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In Vivo Antitumor Activity of 5-Fluorocytosine on Human Colorectal Carcinoma Cells Genetically Modified to Express Cytosine Deaminase

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ABSTRACT

A human colorectal carcinoma cell line, WiDr, was genetically engineered to express the nonmammalian enzyme, cytosine deaminase (CD). Expression of CD in WiDr cells (WiDr/CD) did not alter the growth rate of these cells when grown *in vitro* or as solid tumor xenografts in nude mice. However, expression of CD did increase the sensitivity of these cells to the nontoxic prodrug, 5-fluorocytosine (FCyt), decreasing the 50% inhibitory concentration for FCyt from 26,000 μM in parental WiDr cells to 27 μM in WiDr/CD cells. The increase in sensitivity to FCyt in WiDr/CD cells was the result of the CD-mediated conversion of FCyt to 5-fluorouracil (FUra) and subsequent FUra anabolites. The half-life of the prodrug, FCyt, was determined to be approximately 40 min in nude mice. A single i.p. injection of 500 mg FCyt/kg body weight resulted in a transient FCyt plasma level of approximately 4000 μM while osmotic minipumps or constant tail vein infusions of FCyt achieved continual FCyt plasma levels of 5 μM and 50 μM, respectively, with no overt signs of toxicity. Significant antitumor effects were observed in nude mice bearing tumors derived from WiDr/CD cells when these animals were given 500 mg FCyt/kg i.p. for 10 consecutive days. These antitumor effects were demonstrated by decreases in tumor growth rate, tumor size, tumor weight, and thymidine incorporation into tumor DNA. This antitumor effect was significant but less profound if FCyt was administered by constant tail vein infusion. WiDr and WiDr/CD cells were very sensitive to FUra *in vitro* (50% inhibitory concentration approximately 5 μM). However, no significant antitumor effects were observed in nude mice bearing tumors derived from either WiDr or WiDr/CD cells when these animals were treated with various doses of FUra. Taken collectively, these data indicate that nontoxic plasma levels of FCyt can be attained which can produce profound antitumor effects on tumors engineered to express CD and that these antitumor effects are significantly better than those that can be achieved using FUra. These positive data support the continued development of a gene therapy approach to colorectal carcinoma involving the selective expression of CD in colorectal tumors with subsequent administration of FCyt.

INTRODUCTION

In select cases, chemotherapy targeted against many specific infectious agents is both extremely safe and efficacious. For example, it is well established that penicillin, acyclovir, and FCyt[2] are relatively safe and effective therapies for certain types of bacterial, viral, and fungal infections, respectively (for reviews see Refs. 1–3). These and other such agents achieve therapeutic selectivity by exploiting qualitative differences in the structure, function, or intermediary metabolism between the target organism and humans.

Unlike chemotherapy targeted to infectious agents, chemotherapy targeted to neoplastic disease has generally relied upon subtle quantitative differences between normal and neoplastic cells to achieve therapeutic selectivity. This, of course, has been difficult to effectively achieve and, in many cases, such as CRC, has had little overall success (for reviews see Refs. 4–6).

We are developing a novel gene therapy approach to cancer therapy which involves selectively creating qualitative biochemical differences between normal and neoplastic cells (7–9).[3] This approach, called VDEPT (virus-directed enzyme/prodrug therapy), exploits the transcriptional differences between normal and neoplastic cells. An artificial, chimeric gene is created which is composed of the transcriptional regulatory domain of a tumor-associated marker gene and the protein coding domain of a nonmammalian enzyme. This artificial gene is delivered to and integrated into the tumor cell's genome via a replication-defective retroviral shuttle vector. When selectively expressed, the nonmammalian enzyme can metabolically convert a nontoxic prodrug to a toxic metabolite selectively in the neoplastic cell. We have described specific examples of this approach for primary hepatocellular carcinoma (7, 8) and CRC (9).[3]

For CRC, an artificial, chimeric gene is created which is composed of 5′ transcriptional regulatory sequences of the tumor-associated marker, carcinoembryonic antigen, and the protein coding domain of the nonmammalian enzyme, cytosine deaminase (CD). This chimeric gene will result in the tumor-specific expression of CD which can subsequently convert the nontoxic prodrug, FCyt, to the toxic metabolite, 5-fluorouracil (FUra) (9). Importantly, FUra is the drug of choice in the treatment of CRC (4–6).

To develop this gene therapy approach for CRC, the transcriptional regulatory region of the human carcinoembryonic antigen gene has been cloned and sequenced[3] and is presently being analyzed. In addition, CD has been cloned, sequenced, and expressed in human colorectal tumor cells (9). The next essential step in the development of this approach is the demonstration that plasma levels of FCyt can be obtained which are sufficient to generate an antitumor effect in CD-expressing tumors without generating systemic toxicity. We now describe the pharmacokinetics and *in vivo* antitumor activity of FCyt in nude mice bearing human colorectal tumor xenografts engineered to express CD.

MATERIALS AND METHODS

Cell Lines and Growth Kinetics. WiDr cells were obtained from the American Type Culture Collection (ATCC No. CCL 218). These cells were modified to express CD by transfection of the expression cassette pCMV/CD-1 with subsequent selection on G418 (1 mg G418/ml culture media) as described previously (9). Cellular growth kinetics of control and CD expressing WiDr cells were determined using the stain Hoechst 33342 and a fluorescence assay as described previously (7). Inhibition of cell growth in the presence of varying concentrations of FCyt or FUra was determined as described previously (7). Concentrations of FCyt and FUra yielding IC_{50} were calculated using curve-fitting parameters based on the Marquardt method (7, 10).

Animal Studies. Female athymic nude mice [*nu/nu* (CD-1)BR; Charles River Laboratories] were used in all studies. Drugs were administered either p.o. (in water to a final volume of 0.5 ml), i.p. (in saline to a final volume of 0.5 ml), by a constant tail vein infusion (i.v.) using an indwelling tail vein

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[2] The abbreviations used are; FCyt, 5-fluorocytosine; CRC, colorectal carcinoma; CD, cytosine deaminase; FUra, 5-fluorouracil; AcA, 1 M acetic acid; IC_{50}, 50% inhibitory concentration; LD_{10}, 10% lethal dose (other percentages follow suit); AUC, area under the concentration-time curve.

[3] C. A. Richards, A. Wolberg, and B. E. Huber. The transcriptional control region of the human carcinoembryonic antigen gene: DNA sequence and homology studies, *DNA Sequence*, in press.

catheter for a maximum of 7 days (in saline at flow rates as indicated), or by osmotic minipumps [Alzet (minipump size as indicated)] implanted ip.

Tumor cells (1×10^7 cells) were implanted s.c. in 0.5 ml saline. During the course of an experiment, tumor weights were estimated based upon tumor size as

$$\frac{(\text{Length [mm]}) \times (\text{width [mm]})^2}{2} = \text{Tumor weight [mg]}.$$

Determination of thymidine incorporation into tumor DNA was determined by administering an i.p. injection of 0.5 mCi of [5′-^{3}H]thymidine (14.6 Ci/mmol) 2 h before the animals were killed. Tumors were removed, weighed, and homogenized using a polytron in 12 ml saline/1.5 g tumor tissue. Thymidine incorporation into DNA was determined as described previously (11).

Animals were killed by inhalation overdose with Halothane or CO_2. All animal procedures were performed with approved protocols and in accordance with published recommendations for the proper care and use of laboratory animals (12).

Analytical Methods. For the determination of plasma levels of FCyt in nude mice, animals were dosed as described and samples of blood were obtained by cardiac puncture under CO_2-induced anesthesia using EDTA-treated syringes. Plasmas were prepared by centrifugation and stored at −15°C until analyzed by reverse-phase high-performance liquid chromatography. Samples of each plasma were prepared for analysis by the following solid-phase extraction procedure; 200 μl of plasma was mixed with 300 μl M AcA and the mixture was applied to a Bond Elut SCX column (1 cm^3; Varian) which had been preconditioned with 2 ml AcA. The column was washed with 2 ml AcA and FCyt was eluted with 1 ml 33% acetonitrile in 333 mM ammonium phosphate buffer (pH 7.2). The eluate was evaporated to dryness using a SpeedVac (Savant Instruments, Inc., Farmington, NY), redissolved in 200 μl deionized water, and centrifuged to remove particulates. Using these conditions, the recovery of FCyt from spiked normal mouse plasma was greater than 94%.

Aliquots (100 μl) of each plasma extract were analyzed by high-performance liquid chromatography using an Adsorbosphere C_{18} column (4.6 x 250 mm; Alltech Associates, Inc., Deerfield, IL). The samples were eluted isocratically with 50 mM ammonium acetate, pH 5.5 (Buffer A), for 5 min, followed by a 20-min linear gradient from 0 to 40% acetonitrile in Buffer A. The flow rate was 1.0 ml/min and the solvent breakthrough (elution of nonretained material) was at 2.8 min. The UV absorbance of the column effluent was monitored at 260 nm using an SM4000 programmable wavelength detector (LDC/Milton Roy, Riviera Beach, FL). The retention time for FCyt was about 5.3 min. The column was equilibrated for 10 min in Buffer A prior to injection of each sample. Plasma levels of FCyt were determined by comparison of corresponding peak areas to a calibration curve obtained by least squares linear regression analysis of peak areas *versus* concentrations from identically processed normal mouse plasmas spiked with known concentrations of FCyt.

RESULTS

Effects of CD Expression on Cellular Growth Rate, and Sensitivity to FCyt and FUra. The human colorectal tumor cell line, WiDr, was transfected with the plasmid pCMV/CD-1 which contains the gene for CD in an expression cassette (9). Subsequent to selection with G418, cellular growth rate and sensitivity to FCyt and FUra were determined. There was no significant difference in cellular growth rate between control WiDr cells (WiDr) and WiDr cells expressing CD (WiDr/CD) (Fig. 1*A*). However, the IC_{50} for FCyt shifted from approximately 26,000 μM in WiDr cells to approximately 27 μM in WiDr/CD cells (Fig. 1*B*; Table 1). The IC_{50} value for FUra was approximately 5 μM in both cell lines (Fig. 1*B*; Table 1). The increase in sensitivity to FCyt resulting from expression of CD inWiDr/CD cells has remained stable for the year in which these cells have been in continuous culture. These data indicate that expression of CD in WiDr cells has no direct effect on either the growth rate or the IC_{50} for FUra but does increase the sensitivity to FCyt by approximately 3 orders of magnitude.

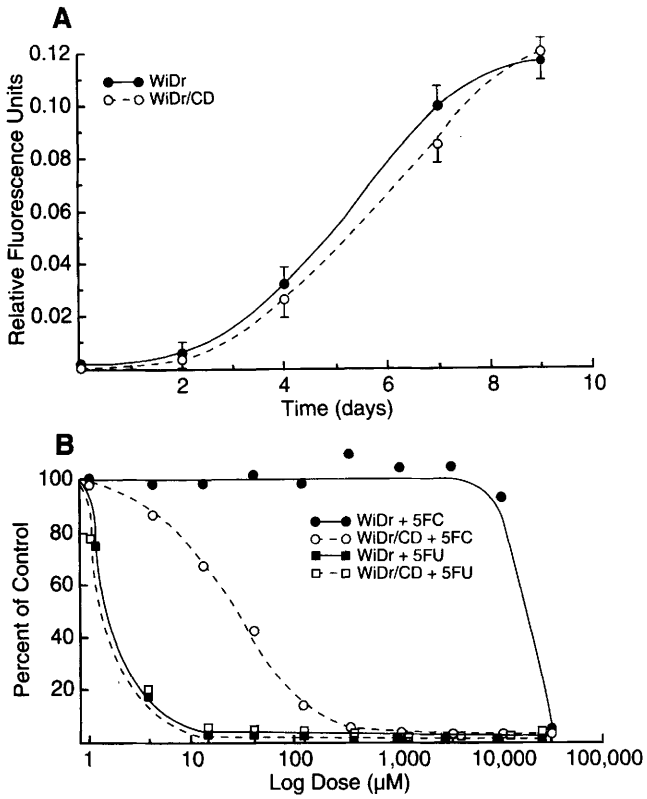

Fig. 1. Cellular growth rate and sensitivity to FCyt (*5FC*) and FUra (*5FU*) in WiDr and WiDr/CD cells. In *A*, control WiDr cells (WiDr) and WiDr cells expressing CD (WiDr/CD) were plated at 3000 cells/well in a 96-well microtiter dish. Growth rate was determined over a 9-day period using a Hoechst dye and quantitating specific fluorescence as described previously (7). Each *point* represents the average ± SE (*bars*) of eight individual determinations. There was no statistical difference between the growth rate of WiDr and WiDr/CD cells. In *B*, log dose-response curves showing the inhibition of growth of WiDr and WiDr/CD cells to various concentrations of FCyt and FUra. Each *point* represents the average of eight individual determinations.

To confirm that the increased sensitivity to FCyt in WiDr/CD was the result of the conversion of FCyt to FUra and subsequent anabolites, WiDr and WiDr/CD cells were cultured for 24 h in the presence of 30 μM [6-^{3}H]FCyt (final specific activity, 166 mCi/mmol). Analysis of cell extracts demonstrated that there was selective conversion of FCyt to FUra anabolites in WiDr/CD cells.[4]

***In Vivo* Toxicity and Pharmacokinetics of FCyt in Nude Mice.** The next series of experiments were designed to determine whether nontoxic plasma levels of FCyt could be obtained which would have significant antitumor effects on tumors expressing CD. Preliminary experiments examined the *in vivo* toxicity and pharmacokinetics of FCyt in nude mice.

Experiments designed to determine the LD_{10}, LD_{20}, LD_{50}, and LD_{90} were performed. FCyt or FUra was administered i.p. or p.o. at varying concentrations on days 1–5 and days 8 and 9. At the highest FCyt dose tested (675 mg FCyt/kg body weight), there was no overt toxicity or weight loss in any of the animals (Table 1). This dose exceeded the solubility limit of FCyt and, as such, FCyt was administered as a suspension. Since FCyt was not lethal over the dose range tested, an estimated LD_{10} could not be determined. To date, approximately 140 mice have received 500 mg FCyt/kg body weight i.p. for 15 to 25 days without obvious signs of drug-related toxicity or significant weight loss. These data are consistent with other reports examining the toxicity of FCyt in other strains and species (13). By

[4] B. E. Huber, E. A. Austin, S. S. Good, and C. E. Richards, submitted for publication.

Table 1 *In vitro and in vivo toxicity of FCyt and FUra*

The IC_{50} for FUra and FCyt in WiDr cells or WiDr cells expressing cytosine deaminase (WiDr/CD). LD in nude mice for i.p. or p.o. FUra and FCyt administered once a day on days 1–5 and 8 and 9. Values are given as mg/kg body weight

Agent	*In vitro* toxicity (IC_{50}; μM)[a]		*In vivo* toxicity (LD)							
	WiDr	WiDr/CD	i.p.				p.o.			
			LD_{10}	LD_{20}	LD_{50}	LD_{90}	LD_{10}	LD_{20}	LD_{50}	LD_{90}
FUra	~5	~5	25	27	33	41	32	35	45	47
FCyt	~26,000	~27	>675[b]				>675			

[a] IC_{50}, that concentration that inhibited growth by 50% compared to untreated control cells; LD, as that concentration (mg/kg body weight) which resulted in the death of 10, 20, 50, or 90% of the treated animals.

[b] FCyt was nontoxic at 675 mg/kg body weight as assessed by animal weight gain and overt signs of toxicity. FCyt was not soluble at doses above 375 mg/kg body weight and, as such, was administered as a suspension.

contrast, FUra was relatively toxic with i.p. and p.o. LD_{10} values of 25 and 32 mg/kg, respectively (Table 1).

The pharmacokinetics of FCyt was investigated in nude mice. Plasma profiles of FCyt in nude mice given multiple i.p. injections or constant tail vein infusions of FCyt were simulated by using a two compartmental model. First order input and zero order input were used in the simulation of i.v. infusion and i.p. administration, respectively. The pharmacokinetic parameters used in the simulations were estimated from fitting of experimental FCyt concentration-time data from nude mice following 500 mg/kg i.p. administration of FCyt. Fig. 2*A* illustrates the average plasma concentration-time profile obtained from 4 mice after a single i.p. dose of 500 mg FCyt/kg. The mean AUC was 3820 μM·hr, the mean maximum plasma concentration achieved was 4133 μM, and the plasma elimination phase half-life was approximately 40 min. Apparent total body clearance for FCyt was estimated to be 1.0 liter/h/kg. Plasma levels of 25 μM or greater were maintained for approximately 4 h after dosing. Plasma profiles of FCyt in nude mice given multiple i.p. injections or constant tail vein infusions of FCyt were simulated by using a two compartmental model. It was estimated that multiple i.p. dosing at 500 mg FCyt/kg body weight every 12 h (twice a day) would yield steady-state peak and trough plasma concentrations of approximately 3900 and 2 μM, respectively (Fig. 2*B*). The corresponding calculations for dosing every 6 h (4 times a day) with 500 mg FCyt/kg body weight were 3900 and 20 μM, respectively (Fig. 2*C*). Hence, there should be no significant accumulation of FCyt but maintenance of plasma levels of greater than 2 and 20 μM would be expected if the drug were to be administered at 500 mg/kg on a twice a day or 4 times a day schedule, respectively.

Since the plasma half-life of FCyt was approximately 40 min, methods for the continual administration of FCyt were investigated. Seven- or 14-day osmotic minipumps (delivering 1 and 0.5 μl/h, respectively) containing 100 mM FCyt were implanted i.p. into nude mice. These pumps should deliver approximately 645 μg FCyt/kg body weight/h for 7 days or 322 μg FCyt/kg body weight/h for 14 days. Following implantation, plasma levels of FCyt were monitored

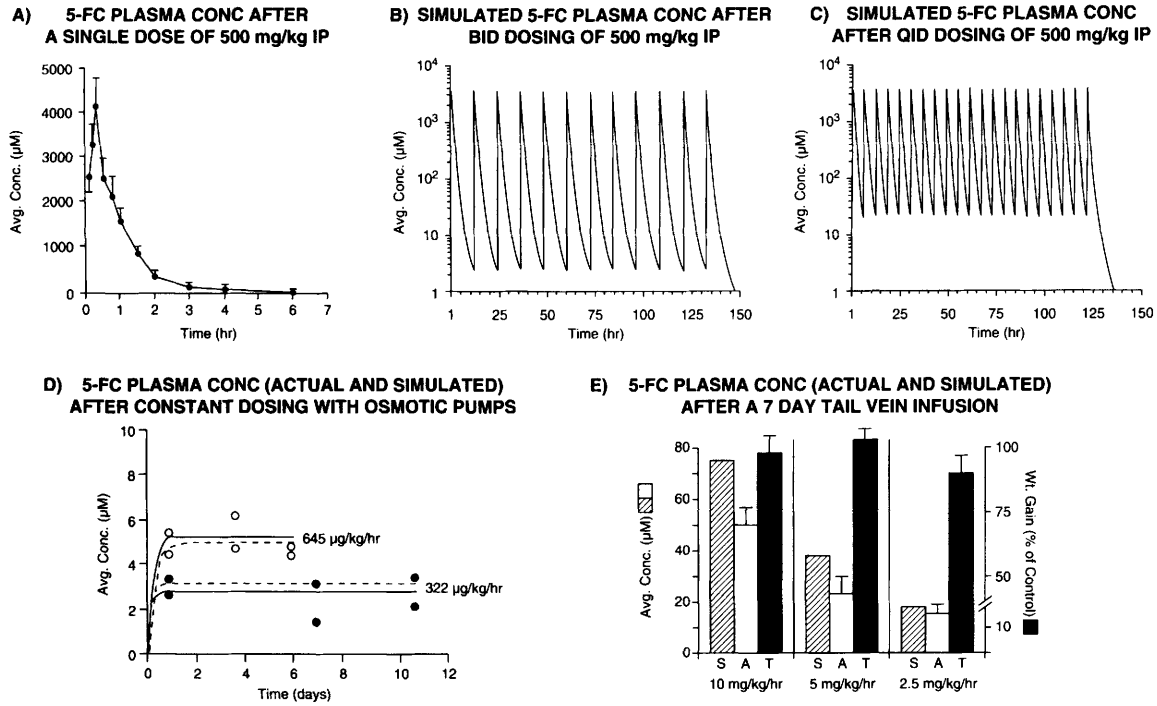

Fig. 2. Pharmacokinetics of FCyt (*5-FC*)in nude mice. In *A*, the plasma concentration time curve for FCyt was determined in nude mice after an i.p. injection of 500 mg/kg body weight. Each *point* represents the average of three individual mice (*bars*, SEM). *B* and *C*, using the pharmacokinetic parameters determined from the data presented in *A*, plasma concentrations of FCyt were computer simulated for twice a day (*bid*) and 4 times a day (*qid*) administration of FCyt. In *D*, 7- and 14-day osmotic minipumps delivering 645 and 322 μg FCyt/kg body weight/h, respectively, were implanted i.p. into nude mice. ○, and ●, actual determinations of FCyt plasma concentrations for the 7- and 14-day pumps, respectively, in individual animals. ——, average plasma concentrations based upon the actual determinations. – – – –, computer-simulated data calculated from the infusion rate of the pumps and the pharmacokinetic parameters determined from the data in *A*. In *E*, FCyt was administered at 10, 5, or 2.5 mg/kg body weight/h by continual tail vein infusions for 7 days. Actual plasma concentrations (*A*; □) and simulated plasma concentrations (*S*; ▨) were determined. Simulated plasma concentrations corresponding to each infusion rate were determined from the pharmacokinetic parameters determined from the data in *A*. ■, estimate of toxicity (*T*) as measured by weight gain of the animals at the end of the infusion, expressed as percentage of control animals receiving no infusion or infusion of vehicle.

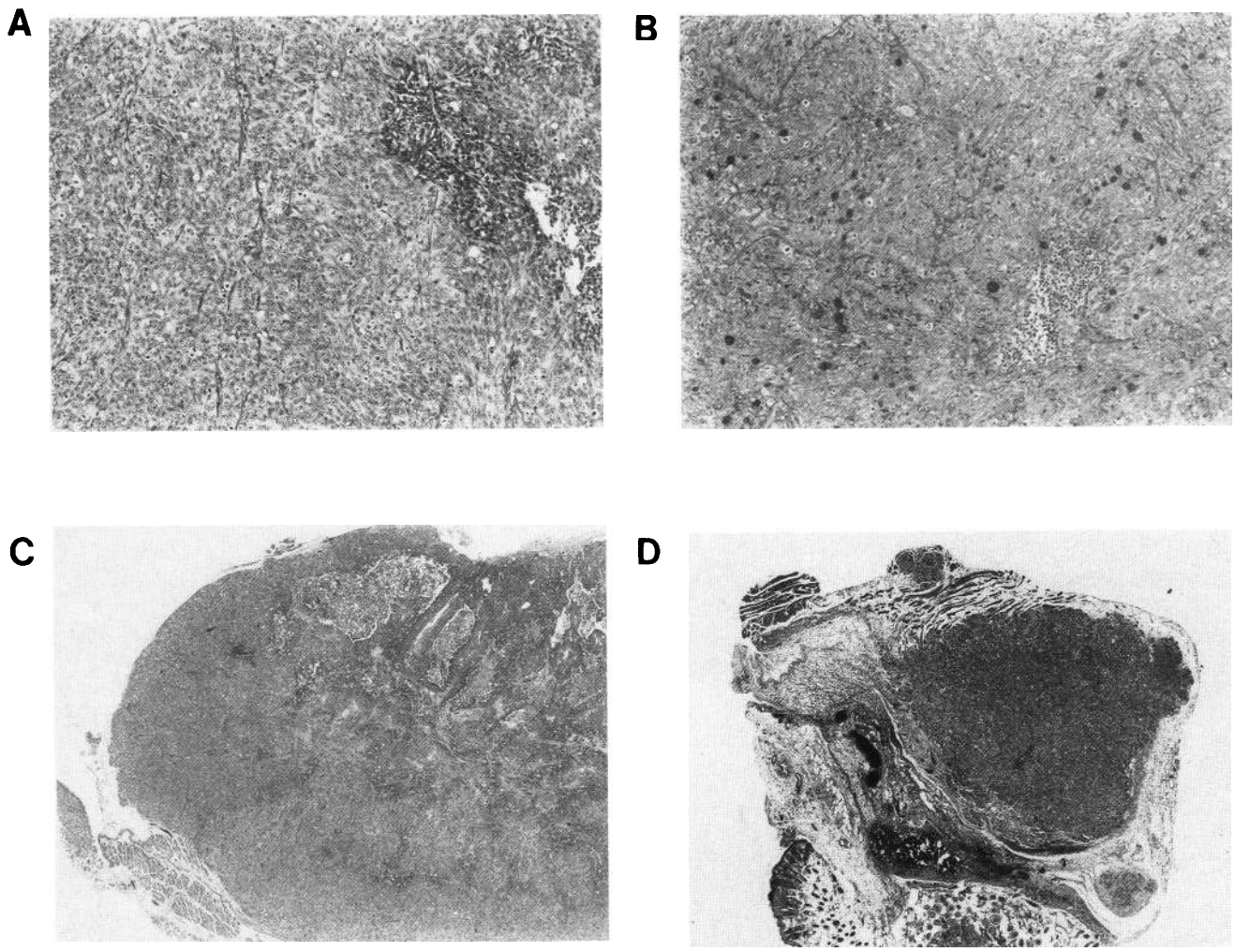

Fig. 3. Tumor histology. *A* and *B*, typical histological architecture of WiDr and WiDr/CD tumors growing s.c. in nude mice. *A*, hematoxylin and eosin staining. × 100. *B*, periodic acid-Schiff staining; 100 × magnification. *C* and *D*, typical histological pattern of WiDr (*C*) and WiDr/CD (*D*) tumors growing s.c. in nude mice on day 24 after i.p. treatment with 500 mg FCyt/kg given intermittently on days 9 through 23. Few necrotic areas are seen in *C* while significant necrotic areas are seen in *D*. × 40.

over time. Pumps delivering 645 and 322 μg FCyt/kg body weight/h produced constant plasma levels of approximately 5 and 2.5 μM, respectively (Fig. 2*D*). These values are identical to the predicted values calculated from the plasma concentration time curve. Administration of FCyt by osmotic minipumps produced no overt signs of toxicity or weight loss.

Since the osmotic minipumps produced relatively low steady-state levels of FCyt, an alternative continual dosing technique was used with a constant 7-day tail vein infusion of FCyt. Infusion rates were 10, 5, or 2.5 mg FCyt/kg body weight/h (Fig. 2*E*). Infusions at these rates produced constant plasma levels of approximately 50, 25, and 14 μM, respectively. These values are slightly less than the predicted values calculated from the infusion rate and the pharmacokinetic parameters determined from the data in Fig. 2*A*. This slight discrepancy may have resulted from the time needed to remove the tail vein infusion apparatus before blood could be drawn for FCyt plasma determinations. There were no overt signs of toxicity or weight loss in any of the animals receiving constant tail vein infusions of FCyt (Fig. 2*E*).

Taken collectively, these data indicate that FCyt is very nontoxic *in vitro* and *in vivo*. No overt toxicity or weight loss was observed when FCyt was administered i.p. or p.o. up to 675 mg/kg for 7 days or by tail vein infusion up to 10 mg/kg/h for 7 days. The half-life of FCyt was calculated to be approximately 40 min in nude mice. After a single i.p. injection of 500 mg FCyt/kg body weight, plasma levels of 25 μM or greater could be maintained for approximately 4 h. Osmotic minipumps implanted i.p. could produce constant plasma levels of 5 and 2.5 μM for 7 and 14 days, respectively, without signs of toxicity. Tail vein infusions of FCyt could attain constant plasma levels of approximately 50 μM for 7 days without signs of toxicity.

***In Vivo* Antitumor Activity of FCyt in WiDr and WiDr/CD Cells.** WiDr and WiDr/CD cells injected s.c. into nude mice form tumors with 100% efficiency (150 tumors/150 injections). Histological evaluation of the tumors revealed typical differentiated, well vascularized adenocarcinomas containing many mitotic figures, few necrotic areas for the size of the tumors, and an overall mature differentiated architecture (Fig. 3*A*). Periodic acid-Schiff staining revealed the presence of many goblet cells which were positive for mucin along with diffuse granules of periodic acid-Schiff-positive material (Fig. 3*B*).

Ten million WiDr or WiDr/CD cells were injected s.c. into the right and left abdominal regions, respectively, of each nude mouse. By day

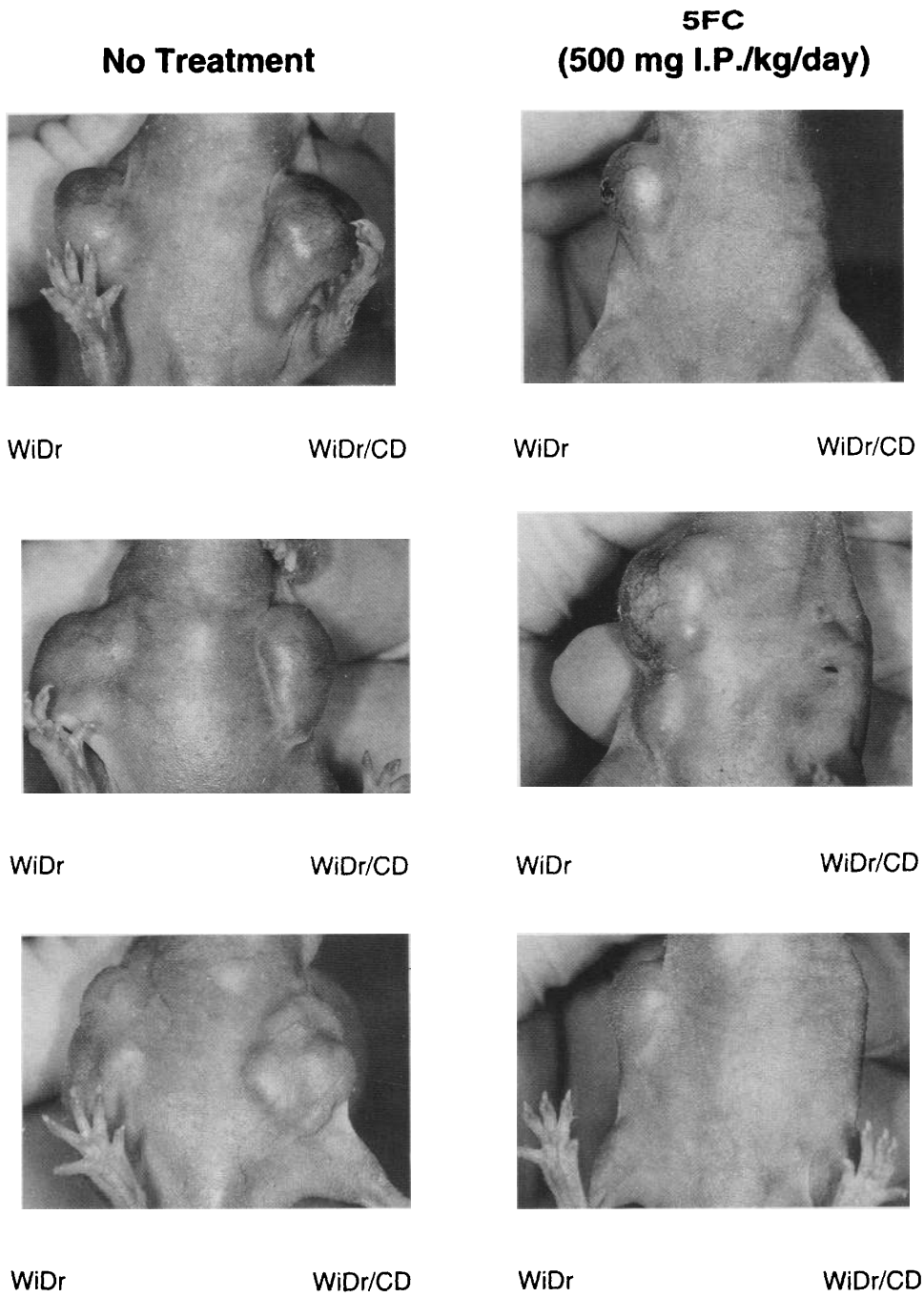

Fig. 4. WiDr and WiDr/CD tumors in nude mice. On day 0, 1×10^7 WiDr or WiDr/CD cells were injected s.c. into the right or left abdominal region, respectively, in nude mice. No treatment was given between day 0 and day 9. On day 9, all tumors weighed approximately 150 mg and the treatment was initiated. On day 9, mice received either no treatment or were treated with either i.p. injections of 500 mg FCyt (*5FC*)/kg body weight or tail vein infusions of 10 mg FCyt/kg body weight/h. Injections of FCyt were administered intermittently i.p. between days 9 and 23 as indicated in Fig. 5. Tail vein infusions of FCyt were administered on days 9 through 16. On day 24, the experiment was terminated due to the size of the control WiDr tumors. Pictured are animals on day 24 which received either no treatment or i.p. administration of 500 mg FCyt/kg body weight. For quantitation of growth, see Fig. 5; for quantitation of day 24 values, see Fig. 6.

9, mice had similar individual tumor weights of approximately 150 mg as determined by tumor size. This tumor burden was approximately 1% of total body weight at the start of treatment and, if extrapolated to a 75-kg human, would represent an approximate 750-g tumor burden. On day 9, mice either received no treatment or were treated with either i.p. injections of 500 mg FCyt/kg body weight or a tail vein infusion of 10 mg FCyt/kg body weight/h (see Fig. 2). Injections i.p. of FCyt were administered intermittently on days 9 through 23 as indicated. Tail vein infusions of FCyt were administered on days 9 through 16. Throughout the course of the experiment, tumor weights were determined based upon tumor size. On day 24, the experiment was terminated due to the size of the control WiDr tumors. At this time, [^{3}H]thymidine was injected i.p. and 2 h later, all animals were killed by anesthesia overdose. Tumor size, tumor weight, animal weight, and thymidine incorporation into tumor DNA were determined.

In untreated animals, tumors generated from injections of WiDr or WiDr/CD cells grew at the same rate throughout the course of the experiment (Figs. 4 and 5*A*). On day 24, there was no significant difference in estimated tumor weight, actual tumor weight, or thymidine incorporation into WiDr tumors compared to WiDr/CD tumors in untreated control animals (Fig. 6). These data are consistent with the *in vitro* growth kinetics presented in Fig. 1*A*.

Profound antitumor effects were observed in tumors generated from WiDr/CD cells when the animals were given FCyt by i.p. injections. This effect was evident in the appearance of the tumors (Fig. 4), the tumor growth rate (Fig. 5*B*), and tumor weights and thymidine incorporation determined on day 24 (Fig. 6). Many WiDr/CD tumors completely regressed and were undetectable on day 24, while others partially regressed or did not increase in weight from day 9 values. Histological evaluation of the WiDr/CD tumor tissue which remained on day 24 in animals treated with i.p. FCyt indicated that this tumor

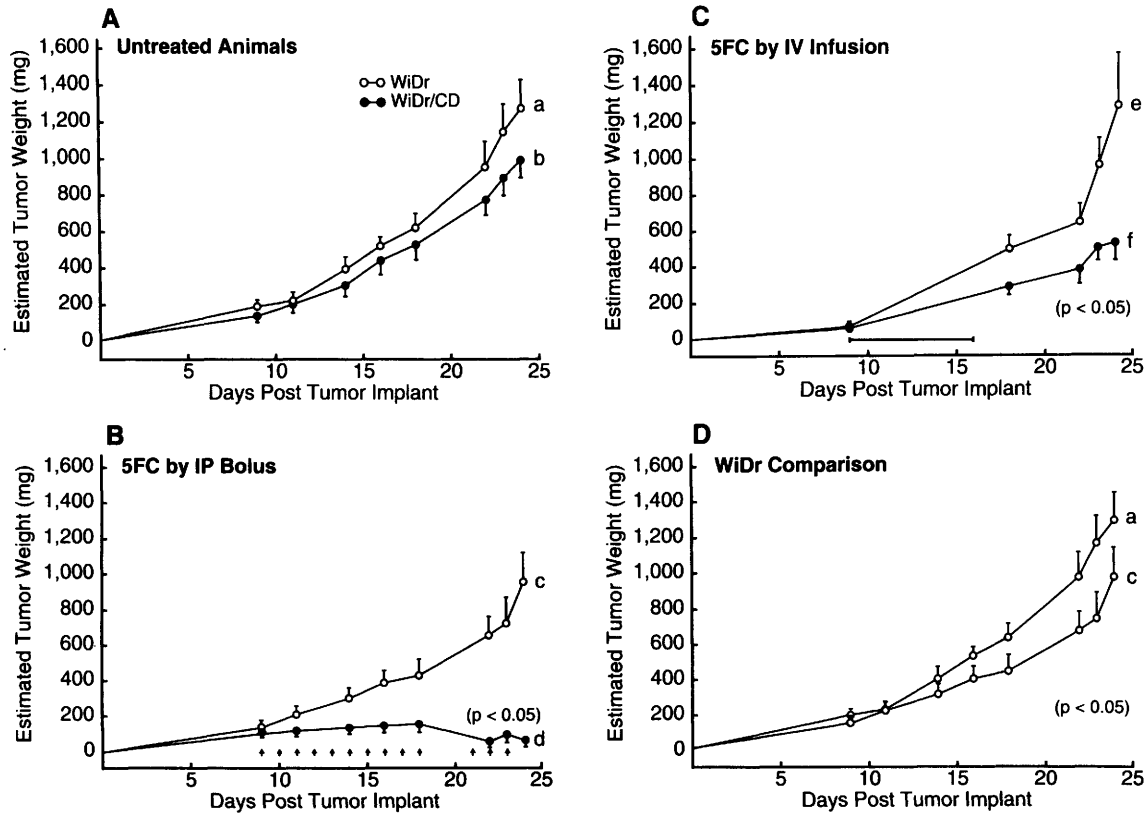

Fig. 5. Tumor growth rate for the tumors described in Fig. 4. *A*, rate of tumor growth for tumors derived from WiDr or WiDr/CD cells growing in right and left contralateral flanks of untreated animals. *B*, rate of tumor growth for tumors derived from WiDr or WiDr/CD cells growing in the right and left contralateral flanks of animals treated with FCyt (*5FC*) at 500 mg/kg i.p. FCyt was administered intermittently between days 9 and 23 (*arrowheads*). *C*, rate of tumor growth for tumors derived from WiDr or WiDr/CD cells growing in right and left contralateral flanks of animals treated by a tail vein infusion of 10 mg FCyt/kg body weight/h. FCyt was continually infused between days 9 through 16 (——). *D*, comparison of *Curve a* in *A* and *Curve c* in *B*. *Curve a*, rate of tumor growth for WiDr-derived tumors in animals receiving no treatment; *Curve c*, rate of tumor growth forWiDr-derived tumors in animals receiving 500 mg FCyt/kg i.p. intermittently between days 9 and 23.

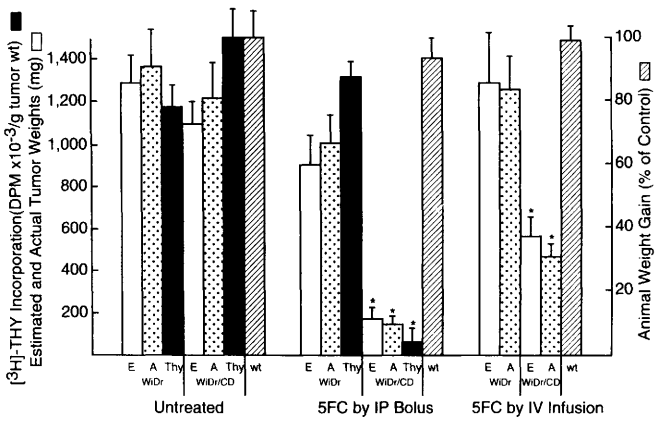

Fig. 6. Day 24 data. Animals bearing WiDr- and WiDr/CD-derived tumors in contralateral flanks were untreated, treated with 500 mg FCyt (*5FC*)/kg body weight i.p., or treated with 10 mg FCyt/kg/h constant tail vein infusion as described in the text and in Figs. 4 and 5. On day 24 post-tumor implantation, the animals were killed and estimated tumor weights (*E*; based on tumor size determinations), actual tumor weights (*A*), thymidine incorporation in tumor DNA (*Thy*) were determined for WiDr- and WiDr/CD-derived tumors in the 3 animal groups. There was no statistical difference in any of these parameters comparing WiDr- and WiDr/CD-derived tumors in untreated animals. There was a statistically significant difference in these parameters comparing WiDr- and WiDr/CD-derived tumors in the animals treated with FCyt by i.p. bolus or iv infusion. Note: thymidine incorporation into tumor DNA was not performed for the i.v. infusion animal group. At the time of sacrifice, animal weights (*wt*) were determined in the 3 groups and are expressed as a percentage of the untreated group. Each determination represents the average of 6 determinations ± SD (*bars*). *Asterisks*, significance ($P < 0.05$) comparing contralateral tumors.

tissue was composed of significant fibrotic and necrotic tissue (Fig. 3*D*). In contrast, adjacent tumors generated from WiDr cells had a histological pattern similar to that of untreated animals with few areas of detectable necrosis (Fig. 3*C*).

Significant but less profound antitumor effects on WiDr/CD tumors were observed if FCyt was administered by constant tail vein infusion. This is illustrated in the tumor growth rate (Fig. 5*C*) and in the tumor weights determined on day 24 (Fig. 6).

Additional observations are illustrated in Fig. 6. (*a*) Day 24 estimated tumor weights calculated by size determinations are statistically the same as day 24 actual tumor weights. These data validate the use of size determination in Fig. 5 to estimate tumor weight. (*b*) there was no apparent drug-related animal toxicity or weight loss in any of the treatment groups.

An additional observation in these series of experiments was that the rate of growth of control WiDr tumors was statistically less in animals treated with FCyt by i.p. injections compared to untreated animals (Fig. 5*D*). There was also a trend in this direction for tumor weights on day 24, but these effects were not statistically significant (Fig. 6). The slight but statistically significant effect on WiDr tumor growth rate illustrated in Fig. 5D could result from either a direct effect of FCyt on control WiDr tumors, an indirect effect caused by significant levels of FUra being produced in WiDr/CD tumors in the contralateral flank, or an immunological effect resulting from the extensive cell killing in WiDr/CD tumors growing in the contralateral flank. To investigate whether FCyt had a direct effect on WiDr-derived tumors, nude mice containing only tumors derived from WiDr cells

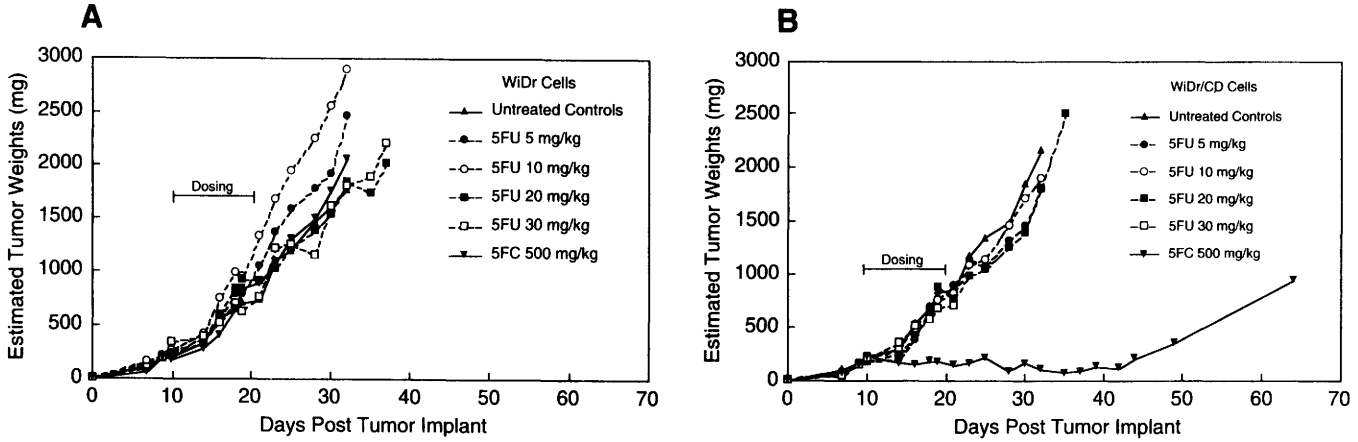

Fig. 7. Comparison of FUra (*5FU*)and FCyt (5FC) antitumor activity targeted to WiDr- and WiDr/CD-derived tumors. Animals were implanted on day 0 with either WiDr (*A*) or WiDr/CD (*B*) cells. On day 9 through 19, animals were either untreated; dosed with 5, 10, 20, or 30 mg FUra/kg; or dosed with 500 mg FCyt/kg. Tumor weight (determined by tumor size) was determined on a daily basis.

were treated with i.p. injections of 500 mg FCyt/kg body weight as described in Fig. 5. No antitumor effects were observed. This indicates that the effects illustrated in Fig. 5*D* are not the result of a direct effect of FCyt on WiDr-derived tumors.

Comparison of the Antitumor Effects of FUra and FCyt. Experiments designed to compare and contrast the *in vivo* antitumor effects of FUra and FCyt in tumors derived from WiDr or WiDr/CD cells were performed. On day 0, nude mice were given a single s.c. injection of either WiDr or WiDr/CD cells. At the start of dosing on day 9, the average tumor weights in all groups were statistically identical (216 ± 19 mg). On day 9, mice were given FUra i.p. at 5, 10, 20, or 30 mg/kg body weight; FCyt i.p. at 500 mg/kg body weight; or were untreated. Mice were dosed once a day for 10 consecutive days and then monitored for an additional 50 days. Tumor weights were determined throughout the course of the experiment.

Doses of FUra ranged from nontoxic doses to the LD_{90}. At 5 and 10 mg FUra/kg, no significant weight loss or drug-related death was observed in any of the animals. At 20 mg FUra/kg, significant weight loss occurred and 1 of 14 animals died a drug-related death. At 30 mg/kg, there was severe weight loss and 13 of 14 animals died a drug-related death.

There was no statistically significant antitumor activity in any of the FUra- or FCyt-treated animals bearing tumors derived from WiDr cells (Fig. 7*A*). Due to the size of the tumors, the FUra-treated mice were terminated between day 30 and day 40 (it should be noted that only one animal survived past day 23 in the treatment regimen of 30 mg FUra/kg).

Likewise, there was no statistically significant antitumor activity in any of the FUra-treated animals growing tumors derived from WiDr/CD cells (Fig. 7*B*; it should be noted that no animals survived past day 21 in the treatment regimen of FUra, 30 mg/kg). However, there was profound antitumor activity in animals treated with 500 mg FCyt/kg for 10 days (Fig. 7*B*). In this treatment group 100% of the tumors regressed. Some WiDr/CD tumors in the FCyt treatment group relapsed between days 40 and 50 but approximately 30% of the tumors appeared to be cured by day 60.

Taken collectively, these data indicate that FUra had no *in vivo* antitumor effect on tumors derived from either WiDr or WiDr/CD cells at doses ranging from nontoxic levels to the LD_{90}. However, both cell lines are very sensitive to the direct effects of FUra *in vitro* as illustrated in Fig. 1*B*. FCyt had profound antitumor effects on tumors derived from WiDr/CD cells at dose levels which were nontoxic systemically. A short dosing period resulted in 100% regressions and some cures. There was relapse in some tumors, most probably resulting from residual viable tumor cells being present after the short dosing period. Additional studies indicated that increasing the dosing period results in an approximate 75% cure rate.[5]

DISCUSSION

CD from *Escherichia coli* was previously cloned, sequenced, and placed into a eukaryotic expression vector system (10). We now report that expression of CD in the human colorectal tumor cell line, WiDr, does not alter the rate of WiDr cell growth when the cells are growing *in vitro* or as a tumor in nude mice. However, CD expression did make the cells very sensitive to the nontoxic prodrug, FCyt, decreasing the IC_{50} from 28,000 μM in parental WiDr cells to 27 μM in WiDr cells expressing CD. This decrease was presumably due to the CD-mediated conversion of FCyt to FUra and subsequent anabolites in WiDr/CD cells.

Next we investigated whether the increased *in vitro* sensitivity of WiDr/CD cells to FCyt could be demonstrated *in vivo* by achieving sufficient nontoxic plasma levels of FCyt to have significant antitumor effects on WiDr/CD-derived tumors. Preliminary experiments determined the *in vivo* toxicity and pharmacokinetics of FCyt in nude mice. FCyt was very nontoxic in nude mice. Administration of 675 mg FCyt/kg body weight i.p. or p.o. for 7 days produced no weight loss or other overt signs of toxicity. Likewise, a constant tail vein infusion of 10 mg FCyt/kg body weight/h for 7 consecutive days produced no overt signs of toxicity or weight loss. These data are consistent with other reports indicating that the IC_{50}s for FCyt administered p.o. or s.c. are >2000 and 1190 mg/kg body weight, respectively, in immunocompetent mice (13).

The plasma half-life of FCyt was estimated to be approximately 40 min. Following an i.p. dose of 500 mg FCyt/kg body weight, the maximum FCyt plasma concentration was approximately 4100 μM, and the AUC was 3820 μM·h. Due to the short half-life, different dosing strategies of FCyt were explored to achieve optimal plasma drug concentrations. Using i.p.-implanted osmotic minipumps, the maximal steady-state plasma FCyt concentration achieved was 5 μM for 7 consecutive days. This was below the IC_{50} estimated from the *in vitro* toxicity data (Fig. 1). A constant FCyt plasma level of ap-

[5] Manuscript in preparation.

proximately 50 μM was achieved using a constant, 7-day tail vein infusion. This level was above the *in vitro* IC_{50} value for FCyt in WiDr/CD cells.

Based on the pharmacokinetics of FCyt, initial antitumor studies were performed in mice bearing adjacent WiDr and WiDr/CD tumors by administering FCyt by either daily i.p. injections or by constant tail vein infusions. Administration of FCyt i.p. was more efficacious than constant tail vein infusions of FCyt (Figs. 5 and 6). In mice receiving FCyt by i.p. injections, the rate of WiDr/CD tumor growth was profoundly decreased, with some tumors showing complete remission. FCyt administered by constant tail vein infusion produced a significant but less dramatic antitumor effect compared to daily i.p. dosing. The difference in antitumor efficacy between i.p. injections and tail vein infusion of FCyt most probably reflects the large differences in the maximal plasma levels of FCyt achieved by the two treatment regimens since there is only a 2-fold difference in total daily dose and only a 3-fold difference in total daily exposure (AUC) to FCyt by the two treatment regimens. These observations suggest that transient, high plasma levels of FCyt are more efficacious than constant levels of FCyt of approximately 50 μM. It should be noted, however, that the optimal administration of FCyt regarding the minimal dose and time to achieve maximal antitumor efficacy is still under investigation. In these studies, an i.p. bolus of 500 mg FCyt/kg was administered, but this may be significantly higher than what is required to achieve maximal efficacy.

Another interesting and unexpected observation was the fact that adjacent WiDr control tumors grew at a slight but statistically significant lower rate in animals given FCyt i.p. compared to untreated control animals (Fig. 5*D*). This effect on tumor growth rate could result from either a direct antitumor effect of FCyt on WiDr control tumors, an indirect effect caused by significant levels of FUra being produced in WiDr/CD-derived tumors growing in the contralateral flank, or an immunological effect resulting from the extensive cell killing in WiDr/CD-derived tumors growing in the contralateral flank. It is very unlikely that this slight, but significant, effect on the growth rate of WiDr tumors results from either the direct effects of FCyt or the indirect effects of FUra production in adjacent WiDr/CD tumors. No antitumor effects were observed in animals containing only WiDr tumors when treated with 500 mg FCyt/kg body weight for 10 consecutive days. In addition, levels of circulating FUra could not be obtained *in vivo* which produced significant antitumor activity on either WiDr or WiDr/CD tumors. Hence, it seems reasonable to hypothesize that this slight, but significant, antitumor effect on WiDr tumor growth rate may have resulted from an immunological effect. This effect may be initiated by the profound tumor cell death in the adjacent WiDr/CD-derived tumors. Athymic nude mice do contain natural killer cells and other immunological functions (14). It is possible that the slight, but significant, antitumor effect on adjacent WiDr tumor growth rate may be mediated by natural killer cells. It remains to be determined if this slight effect has any biological significance in tumor cell killing.

These data demonstrate that nontoxic, *in vivo* plasma levels of FCyt can be obtained that produce profound antitumor effects on tumors expressing CD. Most importantly, these data demonstrate that 100% tumor regressions and some tumor cures can be produced without systemic toxicity by a short treatment course of FCyt. Subsequent experiments have shown that by extending the course of treatment for 9 additional days, it is possible to achieve an approximate 100% regression rate and a 75% cure rate.[5] This is most significant since *in vivo* plasma levels of directly administered FUra cannot be achieved which have any antitumor effects on WiDr- or WiDr/CD-derived tumors, despite the fact that WiDr and WiDr/CD cells are more sensitive to FUra than FCyt *in vitro*. These data indicate that if the gene for CD can be delivered and expressed in tumor cells, then these tumor cells will selectively convert FCyt to FUra, resulting in tumor cell death without systemic toxicity. Current efforts are focused on determination of percentage of tumor cells requiring CD expression for tumor regression and different methods for gene delivery into solid tumors. Taken collectively, these observations support the further development of the virus-directed enzyme/prodrug therapy gene therapy approach for cancer therapy. The introduction of qualitative biochemical differences between normal and neoplastic cells by gene therapy may offer new avenues of safe and efficacious cancer therapy.

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REFERENCES

1. Mandell, G. L., and Sande, M. A. Antimicrobial agents: penicillins and cephalosporins, *In:* L. S. Goodman and A. G. Gilmans (eds.), The Pharmacological Basis of Therapeutics Ed. 6, pp. 1126–1161. New York: MacMillian Publishing Co., 1980.
2. Whitley, R., and Gnann, J. W., Jr. Acyclovir: a decade later. N. Engl. J. Med., *327:* 782–789, 1992.
3. Ashe, W. D., Jr., and Van Reken, D. E. 5-Fluorocytosine: a brief review. Clin. Pediatr., *16:* 364–366, 1977.
4. Gunderson, L. L., Beart, R. W., and O'Connell, M. J. Current issues in the treatment of colorectal cancer. CRC Crit. Rev. Oncol. Hematol., *6:* 223–260.
5. Abbruzzese, J. L., and Levin, B. Treatment of advanced colorectal cancer. Hematol. Oncol. Clin. North Am., *3:* 135–153, 1989.
6. Bleiberg, H. Treatment of advanced colorectal cancer. Anticancer Res., *9:* 1013–1015, 1989.
7. Huber, B. E., Richards, C. A., and Krenitsky, T. A. Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: an innovative approach for cancer therapy. Proc. Natl. Acad. Sci. USA, *88:* 8039–8043, 1991.
8. Richards, C. A., and Huber, B. E. Generation of a transgenic model for retrovirus-mediated gene therapy for hepatocellular carcinoma is thwarted by the lack of transgene expression. Hum. Gene Ther., *4:* 143–150, 1993.
9. Austin, E. A., and Huber, B. E. A first step in the development of gene therapy for colorectal carcinoma: cloning, sequencing, and expression of *E. coli* cytosine deaminase. Mol. Pharmacol., *43:* 380–387, 1993.
10. Marquardt, D. W., and Siam, J. An algorithm for least square estimation of non-linear parameters. J. Soc. Indust. Appl. Math., *11:* 431–441, 1963.
11. Huber, B. E., and Brown, N. A. 12-*O*-Tetradecanoylphorbol-13-acetate actions on macromolecular synthesis, ornithine decarboxylase, and cellular differentiation of the rat embryonic visceral yolk sac in culture. Cancer Res., *43:* 5552–5559, 1983.
12. Guide for the Care and Use of Laboratory Animals. Revised 1985, prepared by the Committee on the Care and Use of Laboratory Animals, United States Department of Health and Human Services. NIH Publications No. 86–23. Washington, DC: United States Government Printing Office, 1988.
13. Windholz, M. (ed.). The Merck Index, Ed. 9, p. 534. Rahway, NJ: Merck and Co., Inc., 1976.
14. Andriole, G. L., Mule, J. J., Hasen, C. T., Linehan, W. M., and Rosenberg, S. A. Evidence that lymphokine-activated killer cells and natural killer cells are distinct based on an analysis of congenitally immunodeficient mice. J. Immunol., *135:* 2911–2913, 1985.