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In Vivo Cancer Gene Therapy by Adenovirus-mediated Transfer of a Bifunctional Yeast Cytosine Deaminase/Uracil Phosphoribosyltransferase Fusion Gene

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ABSTRACT

Direct transfer of prodrug activation systems into tumors was demonstrated to be an attractive method for the selective *in vivo* elimination of tumor cells. However, most current suicide gene therapy strategies are still handicapped by a poor efficiency of *in vivo* gene transfer and a limited bystander cell killing effect. In this study, we describe a novel and highly potent suicide gene derived from the *Saccharomyces cerevisiae* cytosine deaminase (*FCY1*) and uracil phosphoribosyltransferase genes (*FUR1*). This suicide gene, designated *FCU1*, encodes a bifunctional chimeric protein that combines the enzymatic activities of *FCY1* and *FUR1* and efficiently catalyzes the direct conversion of 5-FC, a nontoxic antifungal agent, into the toxic metabolites 5-fluorouracil and 5-fluorouridine-5′-monophosphate, thus bypassing the natural resistance of certain human tumor cells to 5-fluorouracil. Unexpectedly, although the uracil phosphoribosyltransferase activity of *FCU1* was equivalent to that encoded by *FUR1*, its cytosine deaminase activity was 100-fold higher than the one encoded by *FCY1*. As a consequence, tumor cells transduced with an adenovirus expressing *FCU1* (Ad-FCU1) were sensitive to concentrations of 5-FC 1000-fold lower than the ones used for cells transduced with a vector expressing *FCY1* (Ad-FCY1). Furthermore, bystander cell killing was also more effective in cells transduced with Ad-FCU1 than in cultures infected with Ad-FCY1 or Ad-FUR1, alone or in combination. Finally, intratumoral injections of Ad-FCU1 into allo- or xenogeneic tumors implanted s.c. into mice, with concomitant systemic administration of 5-FC, led to substantial delays in tumor growth. These unique properties make of the *FCU1*/5-FC prodrug activation system a novel and powerful candidate for cancer gene therapy strategies.

INTRODUCTION

Transfer into tumors of conditionally toxic "suicide" genes has emerged as an attractive gene therapy strategy for selective elimination of cancer cells. In most cases, such suicide genes encode non-mammalian enzymes that can convert toxicologically and pharmacodynamically inert agents into highly toxic metabolites (1–3). Besides its direct local cytotoxic impact, the potential interest of this strategy was further strengthened by the recent demonstrations that tumor cell killing also contributes to the enhancement of the host antitumor immune response through the local release of cellular debris that can then be processed and presented by the antigen presenting cells (4–6).

One of the most intensely studied suicide genes is the HSV-*TK*[2] gene (7). Expression of HSV-*TK* in tumor cells allows the conversion of prodrugs such as GCV and acyclovir, two acyclic guanosine analogues, into their monophosphate forms, which are then further phosphorylated by cellular kinases into their di- and triphosphate derivatives. Intracellular accumulation of such triphosphate metabolites and their subsequent incorporation into nascent DNA strands inhibit mammalian DNA polymerases and ultimately lead to cell death (7). Interestingly, neighboring tumor cells that do not express the HSV-*TK* gene were also shown to be actively killed in presence of GCV (8, 9). This enhancement of the antitumor activity by a bystander killing effect was demonstrated to be mediated by cellular connexins that allow the transfer of the toxic metabolites from the transduced cells to neighboring cells (10–13).

Another suicide gene that has recently received considerable attention is CDase. CDase, an enzyme present in fungi and bacteria but absent from mammalian cells, deaminates cytosine to uracil (14). CDase can also deaminate the nontoxic prodrug 5-FC to its highly toxic derivative 5-FU. This metabolite is then converted by cellular enzymes into 5-FUTP and 5-fluoro-dUMP (5-FdUMP). 5-FUTP can be incorporated into RNA in place of UTP, resulting in the inhibition of the nuclear processing of the ribosomal and mRNAs, whereas 5-FdUMP irreversibly inhibits thymidylate synthase, preventing DNA synthesis. Interestingly, and in contrast to GCV and its derivatives, 5-FC and 5-FU can penetrate tumor cells by passive diffusion and expand the local toxic effect to neighboring cells, irrespective of the presence or absence of specific cellular connexins. The potential for using the bacterial *Escherichia coli* CDase gene (*codA*) to confer sensitivity to 5-FC has already been demonstrated *in vitro* and *in vivo* in various experimental tumor models (15–20). However, relatively disappointing results were also obtained in cell lines derived from breast and pancreatic tumor cell lines (21). Such cells were found to be relatively resistant to 5-FU as a consequence of possible defects in downstream cellular metabolism of 5-FU. Supporting this hypothesis, expression in mammalian cells of *upp*, the gene encoding the *E. coli* UPRTase, which catalyzes the conversion of uracil and 5-FU into UMP and 5-fluoroUMP (5-FUMP), respectively, was shown to greatly enhance the sensitivity of the cells to 5-FU (22).

We had previously cloned and characterized the *Saccharomyces cerevisiae FCY1* and *FUR1* genes encoding the CDase and UPRTase enzymes (23–25), respectively. In this work, we have investigated *in vitro* and *in vivo* whether the co-expression of these two yeast genes may cooperatively increase the chemosensitivity of tumor cells to 5-FC. Replication-deficient adenoviruses were selected for these studies, given their ability to efficiently transfer and express candidate therapeutic genes in a variety of dividing and postmitotic cell types, including tumor cells, *in vitro* and *in vivo* (26). We report that transduction of tumor cells by adenoviruses expressing the *FCY1* or the *FUR1* genes increases their sensitivity to 5-FC and to 5-FU, respectively, and that co-expression of *FCY1* and *FUR1* further enhances the cells sensitivity to 5-FC by more than 10-fold when compared to the transfer of *FCY1* alone. More intriguing, we describe a novel chimeric protein, designated FCU1, that displays both enzymatic activities. This chimeric protein was generated by fusing the *FCY1* and *FUR1* coding sequences in frame. Its exhibits an UPRTase activity similar to the one encoded by the native *FUR1* gene and,

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[2] The abbreviations used are: HSV-*TK*, herpes simplex virus type-1 thymidine kinase; 5-FC, 5-fluorocytosine; CDase, cytosine deaminase; 5-FU, 5-fluorouracil; UPRTase, uracil phosphoribosyltransferase; 5-FUMP, 5-fluorouridine-5′-monophosphate; GCV, ganciclovir; 5-PRPP, 5-phosphoribosyl-1-pyrophosphate; MOI, multiplicity of infection; GFP, green fluorescent protein; Ad-FCU1, adenovirus expressing *FCU1*; Ad-FCY1, adenovirus expressing *FCY1*; Ad-FUR1, adenovirus expressing *FUR1*; Ad-GFP, adenovirus expressing GFP; Ad-TK, adenovirus expressing HSV-TK; Ad-null, adenovirus carrying no expression cassette; CMV, cytomegalovirus; IU, infectious unit(s).

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unexpectedly, a CDase activity 100-fold more elevated than the one encoded by the *FCY1* gene. In addition, we present data showing *in vitro* and *in vivo* that the *FCU1*/5-FC system is a more effective prodrug activation system than the HSV-*TK*/GCV and *CDase*/5-FC systems. These results suggest that this novel suicide gene may constitute an original and potent candidate therapeutic gene for cancer gene therapy.

MATERIALS AND METHODS

Chemicals. [6-^{3}H]5-FC (4.1 Ci/mmol), [2-^{14}C]cytosine (51.3 mCi/mmol), [2-^{14}C]5-FU (55 mCi/mmol), cytosine, uracil, and 5-FU were purchased from Sigma Chemical Co. (St. Louis, MO). [2-^{14}C]Uracil (60 mCi/mmol) was purchased from NEN Life Science Products (Boston, MA). 5-FC and GCV (Cymevan) were from Roche (Neuilly-sur-Seine, France). 5-PRPP was from ICN (Aurora, IL).

Cell Culture. Human colon cancer cell lines SW480 and LoVo, human breast cancer cell line SK-BR-3, human pancreas cancer cell line PANC-1, human embryonal kidney cell line 293 and murine melanoma cancer cell line B16F0 were obtained from the ATCC (Manassas, VA). SW480, SK-BR-3, PANC-1, 293, and B16F0 cells were grown in DMEM supplemented with 10% fetal bovine serum, and LoVo cell line was maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum. Before use, all cell lines were tested and found to be free of *Mycoplasma* infection.

Plasmid Constructions. The *FCY1* gene was modified from phagemid pRS315-*FCY1* (25) by oligonucleotide-directed mutagenesis. The 5′ primer (5′-GTTAAAAGCTTCATAGGCCATGGTG-3′) was designed to introduce a Kozak sequence for mammalian cell expression, as well as a *Hin*dIII site to facilitate cloning. The 3′ primer (5′-AGTAGAGAATTCAGCACGCTG-3′) introduces an *Eco*RI site after the stop codon. The resulting 490-bp *Hin*dIII-*Eco*RI fragment carrying *FCY1* was subcloned into the plasmid pRS306 (27), and the integrity of the sequence was verified by sequencing. From plasmid pRS306-*FCY1*, the fragment *Xho*I-*Xba*I containing the *FCY1* gene was cloned into the corresponding sites of the mammalian expression vector pCI-neo (Promega, Charbonnieres, France) giving rise to vector pCI-neo*FCY1*. From plasmid pCI-neo*FUR1*-105,[3] the fragment *Eco*RI-*Not*I, containing the *FUR1* open-reading frame starting from the second natural translation initiation codon (24), was inserted into the pCI-neo*FCY1* linearized by *Eco*RI-*Not*I, generating the plasmid pCI-neo*FCY1-FUR1* (Fig. 1*A*). Single-stranded phagemid was prepared from vector pCI-neo*FCY1-FUR1*, and the stop and start codons of *FCY1* and *FUR1* were deleted, respectively, by oligonucleotide-directed mutagenesis (primer, 5′-ATGGTTCCGAAGCCTCACCAATATCT-3′), joining in-frame the two enzymatic moieties (Fig. 1*A*). The resulting plasmid carrying the fusion gene, designated *FCU1*, is called pCI-neo*FCU1*.

Adenovirus Production and Titration. All recombinant adenoviral genomes were generated as infectious plasmids by homologous recombination in *E. coli* as described previously (28). In brief, the *FCY1* (fragment *Xho*I-*Xba*I from pCIneo-*FCY1*), *FUR1* (fragment *Eco*RI-*Mlu*I from pCIneo-*FUR1*), and *FCU1* genes (fragment *Xho*I-*Mlu*I from pCIneo-*FCU1*) were inserted in an adenoviral shuttle plasmid containing a CMV-driven expression cassette surrounded by adenoviral sequences (adenoviral nucleotides 1–458 and nucleotides 3328–5788) to allow homologous recombination with the adenoviral sequences of the backbone vector (28). The resulting full-length viral genomes contain a deletion in E3 (nucleotides 28,592–30,470), whereas the E1 region (nucleotides 459-3327) was replaced by the expression cassette containing, from 5′ to 3′, the CMV immediate-early enhancer/promoter, a chimeric human β-globin/IgG intron, the foreign yeast gene, and the SV40 late polyadenylation signal. The recombinant adenoviruses (Ad-FCY1, Ad-FUR1, and Ad-FCU1; Fig. 1*B*) were generated by transfection into the 293 complementation cell line of the viral genomes released from their respective plasmids by *Pac*I digestion. Virus propagation, purification, and titration of IUs by indirect immunofluorescence of the viral DNA binding protein were carried out as described previously (29). Purified viruses were stored in 1 M sucrose, 10 mM Tris-HCl, pH 8.5, 1 mM $MgCl_2$, 150 mM NaCl, 0.005% Tween 80. The same methods were used to generate the Ad-GFP and Ad-TK vectors, which express GFP and the HSV-TK protein, respectively. Ad-null was used as a control (Fig. 1*B*).

[3] P. Erbs, F. Exinger, and R. Jund, manuscript in preparation.

Adenovirus Infections *in Vitro*. All infections were performed in suspension by a 30-min incubation of cells with virus vectors dilutions in 100 μl of PBS supplemented with 2% FCS, 1% cations. Cells were then plated in complete fresh medium, and analysis was performed at various times postinfection. To determine the *in vitro* transduction efficiency, cells were infected with Ad-GFP, and 24 h later, single-cell suspensions were analyzed by flow cytometry using a FACScan instrument (Becton Dickinson).

Enzymatic Assays. To evaluate the percentage of conversion of cytosine to uracil, of uracil to UMP and of cytosine to UMP, SK-BR-3 human tumor cells (5×10^6 cells) were transduced in suspension by Ad-null, Ad-FCY1, Ad-FUR1, or Ad-FCU1 at a MOI of 100. Twenty-four h later, the cells were resuspended in 30 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% Triton X-100) and incubated for 30 min at 4°C. Cellular debris were removed by centrifugation, and 4 μl of cell lysate were combined with 2 μl of reaction buffer [100 mM Tris-HCl, pH 7.5, 0.4 mM [^{14}C]cytosine (0.02 μCi/μl) for CDase activity; 100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM 5-PRPP, 0.4 mM [^{14}C]uracil (0.02 μCi/μl) for UPRTase activity; and 100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM 5-PRPP, 0.4 mM [^{14}C]cytosine (0.02 μCi/μl) for CDase-UPRTase activities] and incubated at 37°C for 2 h. The enzymatic reactions were stopped by heating at 90°C for 2 min. Aliquots of each reaction (1 μl) were run on polyethyleneimine-impregnated cellulose TLC plate (Schleicher & Schuell, Dassel, Germany) and developed in a mixture of 1-butanol (86%)/water (14%). Scanning of the radioactive spots was achieved with a Molecular Dynamics 445 SI PhosphorImager.

For determination of the different specific enzymatic activities using 5-FC and 5-FU as substrates, SW480, SK-BR-3, and PANC-1 human tumor cells were infected in suspension with each adenovirus vector at a MOI of 20. Twenty-four h after infection, 10 μg of cell lysate proteins (determined by Bio-Rad protein assay) were incubated at 37°C for 20 min with a final concentration of 1 mM 5-FC ([^{3}H]5-FC at 0.01 μCi/μl) for CDase and CDase-UPRTase activities and 1 mM 5-FU ([^{14}C]5-FU at 0.006 μCi/μl) for UPRTase activity. Aliquots of each reaction were analyzed by TLC as described above.

***In Vitro* Cell Sensitivity to 5-FC and 5-FU.** Human tumor cells were transduced in suspension by Ad-null, Ad-FCY1, Ad-FUR1, or Ad-FCU1 at a MOI of 5. A total of 2×10^5 cells/well were plated in 6-well culture dishes in 2 ml of medium containing various concentrations of 5-FC or 5-FU. Cells were then cultured at 37°C for 6 days, and the number of viable cells were counted by trypan blue exclusion. Results are expressed as the ratio between the number of viable cells in plates containing the drugs *versus* the number of viable cells in the corresponding drug-free controls.

***In Vitro* Evaluation of the Bystander Effect.** Human tumor cells were infected in suspension by Ad-null, Ad-FCY1, Ad-FUR1, or Ad-FCU1 at a MOI of 20. After 48 h, cells were rinsed extensively to remove the free virions, trypsinized, mixed with uninfected cells at varying ratios, and plated in 6-well culture dishes at a density of 2×10^5 cells/well. Cells were then cultured at 37°C for 6 days in the presence of 1 mM 5-FC. The number of viable cells was measured by trypan blue exclusion. To determine whether cell-to-cell contact was needed for the observed bystander effect, human cells were infected in suspension with Ad-null, Ad-FCY1, Ad-FUR1, or Ad-FCU1 at a MOI of 20 and then plated in 12-well culture dishes (10^6 cells/well) in 1 ml of serum-containing medium. After 24 h, medium was aspirated, and cells were washed with PBS to remove all free viruses. Cells were then incubated for 48 h in a medium containing 0.01, 0.1, or 1 mM 5-FC. This supernatant was collected, diluted 1:5 with normal medium, and transferred to monolayers of parental cells (2×10^5/well) that were incubated for 6 days. Viable cells were then counted by trypan blue exclusion. To examine whether newly synthesized 5-FU metabolites could passively diffuse into the culture supernatant of transduced cells exposed to 5-FC, cells were infected in suspension with the different vectors at a MOI of 20 and plated in 12-well culture dish (10^6 cells/well). After 24 h, 1 mM [^{3}H]5-FC with an activity of 0.25 μCi/μl was added to the cultures. After an additional 24–48 h, the concentrations of [^{3}H]5-FC and [^{3}H]5-FU in the media were measured using TLC as described above. The data are expressed as the percentage of 5-FU in the media for various incubation times with [^{3}H]5-FC.

Comparison of the FCU1/5-FC and HSV-TK/GCV Prodrug Activation Systems. Mouse and human tumor cells were infected in suspension by Ad-null, Ad-FCU1, or Ad-TK (at a MOI of 50 for B16F0 and a MOI of 1 for

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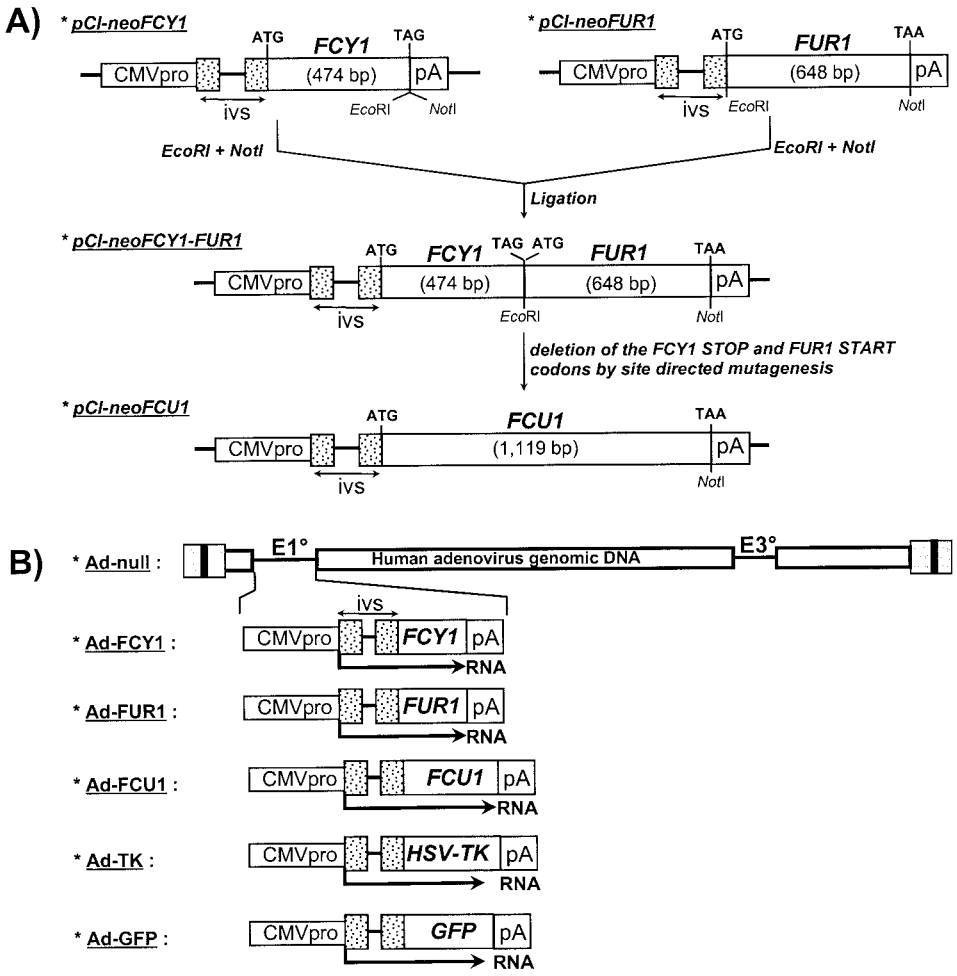

Fig. 1. Schematic representation of the recombinant plasmids and adenoviral vectors. *A, FCU1* was generated by fusing in frame the *FCY*1 and *FUR1* coding sequences. The stop (TAG) and start (ATG) codons of *FCY1* and *FUR1*, respectively, were deleted by site-directed mutagenesis. The 1119-bp open reading frame of *FCU1* encodes a putative polypeptide of 373 amino acid residues. *B,* structures of the E1/E3-deleted adenovirus vectors used in this study. All vectors, except Ad-null, contain in E1 the indicated transgenes under the control of the CMV promoter (*CMVpro*), a chimeric human β-globin/IgG intron (*ivs*), and the SV40 late polyadenylation signal (*pA*).

SW480, SK-BR-3 and PANC-1). A total of 2×10^4 cells/well were plated in 6-well culture dishes in 2 ml of medium containing various concentrations of 5-FC or GCV. Viable cells were counted on day 10 by trypan blue exclusion.

***In Vivo* Experiments.** The mouse B16F0 tumors were established by injecting 3×10^5 cells (in 100 μl PBS) s.c. into the flanks of 6-week-old female immunocompetent B6D2 mice (IFFA-CREDO, L'Arbresle, France). Eight days later, when tumors became palpable, the mice were randomized in a blinded manner (10 mice/group) and were treated with the indicated adenovirus vectors. The test vectors (at a dose of 5×10^8 IU) or the control vehicle used to suspend the adenovirus (10 mM Tris-HCl, pH 7.5, 1 mM MgCl2) were administrated to the tumors at days 8, 9, and 10 after tumor implantation. From day 8 on, the animals were treated twice daily with i.p. injections of 5-FC (500 mg/kg), GCV (50 mg/kg, maximum tolerated dose), or saline (0.9% NaCl). The treatment was maintained for 3 weeks. To evaluate the ability of Ad-FCU1/5-FC to suppress human tumor growth *in vivo*, SW480 and LoVo cells were injected s.c. into athymic BALB/c *nu/nu* mice (6-week-old female; IFFA-CREDO). Each animal was implanted with 10^7 SW480 cells or 5×10^6 LoVo cells suspended in 100 μl of PBS. When tumors had reached 30 mm^3, the mice were randomized in a blinded manner and treated with the indicated vectors at a dose of 10^8 IU (SW480) or 5×10^8 IU (LoVo tumors). The vectors or vehicle were directly injected into the tumor at days 15, 17, and 19 postimplantation for the SW480 tumors and at days 8, 10, and 12 for the LoVo tumors. 5-FC (500 mg/kg) or saline was administered i.p. twice daily for 2 weeks, starting at day 15 (SW480) or at day 8 (LoVo). Tumor size was measured in three dimensions using calipers. Tumor volume were calculated in mm^3 using the formula $(\pi/6)$(length $\times$ width $\times$ height).

Statistical Analyses. Statistical analyses were performed using the nonparametric Mann-Whitney *U* test and STATISTICA 5.1 software (StatSoft, Inc.). A $P < 0.05$ was considered to be statistically significant.

RESULTS

***FCU1* Encodes a Bifunctional Protein That Combines the CDase and UPRTse Activities of *FCY1* and *FUR1*.** The chimeric *FCU1* gene was generated by directly fusing in frame the coding sequences of *FCY1* and *FUR1*, followed by a precise deletion of the translation stop and start codons of *FCY1* and *FUR1*, respectively (Fig. 1*A*). The comparative analysis of the respective enzymatic activities of FCU1, CDase, and UPRTase was first performed *in vitro* in various human and mouse tumor cell lines. For that purpose, the *FCU1*, *FCY1*, and *FUR1* genes were cloned into recombinant E1/E3-deleted vectors derived from the human adenovirus serotype 5 (Fig. 1*B*). Although recombinant adenoviruses constitute currently the most efficient vectors for *in vitro* and *in vivo* expression of therapeutic genes in tumor cells, wide variations in viral susceptibility can be observed among various cell types. To first determine the experimental conditions that allow an efficient transduction of the human (SW480, SK-BR-3, PANC-1, and LoVo) and mouse (B16F0) tumor cells selected for this study, the susceptibility of these cells to virus transduction was analyzed using the replication-deficient Ad-GFP (Fig. 1*B*). These experiments, summarized in Table 1, showed that more than 90% of the human cell populations could be transduced with Ad-GFP at a MOI of 20, whereas only 12% of the mouse B16F0 melanoma cells were transduced at a MOI of 200.

The CDase, UPRTase, and combined CDase/UPRTase activities were first evaluated by the analysis of the enzymatic conversions of [^{14}C]cytosine to [^{14}C]uracil, [^{14}C]uracil to [^{14}C]UMP, and [^{14}C]cy-

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Table 1 *Susceptibility of human and mouse tumor cell lines to adenovirus infection*

Cell line	MOI	Transduction efficiency[a]
SW480	1	34 ± 2.4
	5	74.8 ± 2.2
	10	86.6 ± 1.4
	20	96.2 ± 0.8
SK-BR-3	1	74.5 ± 1.4
	5	94.8 ± 0.6
	10	97 ± 1.2
	20	99 ± 0.4
PANC-1	1	18.6 ± 3.4
	5	53.4 ± 5.1
	10	76.2 ± 4.2
	20	91.7 ± 2.5
LoVo	1	17.4 ± 2.1
	5	48.1 ± 3.1
	10	62 ± 2.6
	20	92.3 ± 1.9
B16F0	50	3.6 ± 0.8
	100	7.1 ± 1.5
	200	12.6 ± 2.5

[a] Tumor cells were infected at the indicated MOI with Ad-GFP, and the percentage of GFP-positive cells was determined by fluorocytometry at 24 h postinfection from scoring a minimum of 5000 cells. The results were obtained from three separate experiments ± SD.

tosine to [^{14}C]UMP, respectively, using lysates prepared from human SK-BR-3 tumor cells infected at a MOI of 100 with the Ad-null, Ad-FCY1, Ad-FUR1, or Ad-FCU1 vector. As expected, no endogenous CDase activity was detectable in the tumor cells, whereas infection with Ad-FCY1 led to an efficient conversion of 20% of the cytosine molecules to uracil (Fig. 2*A*). Unexpectedly however, infection with Ad-FCU1 revealed a higher CDase activity, with a conversion of the entire cytosine pool to uracil (Fig. 2*A*). In contrast to CDase, a low basal endogenous UPRTase activity was detected in the SK-BR-3 cells (conversion of 2% of the uracil molecules to UMP; Fig. 2*B*), but transduction with Ad-FUR1 or Ad-FCU1 further enhanced this UPRTase activity, thus allowing the conversion of the entire uracil pool to UMP. No difference in the UPRTase activities encoded by the *FUR1* and *FCU1* genes was noticed, however (Fig. 2*B*). Co-infection of SK-BR-3 cells with both Ad-FCY1 and Ad-FUR1 vectors led to the direct conversion of 18% of the cytosine molecules to UMP, confirming the ability of Ad-FCY1 and Ad-FUR1 to simultaneously express the complementary CDase and UPRTase enzymatic activities (Fig. 2*C*). Under similar experimental conditions, the chimeric FCU1 enzyme displayed a much more efficient combined CDase/UPRTase activity, with a conversion of the entire cytosine pool into UMP (Fig. 2*C*), as a probable consequence of its higher CDase activity (Fig. 2*A*).

To more precisely characterize the encoded enzymes, SW480, SK-BR-3, and PANC-1 human tumor cells were infected with each vector at a MOI of 20 in presence of 5-FC or 5-FU, and the specific CDase, UPRTase, and CDase/UPRTase enzymatic activities were determined 24 h later as described in "Materials and Methods." Confirming the results described above (Fig. 2), elevated CDase activities were found in cells infected with either Ad-FCY1 or Ad-FCU1, whereas no CDase activity was detectable in noninfected cells or in mock-infected cells (Table 2). The CDase activity was, however, more than 100-fold higher in cells infected with Ad-FCU1 than in cells infected with Ad-FCY1 (Table 2). The reason for this enhanced CDase activity of *FCU1* remains unknown. We can nonetheless exclude variations in the efficiency of cell transduction as a cause because identical experimental conditions were used for all infections. In contrast, the UPRTase enzymatic activity was similar in cells infected with Ad-FCU1 and Ad-FUR1, but significantly higher than in mock-infected cells or in cells infected with Ad-null or Ad-FCY1 (Table 2). Analysis of the direct conversion of [^{3}H]5-FC to [^{3}H]5-FUMP, indicative of the existence of a combined CDase-UPRTase activity, confirmed that *FCU1* does encode a bifunctional enzyme (Table 2). Moreover, this combined enzymatic activity was found to be 10-fold higher in cells infected with Ad-FCU1 than in cells co-infected with Ad-FCY1 and Ad-FUR1.

Taken together, these *in vitro* enzymatic assays demonstrate that *FCU1* encodes a bifunctional enzyme that exhibits a similar UPRTase activity and a much enhanced CDase activity as compared to the parental yeast enzymes encoded by *FUR1* and *FCY1*.

Expression of *FCU1* Enhances the Sensitivity of Human Tumor Cells to 5-FC. To determine whether the combined CDase/UPRTase activities and the higher CDase activity would make of *FCU1* a suitable suicide gene for cancer gene therapy, the sensitivities to 5-FC of the human SW480, SK-BR-3, and PANC-1 tumor cells were analyzed *in vitro* after transduction with the vectors Ad-FCU1, Ad-FCY1, or Ad-FUR1, either alone or in combination (Fig. 3*A*, *C*, and *E*). These results demonstrate that infection with Ad-FCY1 confers to these cells a higher sensitivity to 5-FC, which is further increased by 10-fold when the cells are co-infected with Ad-FUR1 (Fig. 3). This observation supports the hypothesis that the simultaneous expression of CDase and UPRTase can lead to a cooperative effect, resulting in

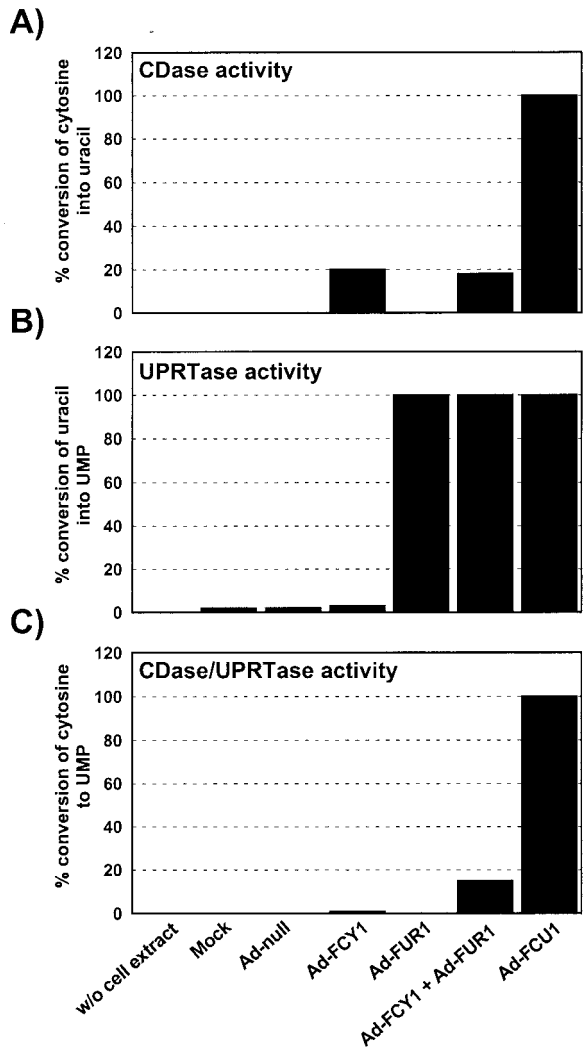

Fig. 2. CDase, UPRTase, and CDase/UPRTase activities in adenovirus-transduced human tumor cells. Efficiencies of conversion of cytosine to uracil (*A*), uracil to UMP (*B*), and cytosine to UMP (*C*) were determined using cell extracts from SK-BR-3 cells transduced *in vitro* with Ad-null, Ad-*FCY1*, Ad-*FUR1*, Ad-*FCU1*, or Ad-*FCY1*+Ad-*FUR1*. The cell extracts, prepared 24 h postinfection, were incubated with [^{14}C]cytosine (*A* and *C*) or [^{14}C]uracil (*B*) for 2 h, and the supernatants were analyzed by TLC as described in "Materials and Methods."

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Table 2 *Specific CDase, UPRTase, and CDase/UPRTase activities in human tumor cell lines*

CDase, UPRTase, and CDase/UPRTase activities are expressed as pmol of 5-FC deaminated/min/mg of protein, pmol of 5-FU phosphorylated/min/mg of protein, and pmol of 5-FUMP produced/min/mg of protein when using 5-FC as substrate, respectively. The indicated enzymatic activities were measured as described in "Materials and Methods." Each value represents the average of three independent experiments ± SD.

Cell line	Vector	CDase 5-FC → 5-FU	UPRTase 5-FU → 5-FUMP	CDase/UPRTase 5-FC → 5-FUMP
PANC-1	Mock	ND[a]	107 ± 16	ND
	Ad-null	ND	114 ± 27	ND
	Ad-FCY1	105 ± 19	105 ± 20	63 ± 9
	Ad-FUR1	ND	833 ± 57	ND
	Ad-FCY1 + Ad-FUR1	105 ± 13	744 ± 110	84 ± 8
	Ad-FCU1	12,496 ± 504	783 ± 126	634 ± 81
SK-BR-3	Mock	ND	86 ± 14	ND
	Ad-null	ND	106 ± 20	ND
	Ad-FCY1	79 ± 12	94 ± 22	55 ± 9
	Ad-FUR1	ND	763 ± 94	ND
	Ad-FCY1 + Ad-FUR1	74 ± 17	810 ± 44	62 ± 7
	Ad-FCU1	10,230 ± 1,225	879 ± 115	758 ± 115
SW480	Mock	ND	78 ± 12	ND
	Ad-null	ND	75 ± 14	ND
	Ad-FCY1	84 ± 10	73 ± 10	53 ± 5
	Ad-FUR1	ND	761 ± 62	ND
	Ad-FCY1 + Ad-FUR1	77 ± 10	831 ± 88	62 ± 8
	Ad-FCU1	11,425 ± 1,086	783 ± 68	636 ± 78

[a] ND, not detected.

an increased sensitivity of the target cells to 5-FC. In agreement with these results, infection with Ad-FCU1 also led similarly to an enhanced sensitivity of the tumor cells to 5-FC. However, cells transduced with Ad-FCU1 died at concentrations of 5-FC that were at least 1000- and 100-fold lower than the concentrations toxic for cells transduced with Ad-FCY1 or co-transduced with Ad-FCY1 + Ad-FUR1, respectively (Fig. 3, *A, C,* and *E*). Consistent with the results from the enzymatic analyses (Table 2), transduction with Ad-FUR1 or Ad-FCU1 increased similarly the sensitivity of the human SW480, SK-BR-3, and PANC-1 cells to 5-FU by approximately 50-fold as compared to the mock-infected cells or to cells infected with Ad-null (Fig. 3, *B, D,* and *F*). As expected, transduction with Ad-FCY1 did not modify the the sensitivity of the cells to 5-FU.

These results corroborate those of the enzymatic assays (Table 2) and further demonstrate the bifunctionality of the FCU1 chimeric protein. More importantly, these studies also show that *FCU1* is much more potent than *FCY1*, or even than the combination of *FCY1* and *FUR1*, in conferring a sensitivity to 5-FC to transduced tumor cells.

Bystander Killing Is More Effective with *FCU1* than with *FCY1* or *FCY1+FUR1*. Given the relatively poor efficiency of gene transfer generally achieved *in vivo*, even with recombinant adenovirus vectors, the major interest of most prodrug activation systems is their ability to extend the prodrug-induced cytotoxicity beyond the tumor area that was effectively transduced. To investigate the existence and the potency of an eventual bystander killing associated with the expression of *FCU1*, human tumor cell lines were infected with Ad-null, Ad-FCY1, Ad-FUR1, and Ad-FCU1 and mixed at different ratios with mock-infected cells. Cell viability was then measured after 6 days of exposure to 1 mM of 5-FC. Consistent with previous reports using the *E. coli* CDase (17, 20), these experiments show that expression of *FCY1* in 10–20% of the cells is required to attain cytotoxicity in the entire cell population (Fig. 4). Similar results were obtained when cells were infected simultaneously with *FCY1* and *FUR1*. In contrast, expression of *FCU1* from as few as 1% transduced cells was sufficient to provoke a near complete eradication of the mixed cell population (Fig. 4).

To examine whether this bystander effect depends on cell-to-cell contact, as described for HSV-*TK* (10–13), or is rather mediated by toxic metabolites liberated from the transduced cells that can then diffuse into the nontransduced cells, SW480, SK-BR-3, and PANC-1 cells were infected with Ad-null, Ad-FCY1, Ad-FUR1, and Ad-FCU1 and were exposed to 5-FC at concentrations of 0.01, 0.1, or 1 mM. Supernatants from these cells were then recovered and added to the cell culture medium of nontransduced cells (Fig. 5). As expected, supernatants recovered from mock-infected cells or from cells infected with Ad-null or Ad-FUR1 displayed no evident toxicity, whereas supernatants from cells infected with Ad-FCY1 or Ad-FCY1 + Ad-FUR1 induced a major cytotoxicity in the treated cells. Consistent with the stronger bystander effect described above for *FCU1*, tumor cells were more efficiently killed when supernatants were collected from cells transduced with Ad-FCU1 than with Ad-FCY1 (Fig. 5). These results indicate that freely diffusible and stable toxic metabolites derived from 5-FC mediate the bystander effect. Analysis of the cell culture supernatant by TLC revealed a progressive increase in the amount of 5-FU in the extracellular milieu of cells transduced with Ad-FCY1 or Ad-FCU1 (Fig. 6). Again, FCU1 was much more effective in transforming 5-FC in 5-FU. Consistent with previous data from Hirschowitz *et al.* (17) describing an adenovirus vector encoding the bacterial CDase, approximately 20% of the ^{3}H-labeled 5-FC was deaminated by the *FCY1*-encoded CDase into 5-FU at 48 h postinfection. In contrast, FCU1 converted close to 100% of the 5-FC into 5-FU during the same period (Fig. 6).

In summary, these results demonstrate that the higher CDase activity of FCU1 results in a much stronger bystander effect as compared to the parental *FCY1*-encoded protein.

***In Vitro* Comparison of the Conditional Cytotoxicity of *FCU1* and HSV-*TK*.** Tumor cells infected with Ad-FCU1 or Ad-TK under similar experimental conditions were compared for their sensitivity toward 5-FC and GCV, respectively (Table 3). The IC_{50} (the concentration of prodrug that inhibits cell growth by 50% as compared to the untreated control cells) were determined for 5-FC and GCV, and the therapeutic indexes were calculated as the ratio of the IC_{50} of cells infected with Ad-null to the IC_{50} of cells infected with Ad-FCU1 or Ad-TK. In all human cell lines tested in this study, the therapeutic index of the *FCU1*/5-FC regimen was higher than that of the *TK*/GCV combination. A similar observation was made for the mouse B16F0 melanoma cells, despite a reduced therapeutic index for both suicide genes, because of the lower susceptibility of murine cells to adenovirus infection (Table 1). These results suggest that the *FCU1*/5-FC prodrug activation system is more efficient at killing tumor cells *in vitro* than the *TK*/GCV system.

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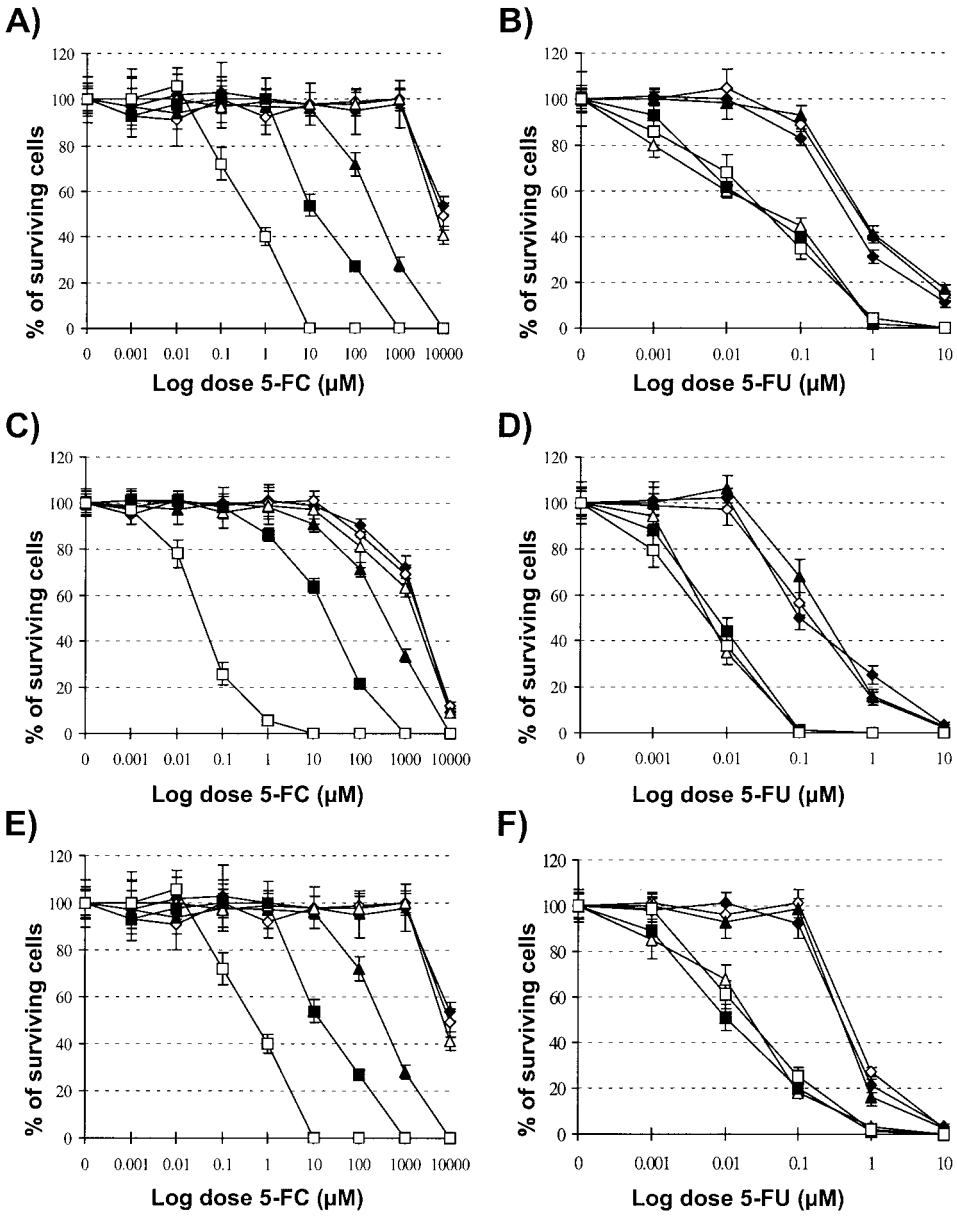

Fig. 3. *In vitro* sensitivities to 5-FC and 5-FU of adenovirus-transduced human tumor cells. SW480 (*A* and *B*), SK-BR-3 (*C* and D), and PANC-1 (*E* and *F*) cells transduced at a MOI of 5 with the indicated vectors (◆, mock-infected; ◇, Ad-null; △; Ad-FUR1; ▲, Ad-FCY1; ■, Ad-FCY1 + Ad-FUR1; □, Ad-FCU1) were exposed to various concentrations of 5-FC (*A*, *C*, and *E*) or 5-FU (*B*, *D*, and *F*), and cell survival was measured 6 days later as described in "Materials and Methods." Results are expressed as the percentage of surviving cells in the presence and absence of the drug. Values are represented as mean ± SD of four individual determinations.

Adenovirus-mediated Transfer of *FCU1* Inhibits Tumor Growth *in Vivo*. The *in vivo* analysis of the *FCU1*/5-FC activation prodrug system was performed either in immunocompetent B6D2 mice implanted s.c. with the mouse B16F0 tumor cell line (Fig. 7) or in immunodeficient BALB/c nude mice implanted with two different human colorectal tumor models (SW480 and LoVo cells; Fig. 8). All test vectors were directly injected into the tumors as indicated in "Materials and Methods," with a concomitant i.p. administration of the appropriate prodrug (5-FC or GCV). The comparative analysis of Ad-FCU1 and Ad-TK in the B16F0 tumor model (Fig. 7), an aggressive, poorly immunogenic tumor cell line, confirmed the higher potency of FCU1. A more sustained control of tumor growth was achieved in mice treated with Ad-FCU1/5-FC than in animals treated with Ad-TK/GCV. As expected, no significant inhibition of tumor growth was observed in any the control groups (Fig. 7). Given the poor susceptibility of mouse cells to adenovirus infection, similar *in vivo* gene transfer experiments were performed in BALB/c nude mice implanted with the more susceptible human SW480 and LoVo colon cancer cells. In both cases, administration of Ad-FCU1/5-FC resulted in a statistically significant suppression of tumor growth, whereas no modification in tumor growth was observed in the control groups (Fig. 8). At 4 weeks after delivery of Ad-FCU1/5-FC, the SW480 and LoVo tumors were 60–65 and 50–55% smaller, respectively, compared to the controls (all controls averaged; Fig. 8). In parallel, control experiments were also performed to determine the *in vivo* antitumor effect of 5-FU in nontransduced tumors derived from SW480 and LoVo cells. Despite the administration of doses of 5-FU that were at the maximum tolerated concentrations (i.p. injection of 10 mg of 5-FU/kg twice daily during 3 weeks), no statistically significant inhibition of tumor growth was observed (data not shown). This result supports the notion that a local production of high concentrations of 5-FC derivatives is necessary to achieve a significant antitumor effect in absence of noticeable toxicity.

DISCUSSION

Previous studies have established that transduction of the *E. coli* CDase gene, *codA*, into either human or rodent tumor cells renders

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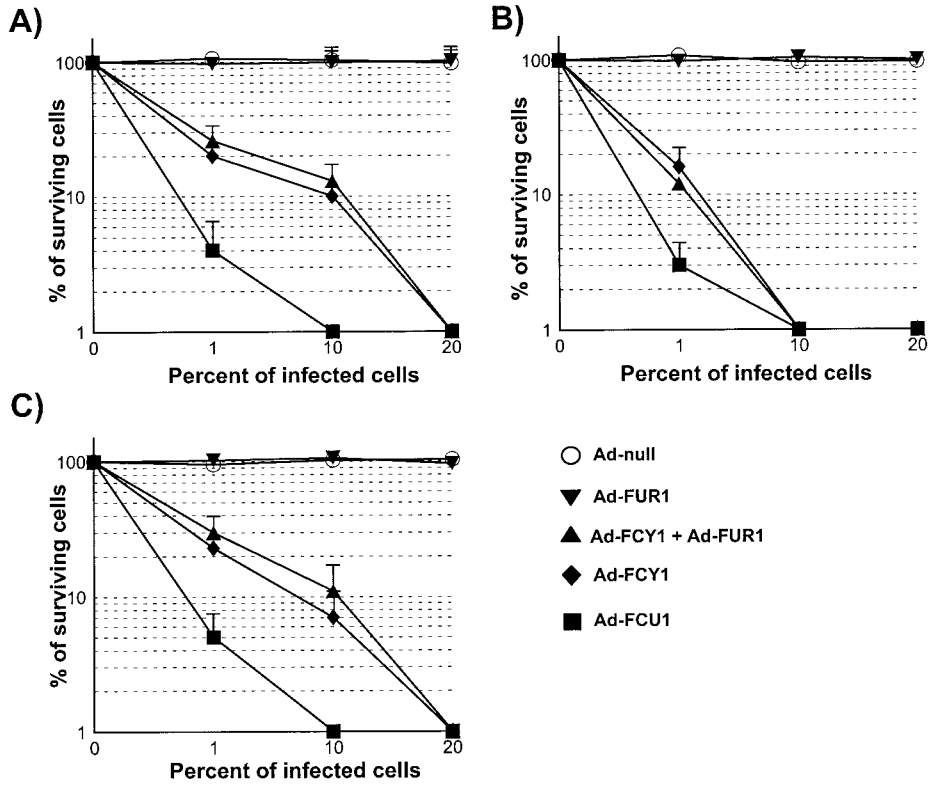

Fig. 4. *In vitro* bystander effect in the human SW480 (*A*), SK-BR-3 (*B*), and PANC-1 (*C*) cells. Cells were infected with the indicated vectors at a MOI of 20 and then mixed at various ratios with uninfected cells in 5-FC-containing media (1 mM). The data are expressed as the percentage of surviving cells compared to non-infected cells. Each data point represents the mean ± SD of four individual determinations.

these cells highly sensitive to 5-FC (15–20). Extending these observations, we demonstrate first in this report that the adenovirus-mediated transfer into tumor cells of the yeast *FCY1* gene can similarly sensitize these cells to 5-FC and suppress their growth *in vitro*. *FCY1* encodes a polypeptide of 158 amino acid residues that shares common motifs with cytosine-nucleoside (cytidine and deoxycytidine) and cytosine-nucleotide (dCMP) deaminases (25) but bears no homology with the *E. coli* CDase (25). SDS gel electrophoresis and amino acid analysis have indicated that the *S. cerevisiae* CDase is a homodimer composed of two 17-kDa subunits (30). Similar to the *codA*/5-FC system, the *FCY1*/5-FC combination described in this report also exhibits a strong bystander killing effect because expression of *FCY1*

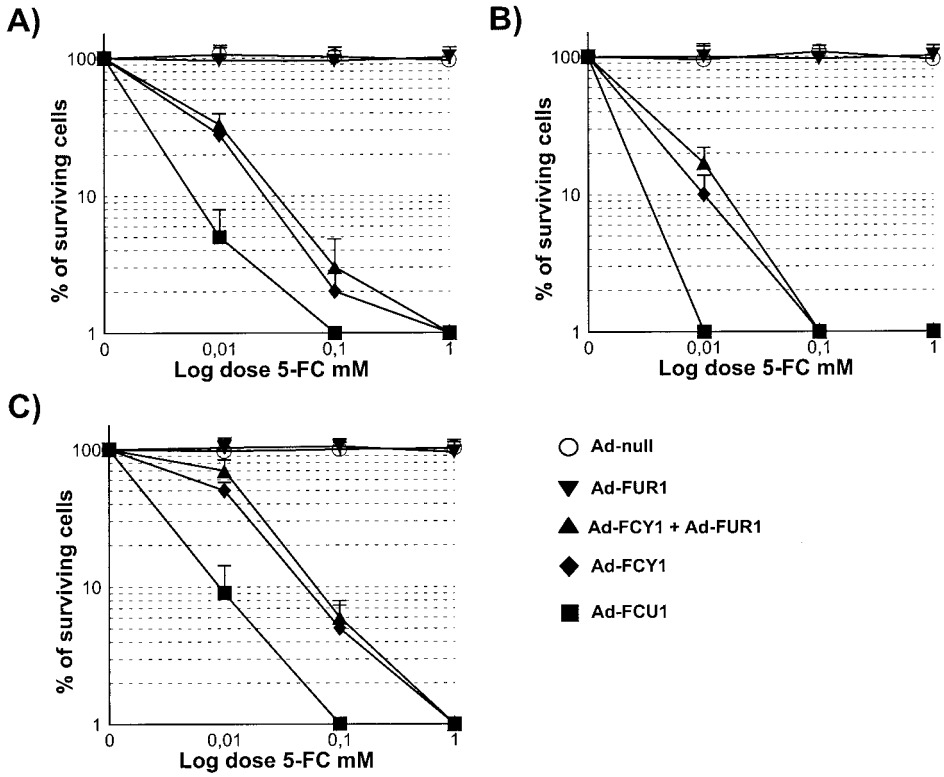

Fig. 5. Cytotoxicity of supernatants from transduced human cells treated with 5-FC. SW480 (*A*), SK-BR-3 (*B*), and PANC-1 (*C*) cells were infected with the indicated vectors at a MOI of 20 and were grown in presence of various concentrations of 5-FC (0.01, 0.1, and 1 mM) in the medium. Media were collected 48 h later, diluted 1:5, and added to uninfected parental cells. Survival was determined at day 6 by trypan blue exclusion. The data are expressed as the percentage of surviving cells in presence and absence of 5-FC. Values are represented as mean ± SD of four individual determinations.

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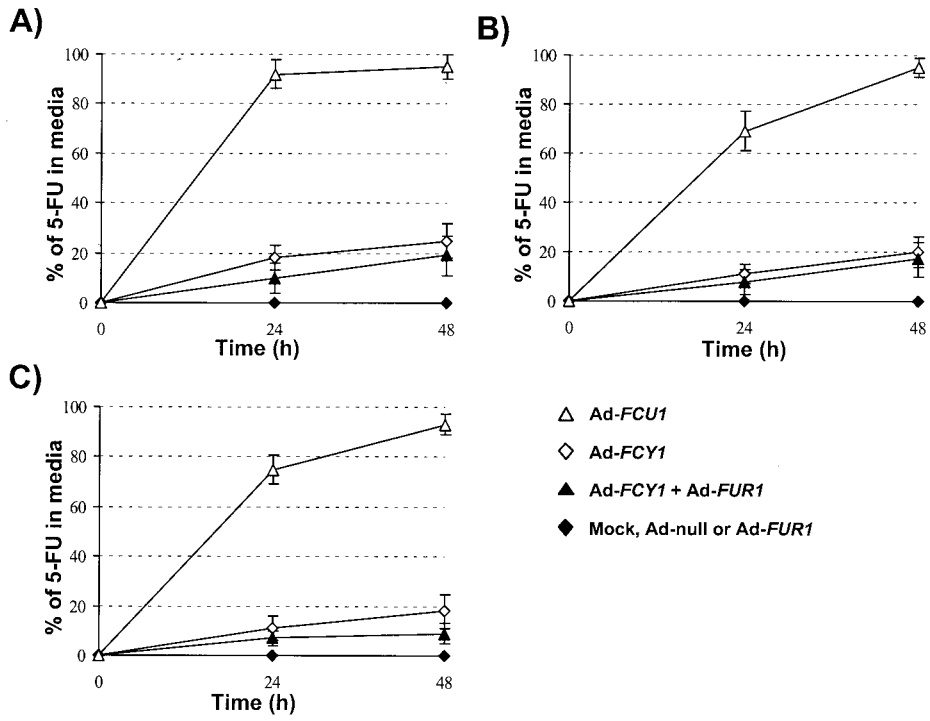

Fig. 6. Conversion of 5-FC to 5-FU and release of 5-FU in the cell culture supernatant. SW480 (*A*), SK-BR-3 (*B*), and PANC-1 (*C*) cells were infected with the indicated vectors at a MOI of 20 and were incubated with 1 mM [^{3}H]5-FC. Twenty-four and 48 h later, the relative concentrations of [^{3}H]5-FC and [^{3}H]5-FU in the media were measured by TLC. The data are expressed as the percentage of [^{3}H]5-FU in the media relative to the total amount of [^{3}H]5-FC + [^{3}H]5-FU. Each *data point* represents the mean ± SD of triplicate determinations.

in only 10–20% of the tumor cells is sufficient to eliminate the entire cell population. In contrast to the HSV-*TK*/GCV system (10–13), this bystander killing does not require cell-to-cell contact given the free diffusibility of 5-FU through cell membranes. In similar studies, Kievit *et al.* (31) and Hamstra *et al.* (32) have recently demonstrated that human and murine cancer cells transduced with the yeast *FCY1* gene were significantly more sensitive *in vitro* and *in vivo* to 5-FC than tumor cells transduced with the bacterial *codA* gene. Using purified enzymes, these investigators observed that yeast and bacterial CDases had comparable K_m and V_{max} values for cytosine, but yeast CDase had a 22-fold lower K_m and a 4-fold higher V_{max} for 5-FC than bacterial CDase. These results may explain the higher sensitivity to 5-FC of tumor cells transduced with the *S. cerevisiae* CDase and strengthen the interest of cancer gene therapy strategies based on the use of the yeast *FCY1* gene.

To exert its cytotoxic effect, the 5-FU produced by the yeast CDase must subsequently be converted by cellular enzymes into one of its active metabolites, 5-fluoroUTP (5-FUTP) or 5-fluoro-dUMP (5-FdUMP). 5-FUTP can be incorporated into the cellular RNAs, whereas 5-FdUMP is a potent inhibitor of the cellular thymidylate synthase, a key enzyme in the *de novo* synthesis of dTMP, which is a precursor for DNA synthesis. The conversion of 5-FU to 5-FUMP can be directly catalyzed by orotate phosphoribosyltransferase using 5-PRPP as the cosubstrate or indirectly in a sequence of reactions in which 5-FU is first converted to 5-fluorouridine by a pyrimidine nucleoside phosphorylase using ribose-1-phosphate as the cosubstrate and then phosphorylated to 5-FUMP by uridine kinase (33). 5-FU can also be converted by a cellular pyrimidine nucleoside phosphorylase into 2′deoxy-5-fluorouridine, with deoxy-Rib-1-P as the cosubstrate,

Table 3 *In vitro therapeutic index of the FCU1/5-FC and HSV-TK/GCV systems*

The therapeutic index is defined as the ratio of the concentrations of prodrug (5-FC or GCV) necessary to kill 50% of the total cell population (IC_{50}) when infected with Ad-null to the concentration of prodrug required when cells are infected with the vector expressing the indicated suicide gene. Cell survival was measured 10 days postinfection by trypan blue exclusion.

Cell line	MOI	Ad-FCU1/5-FC	Ad-TK/GCV
SW480	1	300	100
SK-BR-3	1	3000	1000
PANC-1	1	1000	150
B16F0	50	30	10

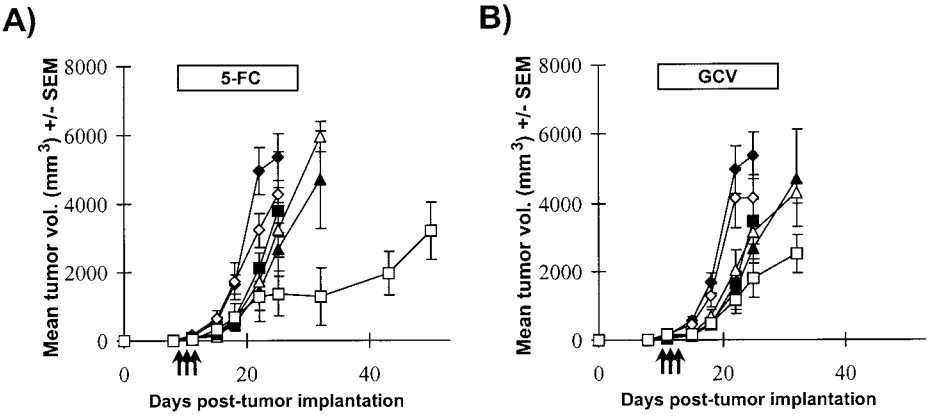

Fig. 7. Growth suppression of murine melanoma B16F0 tumors after three *in vivo* intratumoral injections of Ad-*FCU1* plus 5-FC (*A*) or Ad-*TK* plus GCV (*B*). 3 × 10^5 B16F0 melanoma cells were implanted s.c. into B6D2 immunocompetent mice, and palpable tumors were randomized to be treated at days 8, 9, and 10 with either the vehicle alone (◆, vehicle + saline; ◇, vehicle + 5-FC or GCV) or with 5 × 10^8 IU of the indicated adenovirus vectors (□, Ad-FCU1+5-FC or Ad-TK + GCV; ■, Ad-FCU1 + saline or Ad-TK + saline; △, Ad-null + 5-FC or Ad-null + GCV; ▲, Ad-null + saline). The animals were then treated twice daily from day 8 to day 28 with i.p. administrations of saline, 5-FC (1,000 mg/kg/day), or GCV (100 mg/kg/day). Tumors were measured in three dimensions, and tumor volumes were calculated.

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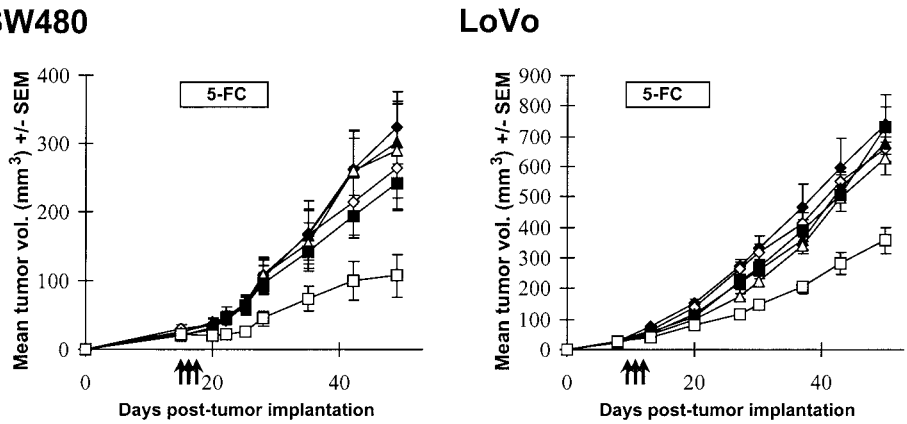

Fig. 8. Growth suppression of human colorectal tumors after three *in vivo* injections of Ad-FCU1 plus 5-FC. SW480 cells (10^7) or LoVo cells (5×10^6) implanted s.c. into nude mice were injected at days 15, 17, and 19 (SW480) or at days 8, 10, and 12 (LoVo) with the vehicle alone (♦, vehicle + saline; ◇, vehicle + 5-FC) or with either 1×10^8 IU (SW480) or 5×10^8 IU (LoVo) of the indicated viruses (▲, Ad-null + saline; △; Ad-null + 5-FC; ■, Ad-FCU1 + saline; □, Ad-FCU1+5-FC). The animals were then treated twice daily from day 15 to day 28 (SW480) or from day 8 to day 21 (LoVo) with i.p. administrations of saline or 5-FC (1,000 mg/kg/day). Tumors were measured in three dimensions, and tumor volumes were calculated. Differences in tumor sizes between the group treated with Ad-FCU1+5-FC and the other groups were shown to be statistically significant ($P < 0.01$).

and then by a cellular thymidine kinase to 5-FUMP (33). However, certain cancer cells may become relatively resistant to 5-FU, because of a poorly efficient conversion of 5-FU to its toxic metabolites (21). The existence in *E. coli* and *S. cerevisiae* of genes (*upp* and *FUR1*, respectively) encoding an enzyme, UPRTase, that can actively convert uracil to UMP, suggests that transfer into tumor cells of these genes should in principle induce a more efficient conversion of 5-FU to 5-FUMP, thus restoring the cells sensitivity to 5-FU. This hypothesis is supported by our demonstration that the adenovirus-mediated transduction of *FUR1* into tumor cells enhances their sensitivity to 5-FU by 50-fold. These results are consistent with a recent report from Kanai *et al.* (22) showing that expression of the bacterial UPRTase gene similarly enhances the cells sensitivity to 5-FU. These results support the notion that in some tumor cells the conversion of 5-FU to 5-FUMP may be a rate-limiting step that can be bypassed by the expression of the *FUR1* or *upp* genes. Interestingly, we also demonstrate in this report that *FUR1* gene transfer can substantially augment the sensitivity of tumor cells to 5-FC when such cells do simultaneously express *FCY1*.

These demonstrations of the cooperativity between the *FCY1*- and *FUR1*-encoded enzymes prompted us to investigate whether a direct channeling of 5-FC to 5-FUMP could be achieved through the generation of a bifunctional protein that combines the two enzymatic activities in one single polypeptide. This fusion protein, designated FCU1, was constructed by fusing in frame the *FCY1* and *FUR1* coding sequences and was demonstrated *in vitro* to exhibit both enzymatic activities. However, although the UPRTase activity of FCU1 was comparable to that encoded by the parental *FUR1* gene, the CDase activity was found to be 100-fold higher than the activity encoded by the parental *FCY1* gene. These unexpected results were confirmed by the 1000-fold and 100-fold higher *in vitro* sensitivity to 5-FC of the *FCU1*-transduced tumor cells as compared to cells transduced with *FCY1* or with both *FCY1* and *FUR1*, respectively. The mechanism whereby the enzymatic activity of the CDase moiety was improved in FCU1 is currently unknown, but future studies using purified CDase, UPRTase and FCU1 proteins are under consideration to better characterize the properties of these proteins. The construction of a similar fusion protein combining the CDase and UPRTase enzymatic activities of the bacterial *codA* and *upp* genes was recently reported (34). Interestingly, although this chimeric bacterial protein also exhibited both enzymatic activities, their efficiencies were comparable to the activities of the proteins encoded by the native *codA* and *upp* genes (34).

Because one of the major limitation of current cancer gene therapy strategies is the poor efficiency of *in vivo* gene transfer, successful application of a suicide gene will rely on the potency of its bystander killing effect. A direct consequence of the increased CDase activity of FCU1 is the very rapid intracellular conversion of 5-FC to 5-FU and the release of 5-FU into the extracellular milieu. This very fast kinetics of production and release of 5-FU and the diffusible nature of the metabolite (35) may both contribute to the observed powerful bystander effect induced by FCU1, because bystander killing was more effective in cells transduced with *FCU1* than in cells expressing *FCY1* alone or in combination with *FUR1*. *FCU1* was also more effective than HSV-*TK* both *in vitro* and in implanted mouse tumor models. This observation confirms and extends previous studies showing that the bacterial CDase/5-FC system was more potent than the HSV-*TK*/GCV system (36, 37). As discussed above, a significant disadvantage of the HSV-*TK* suicide gene as compared to *FCU1* is the requirement for direct cell-to-cell contact and active transport of the toxic GCV metabolites to neighboring cells through the gap junctions (10–13). An increased expression level of HSV-*TK* does not necessarily correlate with more efficient bystander killing because the transport of GCV may become limiting (37). In contrast to GCV, diffusible metabolites such as 5-FU should exert a more potent tumoricidal effect. Any increase in enzymatic CDase activity, as is the case with FCU1, should directly translate into higher local and more toxic concentrations of 5-FU in the tumor area. Another important advantage of the *FCU1*/5-FC system is its impact on both dividing and nondividing cancer cells. Although sensitization to GCV mediated by HSV-*TK* expression occurs only when the target cells are in the S-phase of the cell cycle (7), the incorporation of 5-FU into RNA is independent of the cell cycle (38).

Confirming the *in vitro* properties of *FCU1*, adenovirus-mediated transfer of *FCU1* to tumors derived from mouse melanoma cells or from human colon carcinoma cells could also effectively suppress *in vivo* the growth of the tumors upon systemic administration of 5-FC to the animals. These results are encouraging because such human tumor models were shown to be relatively resistant to 5-FU treatment, and adenovirus injection was found in these experimental conditions to transduce only a few percent of the implanted tumor cells (data not shown).

Thus, the unique combination of an enhanced CDase activity, the presence of an UPRTase activity, and the powerful bystander killing effect mediated by diffusible metabolites makes *FCU1* a promising novel candidate for cancer gene therapy applications, even for 5-FU-resistant target tumors. Moreover, *FCU1* may also be considered for combinations with other therapeutic genes, such as genes encoding immunomodulatory molecules (4–6). Similarly, 5-FU being a radiosensitizer (39), the overall antitumor effect of the *FCU1*/5-FC treatment may be further increased by radiotherapy, as suggested by recent studies showing a better response to radiation when associated with

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CDase/5-FC gene transfer (40, 41). Indeed, our ongoing animal studies have confirmed that co-transfer of *FCU1* with either the interleukin 2 or IFN-γ cytokine genes further improves the *in vivo* antitumor response in immunocompetent mice implanted with the B16F0 melanoma tumor model.

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