cell lines derived from same the genetic background but with different p53 status. We transfected Saos-2 cells with pCMV-P53-Myc or pcDNA3.1 and selected stably transfected cell lines with G418. Saos-2 cells stably transfected with pcDNA3.1 served as p53-negative controls (designated as Saos-2/p53−). Several clones stably transfected with pCMV-P53-Myc expressed p53, as detected by Western blotting; we designated these clones as Saos-2/p53+. Both Saos-2/p53− and Saos-2/p53+ cells were cotransfected with a TK-expressing plasmid along with empty vector or K2–5F–expressing plasmid. As shown in Fig. 8A, the chromosome-integrated endogenous p53 induced the expression of K2–5F, which then inhibited HSV-TK expression from pRL-2MDR-TKp-TK. The p53 in this Saos-2/p53+ clone is at a physiological level because it approximates the p53 level in U-2OS cells, a p53 wild-type osteosarcoma cell line that is similar to SAOS-2 (Fig. 8B).

Regulation of the Response to GCV by Endogenous p53-Induced K2–5F. We next investigated the pharmacological effect of K2–5F induced by endogenous p53 in stably
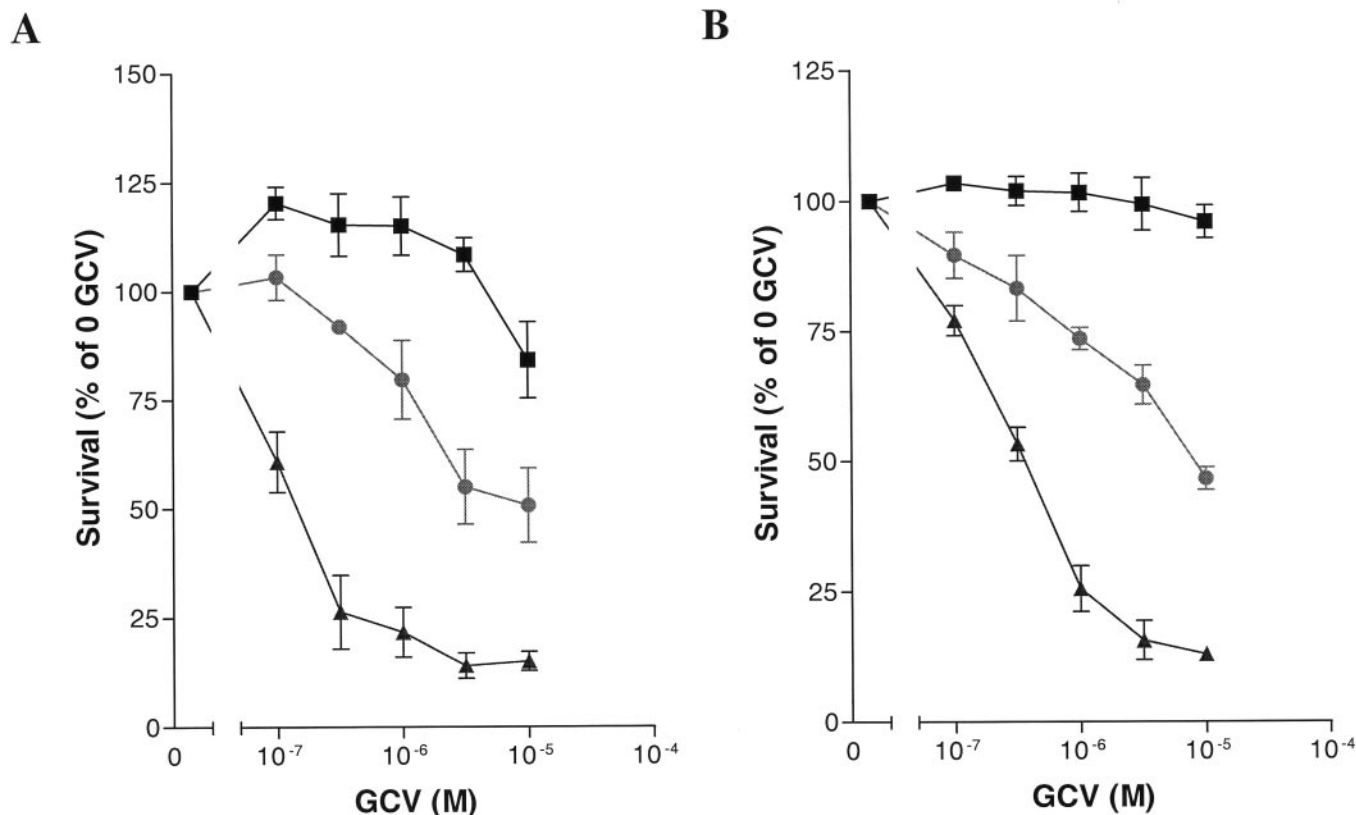

Fig. 4. Regulation of the response to GCV by constitutively expressed K2–5F. Cells (HEK 293T) were cotransfected with equal amounts of the following plasmids: pRL-2MDR-TKp-TK and pcDNA3.1A (▲), pRL-2MDR-TKp-TK and pcK2–5F (●), or pcDNA3.1A and pRL-TK (■). All cells were also cotransfected with a luciferase-expressing plasmid (A) or a β-galactosidase–expressing plasmid (B) as a marker. After 24 h, cells in each well were replated evenly into six-well plates, and in another 24 h, GCV was administered in varying concentrations. Luciferase (A) or β-galactosidase (B) activities were measured after 4 days of treatment by GCV. Results are expressed as a percentage of the enzymatic activities at 0 GCV for each set of plasmids.
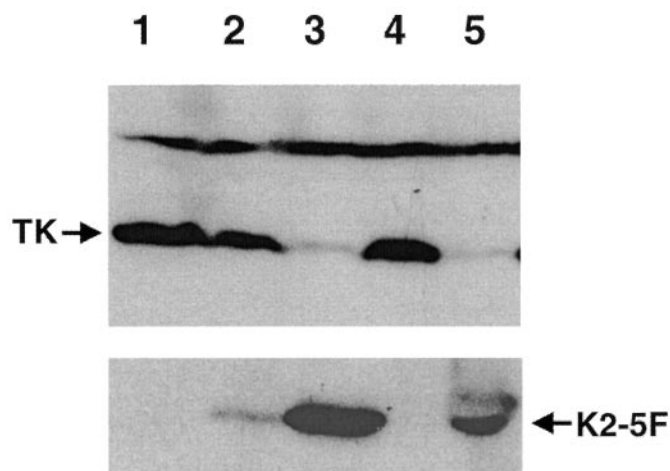
Fig. 5. Repression of TK by p53-induced K2–5F measured by Western blot. Cells (HEK 293) were cotransfected with the following plasmids: pRL-2MDR-TKp-TK (lane 1), pRL-2MDR-TKp-TK and pFR-2p21-K2–5F (lane 2), pRL-2MDR-TKp-TK, pFR-2p21-K2–5F and pCMV-P53-Myc (lane 3), pRL-2MDR-TKp-TK and pCMV-P53-Myc (lane 4), or pRL-2MDR-TKp-TK and pcK2–5F (lane 5). The total DNA amount was adjusted by pcDNA3.1A and/or pRL-TK. The ratio between pRL-2MDR-TKp-TK, pFR-2p21-K2–5F, and pCMV-P53-Myc was 1:1.5:0.1. Expression of HSV-TK and K2–5F was detected by Western blotting using monoclonal anti-His antibody or monoclonal anti–c-myc antibody, respectively.

transfected Saos-2 cells. Saos-2/p53− and Saos-2/p53+ cells were cotransfected with TK-expressing plasmid and K2–5F expressing plasmid or empty vector. The cells were then treated with GCV, and cell viability was evaluated with the use of a colony-formation assay. This assay was chosen so as to validate the pharmacological response using an alternative to the biochemical reporter assays described above. As seen in Fig. 9, the repression of HSV-TK by expression by K2–5F driven by endogenous p53 resulted in a distinct protection against the toxic action of GCV. Thus, in the Saos2/p53− cells, the presence of the K2–5F–expressing plasmid had no effect on the dose-survival curve for GCV. However, in the Saos 2/p53+ cells, the presence of the K2–5F–expressing plasmid resulted in a substantial protective effect, especially at the higher concentrations of GCV. This suggests that the suicide gene system can be regulated by endogenous levels of p53.

Discussion

The ability to selectively kill tumor cells while protecting normal tissue is one of the key goals of cancer therapeutics. This has been attained to varying degrees by conventional cytotoxic chemotherapy and perhaps to a greater degree by
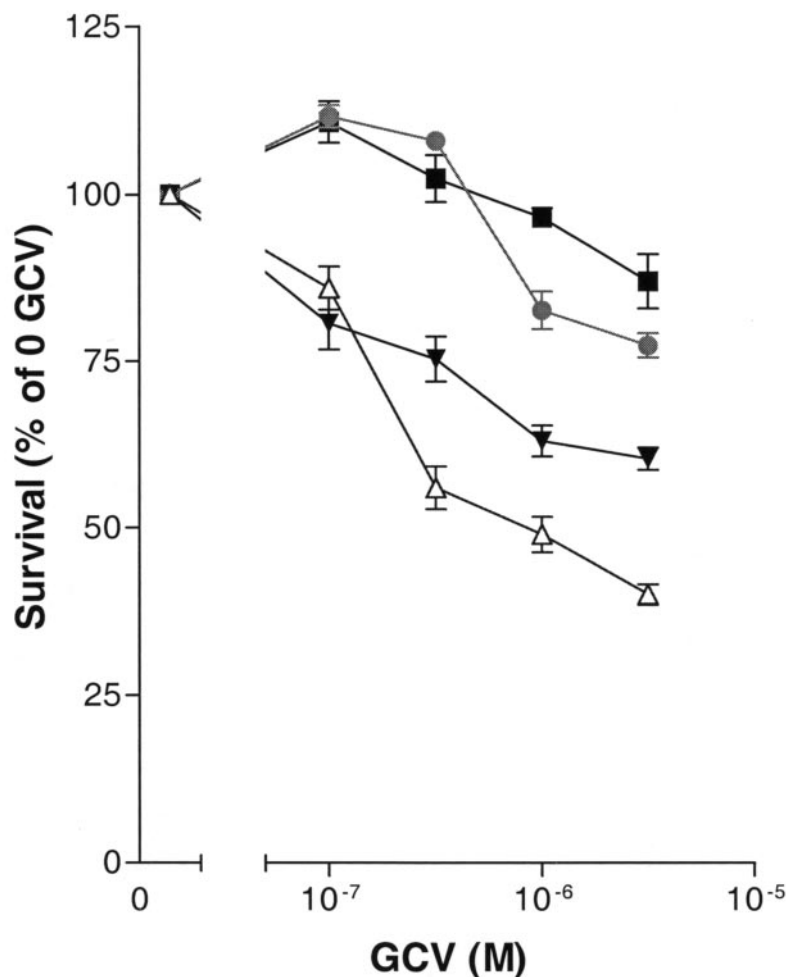

Fig. 6. Regulation of the response to GCV by p53-induced K2–5F. Cells (HEK 293) were cotransfected with the following plasmids: pRL-2MDR-TKp-TK and pFR-2p21-K2–5F (△); pRL-2MDR-TKp-TK, pFR-2p21-K2–5F, and pCMV-P53-Myc (▼); or pRL-2MDR-TKp-TK and pcK2–5F (●). The total DNA amount was adjusted by pcDNA3.1A and/or pRL-TK. A ratio of 1:20:1 between pRL-2MDR-TKp-TK, pFR-2p21-K2–5F, and pCMV-P53-Myc was used. ■, cells transfected with both control vectors. After 24 h, cells in each well were replated uniformly into six-well plates, and after another 24 h, GCV was administered in varying concentrations. Luciferase activities were measured after 4 days of treatment with GCV. Results are expressed as a percentage of the luciferase activities at 0 GCV.

more recent strategies including the use of monoclonal antibodies (Cuello et al., 2001; Ichikawa et al., 2001), drugs that target unique molecular activities in tumors (Attoub et al., 2002), and gene therapy approaches (Tamura et al., 1998; Mohr et al., 2001; Qiao et al., 2002). A major thrust in tumor-selective therapy has been to exploit the altered p53 status of many types of tumor cells. One approach has involved the use of an adenoviral vector defective in the E1B protein that inactivates p53 (Bischoff et al., 1996). Early studies indicated that this virus (ONYX-015) would only replicate in cells with defective p53; however, more recent studies provide a more complex picture showing that viral replication may be more affected by the overall status of the

p53 apoptosis pathway rather than by p53 itself (Harada and Berk, 1999; Ries and Korn, 2000). Despite these controversies, ONYX-015 is progressing through clinical trials and is showing some indications of having an impact on human tumors, especially when combined with chemotherapy (Ries and Korn, 2002). Other approaches have involved the delivery of vectors expressing p53 itself (Abe et al., 2002); indeed, some of these studies have progressed as far as phase III
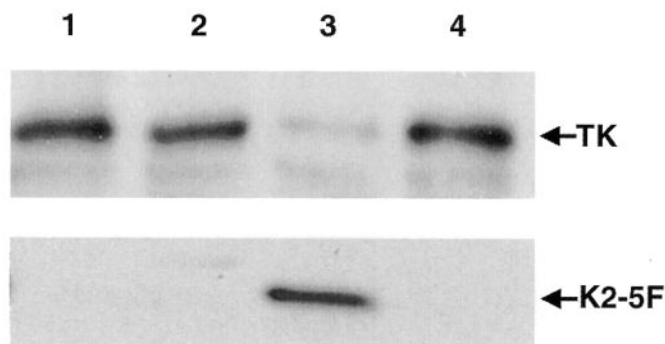

Fig. 8. A, repression of HSV-TK by K2–5F induced by endogenous p53. Saos-2 stable cell lines that were p53-negative (Saos-2/p53−) (lanes 1 and 2) or p53-positive (Saos-2/p53+) (lanes 3 and 4) were transiently transfected with plasmid pRL-2MDR-TKp-TK without (lanes 1 and 3) or with (lanes 2 and 4) cotransfection with pFR-2p21-K2–5F. Expressed HSV-TK was pulled down by nickel-beads and detected by Western blotting using monoclonal anti-His antibody. Expressed K2–5F and p53 were pulled down by immunoprecipitation with monoclonal anti–c-myc antibody and detected by Western blotting using biotin-labeled monoclonal anti–c-myc antibody. B, levels of p53 in SAOS-2 stable cell lines versus U-2OS osteosarcoma cells. Stably transfected Saos-2/p53+ cells (lane 1) have comparable levels of p53 (top band) as U-2OS cells (bottom band) (lane 2). Lane 3 represents U-2OS cells transfected with pCMV-p53Myc. p53 was detected by Western blotting using the monoclonal anti-p53 antibody, DO-1. Note that the epitope-tagged p53 runs at a slightly higher position on the gel than native p53.

Fig. 7. Repression of TK by p53-induced K2–5F in Saos-2 cells. Cells were cotransfected with the following plasmids: pRL-2MDR-TKp-TK (lane 1); pRL-2MDR-TKp-TK and pFR-2p21-K2–5F (lane 2); pRL-2MDR-TKp-TK, pFR-2p21-K2–5F, and pCMV-P53-Myc (lane 3); or pRL-2MDR-TKp-TK and pCMV-P53-Myc (lane 4). The total DNA amount was adjusted by pcDNA3.1A and/or pRL-TK. Expressed HSV-TK and K2–5F were purified by nickel-bead affinity or immunoprecipitation with monoclonal anti–c-myc antibody and detected by Western blotting using monoclonal anti-His antibody or biotin-labeled monoclonal anti–c-myc antibody, respectively.
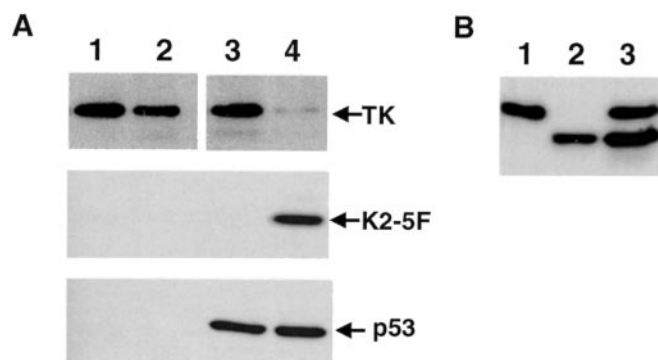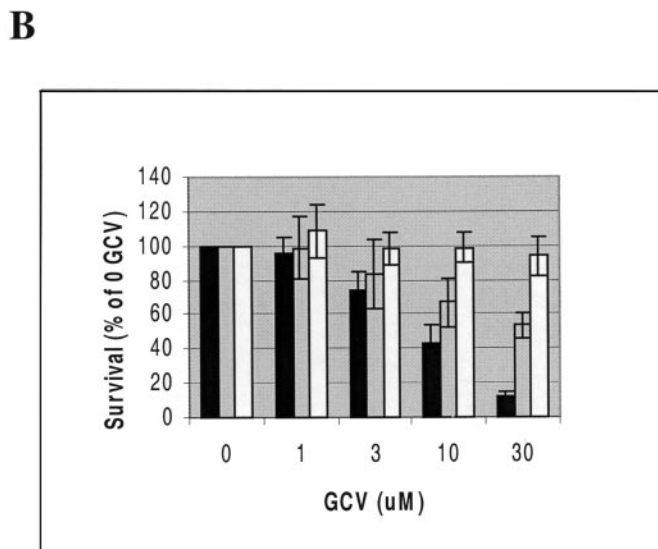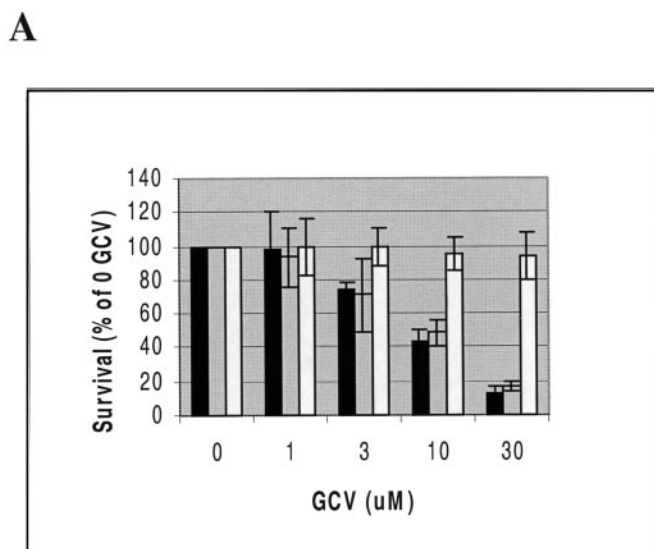

Fig. 9. Regulation of the response to GCV by K2–5F induced by endogenous p53. Saos-2/p53− (A) and Saos-2/p53+ (B) cells were cotransfected with plasmid pRL-2MDR-TKp-TK without (■) or with (▨) cotransfection of pFR-2p21-K2–5F. □, cells transfected with empty vectors. All cells were also transfected with a β-galactosidase–expressing plasmid. After 24 h, 600 cells were replated in 10-cm plates, and after another 24 h, GCV was administered in varying concentrations. After 10 days, surviving blue colonies larger than 50 cells were counted. Survival was expressed as a percentage of colonies formed at 0 GCV. Note that Saos-2 cells require a higher concentration of GCV to attain toxicity than do the HEK 293 cells used in previous figures. The x-axis indicates the concentration of GCV in micromoles. The y-axis indicates the percentage of cell survival compared with cells not treated with GCV.

clinical trials (Anklesaria, 2000). One problem with these approaches is that they involve continuous activity of a potential toxic gene or virus with little opportunity for control after initial administration.

Another strategy for p53-dependent tumor therapy is to use p53 status to regulate the expression of an enzyme capable of activating a prodrug. This then allows pharmacological control of the therapy after the initial administration of vectors. In the work presented here, we used p53 status to regulate the levels of a powerful mammalian repressor protein, K2–5F, which in turn controls the levels of the HSV-TK enzyme. Cytotoxicity is attained by using the HSV-TK to convert the prodrug ganciclovir to its active, phosphorylated form that then inhibits DNA synthesis. A somewhat similar approach has been used by Lipinski et al. (2001), who developed a vector that allowed p53 to regulate the expression of bacterial lac repressor, which in turn regulated the expression of bacterial nitroreductase, an enzyme able to activate CB1954, an alkylating agent prodrug. Detailed dose-response experiments were not provided in this latter study.

In our studies, we found that high levels of the K2–5F–designed repressor protein could strongly inhibit HSV-TK expression and produce an approximately 2-log right shift in the cell-killing curve for GCV. The levels of K2–5F expression attained by cotransfection of cell lines with exogenous p53 also produced significant repression of HSV-TK and a major right shift in the concentration-response curve for GCV-mediated killing. In terms of effects mediated by endogenous p53, there was clearly p53-dependent regulation of TK levels and a modest but distinct protection against GCV toxicity. The results obtained thus far using approximately physiological levels of p53 to drive the expression of K2–5F and TK may not be sufficient for in vivo therapeutic use, because only approximately a 3- to 5-fold shift in the GCV response was attained. However, both pFR-2p21-K2–5F and pRL-2MDR-TKp-TK represent first-generation vectors. It seems likely that manipulations of the promoter sequences driving K2–5F and/or TK expression could result in vectors that provide a more robust response to physiological levels of p53. In addition, the activation status of p53 in the current experiments is likely less than optimal. p53-driven transcription is greatly enhanced by the up-regulation and activation of p53 that follows DNA damage (Prives, 1998). Whereas the transfection reagents used here may have resulted in some degree of p53 activation, no other stimuli were used. In a therapeutic context, vector-based approaches, such as those described here, would likely be accompanied by standard chemotherapy using DNA-damaging agents, and thus greater endogenous p53 activity might be present.

The approach described here would need to be coupled to viral or nonviral approaches to gene delivery to be used for therapy. The expression cassettes used here each total approximately 3 kB and thus could be accommodated easily in either adenoviral or adeno-associated virus vectors (Rabinowitz and Samulski, 2000; Amalfitano and Parks, 2002), both of which have broad cellular tropisms. However, it may be desirable, for the sake of simplicity, to have both the K2–5F and TK expression cassettes in a single vector; this would require an adenoviral platform, because it can accommodate larger inserts.

In summary, in a model system, we used a designed transcriptional repressor to obtain p53-dependent expression of HSV-TK and p53-regulated responses to the prodrug ganciclovir. Further refinement of this approach may evolve into a useful adjunct for cancer therapy.

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