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Converting cancer genes into killer genes

(gene therapy/oncogene/tumor suppressor gene/p53)

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ABSTRACT **Over the past decade, it has become clear that tumorigenesis is driven by alterations in genes that control cell growth or cell death. Theoretically, the proteins encoded by these genes provide excellent targets for new therapeutic agents. Here, we describe a gene therapy approach to specifically kill tumor cells expressing such oncoproteins. In outline, the target oncoprotein binds to exogenously introduced gene products, resulting in transcriptional activation of a toxic gene. As an example, we show that this approach can be used to specifically kill cells overexpressing a mutant p53 gene in cell culture. The strategy may be generally applicable to neoplastic diseases in which the underlying patterns of genetic alterations or abnormal gene expression are known.**

The success of a therapeutic strategy is critically dependent on its specificity. Therapies for infectious diseases achieve high specificity because they target metabolic pathways that differ between the pathogen and the host. The applicability of this concept to cancer therapy, however, has been limited by the difficulty in defining metabolic features that are specific to tumor cells. As a result, current cancer therapeutic agents have largely been found through empirical screening programs rather than through rational design.

Knowledge of the genetic alterations that drive neoplasia has revolutionized cancer research over the past two decades (1). Theoretically, this knowledge provides a large number of potential therapeutic targets in the form of mutant oncoproteins in the resultant tumors. In general, however, it has not been clear how to use such knowledge to design new therapeutic strategies (2).

In this study, we describe a gene therapeutic strategy for cancer in which genetic alterations integral to tumor development are used to selectively target tumor cells for death. As schematically depicted in Fig. 1, a novel intracellular protein complex is created by binding of an endogenous oncoprotein to exogenously introduced nuclear proteins. The resultant complex then activates expression of a toxic gene that kills the cell. Because the rate of complex formation should be directly related to the cellular concentration of the oncoprotein, the strategy can potentially target not only novel oncoproteins but any protein which is overexpressed as a direct or indirect result of the genetic alterations responsible for tumorigenesis. We present a prototypical *in vitro* application of this strategy to the killing of cancer cells expressing high levels of mutant p53 protein.

MATERIALS AND METHODS

Plasmid Construction. "Trigger" plasmids contained the DNA-binding domain (DBD) of GAL4 (aa 1–147) (3) fused in frame to a protein domain that could bind p53 (p53BD). In trigger TA, the p53BD consisted of aa 84–708 of the simian virus 40 (SV40) large tumor (T) antigen (from pTD1; Clontech), separated from the DBD by the 7-aa spacer PEFGLRP. In trigger P5, the p53BD consisted of aa 305–393 of human p53 (from pCEP4-p53WT) (4) separated from the DBD by the 5-aa spacer PEFPG. In both cases, the p53BDs were inserted into a vector derived from pSGVP (5) containing the GAL4 DBD under the control of an SV40 promoter. The "weapon" plasmid contained the bacterial *DeoD* gene, coding for purine nucleoside phosphorylase (PNP) (6), amplified from *Escherichia coli* genomic DNA and modified to contain a consensus eukaryotic translation start sequence (7). The *DeoD* gene was placed under control of a GAL4-responsive promoter obtained from pG5E1b-CAT (5) to create UAS_G-PNP (UAS, upstream activating sequence). The GAL4-responsive UAS_G-β-gal construct was identical to UAS_G-PNP except that the *DeoD* gene was replaced with the β-galactosidase (β-gal) gene from pCMV-β-gal (Clontech). The "killer" plasmids were pCEP4-p53 (wild type or mutants R175H, R248W, or R273H), each containing the complete p53 coding sequence under control of the cytomegalovirus immediate-early promoter (4). Similar levels of p53 expression following transient transfection of these vectors have been demonstrated (4). (Full details of the construction of these plasmids, generally performed by PCR amplification of the indicated templates, are available from the authors upon request.)

Cell Culture and Transfections. The 293 cell line (8) was obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum and antibiotics (FBSA). H1299 cells were obtained from A. Fornace (9) and grown in Ham's F-12 medium (Life Technologies) with FBSA. For transfections, cells were plated at a density of $5–8 \times 10^4$ cells per well of a 24-well tissue culture plate. Transfections were performed 36–60 h after plating, when cells were almost confluent, essentially as described in ref. 10. Briefly, cells were washed once with Opti-MEM (Life Technologies), and 400 μl of Opti-MEM was added to each well. Two micrograms of each plasmid in 3 mM Tris/0.2 mM EDTA was mixed with a sufficient amount of pBluescript (Stratagene) and NaCl (150 mM) to constitute 6 μg of plasmid DNA in a total volume of 50 μl. A mixture of 12 μl of polyethylenimine (0.9 mg/ml) and 38 μl of NaCl (150 mM) was then added to the DNA. After a 10–30-min incubation at room temperature, the DNA–polyethylenimine mix was added to each well. Two to 4 h later, the liquid in the wells was aspirated and replaced with culture medium.

β-gal Assays. Cells used for β-gal assays were harvested 24–48 h after transfection. Cells were washed once with Hanks' balanced saline solution and subjected to three cycles of freezing and thawing in 100 μl of phosphate-buffered saline

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Abbreviations: β-gal, β-galactosidase; DBD, DNA-binding domain; MeP-dR, 6-methylpurine deoxyriboside; p53BD, p53-binding domain; PNP, purine nucleoside phosphorylase; TAD, transactivation domain; UAS, upstream activating sequence; SV40, simian virus 40.

(PBS). Cell lysates were transferred to microfuge tubes containing 11 μl of 10 mM dithiothreitol in PBS, pelleted for 10 min at 4°C, and stored at −20°C until assayed for β-gal activity. In a typical assay, 50 μl of cell lysate was added to 450 μl of reaction buffer (60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1 mM $MgSO_4$, pH 7.0/0.27% 2-mercaptoethanol). After a 5-min preincubation at 28°C, reactions were initiated by addition of 100 μl of a 4 mg/ml solution of *o*-nitrophenyl galactoside in reaction buffer (11). Reactions were stopped by addition of 250 μl of 1 M Na_2CO_3, and absorbances at 420 nm were measured.

Cellular Viability Assays. One day after transfection, 6-methylpurine deoxyribose (MeP-dR) was added to the culture medium to final concentrations of 0, 3, 10, or 30 μM. Five days later, cells were harvested by trypsinization and suspended in a final volume of 600 μl. A 50-μl aliquot of cells was mixed with equal volumes of 1% Nonidet P-40, 6.7% formaldehyde, and 20 μg/ml Hoechst 33258 in PBS for counting and assessing nuclear morphology. Over 90% of cells with a normal nuclear morphology were found to exclude dye (12) in control experiments. For colony-forming assays, aliquots of 400, 40, or 4 μl were plated in 25-cm^2 tissue culture flasks in 5 ml of the appropriate medium. After 7 days, colonies were stained with crystal violet and counted.

RESULTS

Strategy. The strategy adopted in this study to achieve tumor cell-specific killing draws on two previous advances. First, powerful transcription factors can be generated from the interaction of two separate nuclear proteins, one with a DNA binding domain and the second with a transcriptional activation domain (13–15). Second, prokaryotic or viral enzymes that metabolize a nontoxic prodrug into a toxic derivative can be introduced into tumor cells through gene transfer. Because the toxic derivative can diffuse from the gene-transduced tumor cell to surrounding tumor cells, the efficiency of cell killing can greatly exceed the efficiency of gene transfer (the so-called "bystander effect") (16–19).

The most general form of our strategy is depicted in Fig. 1. Exogenous genes encoding "weapons" and "triggers" are delivered into cells. The weapon is encoded by a foreign, toxic gene whose expression is under the control of an inducible UAS. The gene will not be expressed unless a functional, UAS-binding transcription factor is present in the cell. The trigger is a crippled transcription factor which can bind to the UAS through its DBD but cannot activate transcription because its TAD has been replaced with an amino acid sequence that interacts with the target oncoprotein (the killer binding domain). In cells expressing adequate levels of the oncoprotein (termed the "killer"), a protein complex forms that can both bind to the UAS and activate transcription of the weapon through a TAD. The TAD can either be naturally present within the oncoprotein or, if the oncoprotein lacks a TAD, within another exogenously introduced protein that independently binds to the oncoprotein (see Fig. 1). The weapon will be expressed at high levels only in cells expressing high levels of the killer, resulting in cell death.

To test this strategy in a model system, we made the following choices (Fig. 2). For the killer, we chose the p53 gene, which has been shown to be genetically altered in numerous tumor types (20). Immunohistochemical studies have shown that the resultant mutant p53 proteins are expressed at high levels in tumor cells but p53 is undetectable in most normal cells (21). The p53 protein also contains a strong TAD at its amino terminus (22, 23). This TAD is preserved, though other properties of p53 are disrupted, in most p53 mutants (24). For the trigger, we chose protein domains known to bind to the carboxyl terminus of p53, either from p53 itself (25–27) or from the large T antigen of SV40 (28, 29). These domains were fused in frame to the DBD of GAL4. For the weapon, we chose the *E. coli* gene encoding PNP, which can convert the nontoxic prodrug MeP-dR to the diffusible, toxic

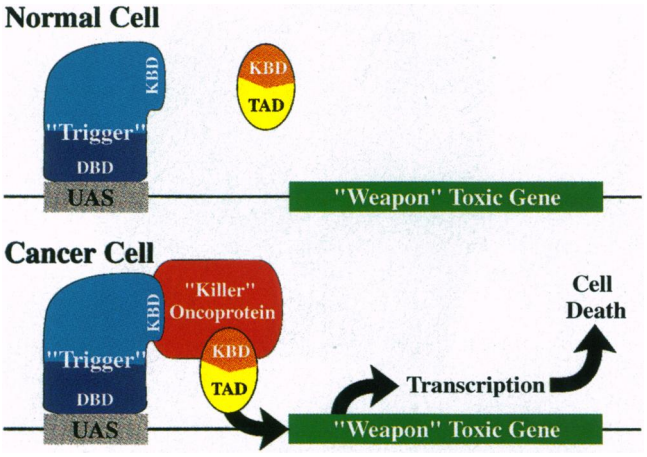

FIG. 1. General strategy to convert cancer genes into killer genes. A weapon, a trigger, and a transactivation domain (TAD)-containing construct are introduced into cells by gene transfer. The weapon is under control of a UAS, which binds to the trigger through a specific DBD. In normal cells (*Upper*), the trigger cannot stimulate transcription of the weapon, whereas in cancer cells (*Lower*) an oncoprotein (the "killer") interacts with the trigger and TAD through killer-binding domains to form a novel UAS-specific transcription factor. When a TAD is naturally present in the oncoprotein, there is no need to introduce it exogenously. Transcriptional activation of the weapon by the complex results in cell death.

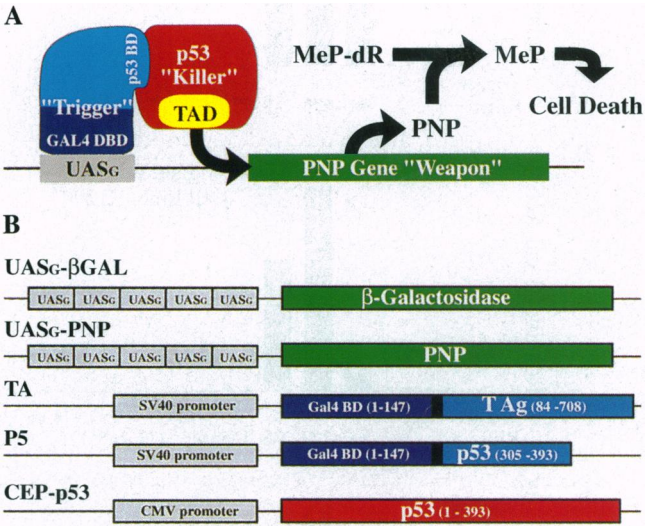

FIG. 2. Strategy used to specifically kill cells expressing high levels of mutant p53. (*A*) Schematic. A novel transcription factor is generated when the trigger interacts with p53 through its p53BD. The trigger-p53 heterodimer binds the GAL4-responsive UAS_G sequences in the PNP gene promoter through the trigger's GAL4 DNA binding domain (GAL4BD) and it stimulates expression of PNP via the transactivation domain of p53. PNP catalyzes the conversion of the nontoxic prodrug MeP-dR to the toxic 6-methylpurine, leading to cell death. (*B*) Constructs. UAS_G-β-gal and UAS_G-PNP are reporter and weapon constructs, respectively. They share a GAL4-responsive promoter that includes five tandem copies of the GAL4 binding site (UAS_G). TA and P5 are trigger constructs, each formed by fusion of the GAL4 DBD to a p53BD. The p53BD in trigger TA consists of aa 84–708 of the SV40 large T antigen, while that in P5 consists of aa 305–393 of human p53. Trigger gene expression is driven by a promoter from SV40. Killer plasmids contain the coding region of human p53 genes under control of a constitutive cytomegalovirus promoter.

6-methylpurine (18). The PNP gene was placed under the control of a GAL4 responsive promoter, UAS_G.

Trigger Testing. We first tested the ability of various triggers to stimulate transcription of a UAS_G-controlled reporter gene in a p53-dependent manner. Two different human cell lines were used. The p53-null H1299 cell line is derived from a lung cancer (9) and the 293 line is derived from human embryonic kidney cells transformed with adenovirus DNA (8). The TAD of the endogenous p53 in 293 cells is inactivated by the adenovirus-encoded E1B protein (30). Several trigger constructs were evaluated in these lines, and the most efficient chosen for further experiments (Fig. 2*B*). When H1299 cells were transfected with the TA trigger plus a UAS_G-controlled β-gal reporter, only low levels of β-gal activity could be detected. However, when p53 was overexpressed in these cells as a result of p53 gene transfer, β-gal levels were increased by up to 30-fold (Fig. 3). Three of the most common p53 mutants were tested in these assays. The R248W and R273H mutants were effective, whereas the R175H mutant was not. These results were consistent with the fact that the R175H mutant is expected to alter proper folding of the p53 protein, while the other mutants tested only affect DNA-binding residues (22, 23, 31). As expected, the wild-type p53 protein also showed activity in this system, though less than the R248W and R273H mutants, perhaps because of the greater protein stability and decreased cellular toxicity associated with mutant forms of p53 (24, 32). The P5 trigger also proved effective at conferring a p53-dependent expression of the reporter, in both H1299 and 293 cells. The results with P5 confirm those of Oren and coworkers (33), demonstrating the ability of the C-terminus of p53 to bind intact p53 and recruit its transactivation domain.

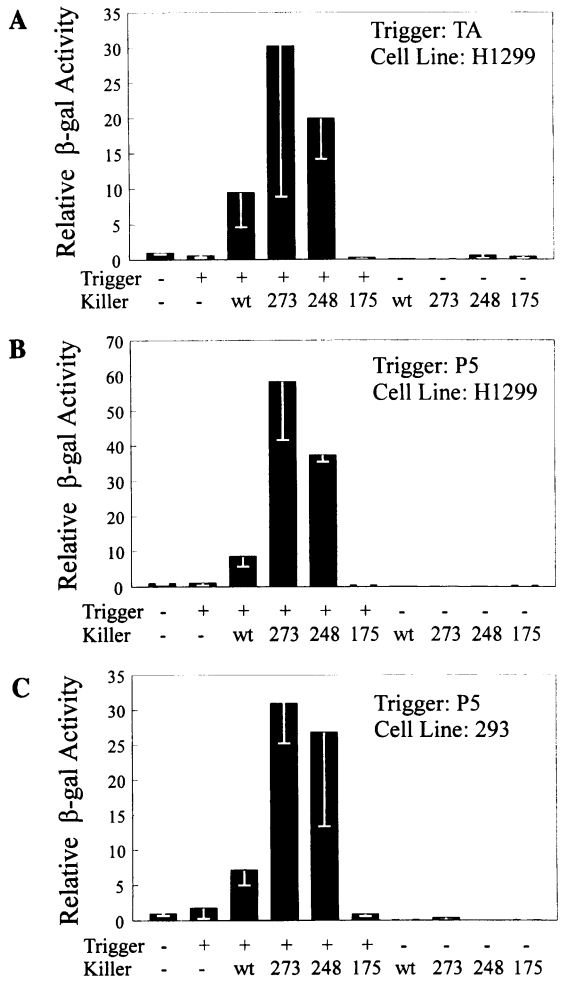

FIG. 3. Trigger evaluation with a reporter construct. Relative β-gal activities in cells transfected with UAS_G-β-gal reporter plus the indicated constructs are presented. (*A*) Cell line H1299, trigger TA. (*B*) Cell line H1299, trigger P5. (*C*) Cell line 293, trigger P5. Bars and brackets represent means and standard deviations, respectively, from triplicate assays, normalized to the activity of the reporter alone.

p53-Dependent Killing. We next sought to determine whether this p53-induced expression of a UAS_G-controlled gene could be translated into differential killing of p53 overexpressing cells. We chose H1299 cells for these experiments, as the absence of all endogenous p53 in this line simplified interpretation. H1299 cells were transfected with both a trigger (P5) and a weapon (UAS_G-PNP), with or without a killer (p53-R273H). Various amounts of MeP-dR were added to the culture medium 1 day after transfection and cell number and viability assessed 5 days later. A dramatic difference in MeP-dR sensitivity was observed between cells transfected with or without the killer (Figs. 4 and 5). At drug concentrations of 3–10 μM, most cells without p53 remained healthy and continued to proliferate. In contrast, after cotransfection with the killer, only a relatively small number of cells remained (Fig.

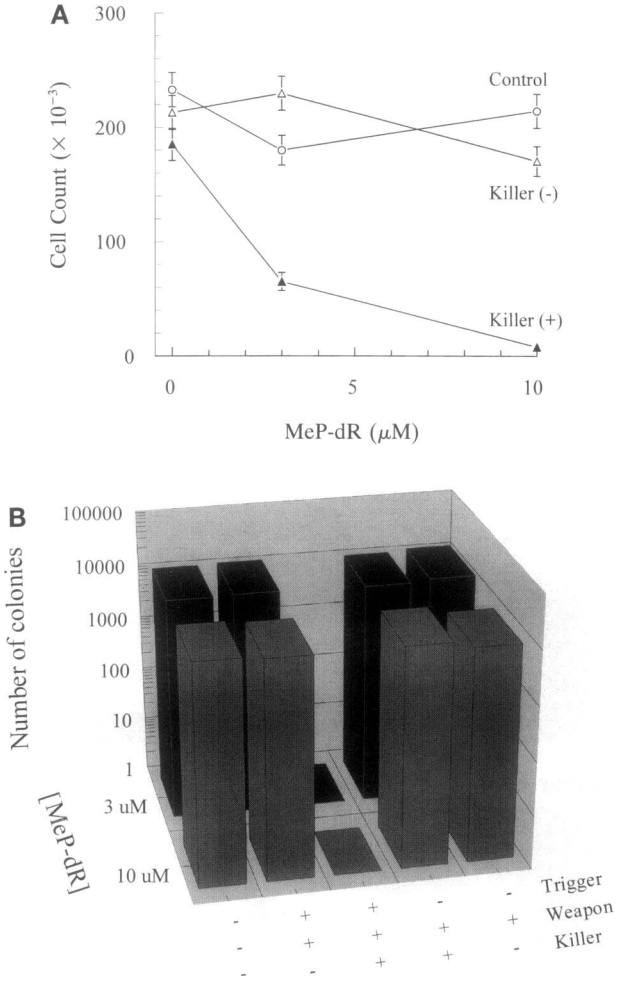

FIG. 4. Selective cell killing using a weapon construct. (*A*) Cells were transfected with the following constructs: Control, pBluescript alone; Killer(−), trigger P5 plus weapon UAS_G-PNP; and Killer(+), trigger, weapon, and killer pCEP4-p53R273H. The number of morphologically intact cells was assessed 5 days after adding MeP-dR to the indicated concentrations. Bars and brackets indicate the means and standard deviations, respectively, the latter calculated as the square root of the number of counted cells, assuming a Poisson distribution. (*B*) After drug treatment, cells were harvested and various dilutions seeded in new flasks, as described. The number of colonies derived from 67% of a well, observed 7 days after replating, is shown.

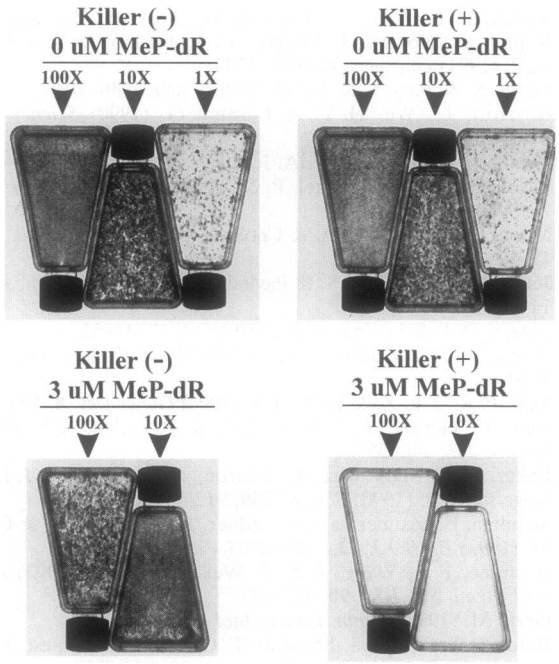

FIG. 5. Representative examples of colony-forming assays after drug treatment. H1299 cells were transfected with weapon and trigger constructs, with or without killer (p53R273H), as indicated. Five days after treatment with MeP-dR, the cells were harvested and seeded into new 25-cm^2 flasks. Sixty-seven percent, 6.7%, and 0.67% of the cells from each transfected well were seeded in the flasks marked 100×, 10×, and 1×, respectively. Seven days later, the colonies were stained with crystal violet and photographed.

4*A*). Cells transfected with the killer and weapon, but without the trigger, also remained healthy. These and additional controls demonstrated that only the combination of weapon, trigger, and killer resulted in substantial cell death at low drug concentrations.

The number of morphologically intact cells may not be an optimal measure of MeP-dR toxicity. When cells were harvested and replated after drug treatment, many of the apparently intact, dye-excluding cells proved incapable of forming colonies (compare Fig. 4 *A* and *B* at 3 μM MeP-dR). Accordingly, there was a striking difference in colony-forming ability between cells expressing killer, trigger, and weapon versus those expressing only trigger and weapon following exposure to drug. Representative examples of the colonies formed after MeP-dR treatment of such cells are shown in Fig. 5 and the results quantitated in Fig. 4*B*. At 3 or 10 μM MeP-dR, there was over a 1000-fold greater effect in the p53-expressing cells compared to the cells without p53. It is also important to note that the polyethylenimine protocol transduced <3% of the H1299 cells, as assessed by β-gal staining. The fact that virtually all H1299 cells were incapable of forming colonies following appropriate transfection and MeP-dR treatment attests to the powerful bystander effect of this system.

DISCUSSION

The results reported above show that in a model system, the strategy outlined in Figs. 1 and 2 can result in substantial toxicity selective for cells overexpressing the p53 gene. This strategy should be applicable to other genes involved in neoplasia. Many oncogenic proteins, like p53, have intrinsic TADs, simplifying the approach. Oncogenic proteins normally present in the cytoplasm can likely be translocated to the nucleus through interaction with triggers containing nuclear localization signals, thus potentially extending the targets to nonnuclear proteins (5). Powerful techniques for identifying polypeptides that interact with given proteins have been developed in the past few years (5, 14, 15, 34), facilitating the design of killer binding domains for trigger and TAD constructs. Novel domains, such as the ones found in foreign—e.g., viral—or mutant proteins represent the most attractive targets, but there are others. Any gene product which is overexpressed in a tumor—e.g., telomerase, cyclins, myc—represents a potential target. The killing efficacy should be directly related to the concentration of the target protein in the cancer cells, with toxicity limited by the concentration of this protein in nonneoplastic cells. The general strategy should also be applicable to selected viral diseases.

It is important to note that our results were obtained only in a model system, and that many technologically demanding hurdles must be overcome for this strategy to reach a clinically testable stage. Some of these hurdles are particular to this strategy, while others pertain to all gene therapy approaches. The first particular obstacle is the optimization of therapeutic index. Our results demonstrate substantial killing in the presence of high levels of exogenously introduced p53. Most normal cells do not express p53, and those that do may be destined for programmed cell death (35). We therefore do not believe that the expression of p53 in nonneoplastic cells poses the major problem for implementation of this strategy. But can the system be designed to be effective at the levels of p53 present in tumors *in situ*? To begin to address this issue, we have transfected weapon and trigger genes into cancer cells with endogenous mutant p53 genes. This resulted in significant toxicity compared to cells devoid of p53 genes, but the doses of MeP-dR required for toxicity were higher, and the differential effect lower, than following transfection with exogenous p53 (unpublished data). An additional issue is that the weapon exerts some toxicity when transfected alone, lowering the therapeutic index expected *in vivo*. Improvement of the weapon and trigger may be possible by engineering them to be maximally sensitive and specific. A variety of genes encoding enzymes not found in human cells, coupled with appropriate engineering of such genes, may also be useful for improving the weapon (19).

The generic problems inherent to gene therapy apply equally to the strategy described here. No method for effectively delivering genes to disseminated tumors in humans has yet been described, though there are some encouraging results in animal models (36–40). Delivery of even standard chemotherapeutic drugs poses problems for treatment of solid tumors, and delivery of large agents—e.g., DNA, viruses—is orders of magnitude more difficult (41). The advantage of bystander systems such as that afforded by PNP is obvious in light of these practical difficulties. We were encouraged by the efficacy of p53-specific cell killing observed when only a very small fraction of cells was successfully transfected (Fig. 5), but it is impossible to predict whether similar bystander effects will be observed in the much different microenvironment present in tumors *in vivo*.

In summary, the general strategy described here and evaluated in the p53 model system represents a promising first step towards a new therapeutic approach. It makes practical use of pathogenically-related genetic alterations found in a wide variety of tumors, and therefore can legitimately be called "rationally designed." However, much future experimentation will be required to determine whether such an approach will ever prove useful in the clinic.

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