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Retroviral Coexpression of Thymidylate Synthase and Dihydrofolate Reductase Confers Fluoropyrimidine and Antifolate Resistance

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Retroviral gene transfer of dominant selectable markers into hematopoietic cells can be used to select genetically modified cells *in vivo* or to attenuate the toxic effects of chemotherapeutic agents. We show that retroviral gene transfer of thymidylate synthase (TS) confers resistance to TS directed anticancer agents and that co-expression of TS and dihydrofolate reductase (DHFR) confers resistance to TS and DHFR cytotoxic agents. Retroviral vectors encoding *Escherichia coli* TS, human TS, and the Tyr-to-His at residue 33 variant of human TS (Y33HhTS) were constructed and fibroblasts transfected with these vectors conferred comparable resistance to the TS-directed agent fluorodeoxyuridine (FdUrd, approximately 4-fold). Retroviral vectors that encode dual expression of Y33HhTS and the human L22Y DHFR (L22YhDHFR) variants conferred resistance to FdUrd (3- to 5-fold) and trimetrexate (30- to 140-fold). A L22YhDHFR-Y33HhTS chimeric retroviral vector was also constructed and transduced cells were resistant to FdUrd (3-fold), AG337 (3-fold), trimetrexate (100-fold) and methotrexate (5-fold). These results show that recombinant retroviruses can be used to transfer the cDNA that encodes both TS and DHFR and dual expression in transduced cells is sufficiently high to confer resistance to TS and DHFR directed anticancer agents.

The transfer of genes that confer drug resistance into hematopoietic cells can be utilized in two gene therapy settings. First, it has been proposed that drug resistant bone marrow may increase the therapeutic index of antineoplastic agents by allowing more aggressive dosing of chemotherapy without the complications of myelosuppression. To date, the most well characterized chemoprotecting agents are p-glycoprotein (MDR-1) (1-3) and dihydrofolate reductase (DHFR) (4-7), and gene transfer of MDR-1 into hematopoietic cells to attenuate myelosuppression has advanced to clinical trials (8-10). Second, drug resistance genes can function as dominant selectable markers and allow for the selection and expansion of genetically modified cells (1,6). In light of results from clinical trials which routinely show low level transduction of cells capable of hematopoietic reconstitution, *in vivo* or *ex vivo* selection and expansion of genetically modified cells may be necessary for the successful application of gene therapy for hematopoietic disorders (11-14).

Knowledge of the molecular mechanisms that result in drug resistance allow for the predictive use of chemoprotective genes other than MDR-1 and DHFR. Resistance to nitrosoureas was achieved by retroviral transfer of the cDNA that encodes the bacterial ada or human methylguanine methyltransferase enzymes (15,16) and gene transfer of the aldehyde dehydrogenase cDNA resulted in resistance against DNA alkylating agents (17-19). There is, however, still a need to adapt such technology to the attenuation of side effects of other antineoplastic agents.

The intracellular requirement of TMP for cell division has made thymidylate synthase (TS) a logical target for chemotherapeutic drug design, and TS currently holds prominence as a target for the rational design of new antifolates for cancer chemotherapy. TS is an excellent candidate for drug resistance gene therapy because: 1) it catalyzes a critical reaction in the *de novo* synthesis of TMP (reviewed in 20), 2) the recombinant enzyme has been well characterized (20,21), 3) variants that confer *in vivo* drug resistance have been identified (22,23), 4) TS consists of two identical subunits, each having a molecular weight of approximately 35,000 and, therefore, the cDNA that encodes TS is relatively small (<1,000 bp) (24), and 5) it is the target of mainstream and novel anticancer agents (25). Inhibitors of TS continue to be created and have demonstrated significant activity in clinical trials against several types of cancer. Myelosuppressive side effects encountered in

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clinical trials utilizing novel TS inhibitors also demonstrate that TS is a rational choice as a chemoprotective agent in retroviral gene transfer investigations (26,27).

By constructing a series of retroviral vectors we show that enforced overexpression of TS confers resistance to TS directed cytotoxic agents. Resistance was conferred to both nucleotide and antifolate analogs. Dual expression of TS and DHFR employing separate promoters or by a single promoter expressing a DHFR-TS fusion product confers resistance to both TS and DHFR directed antineoplastic agents. The DHFR-TS chimera conferred nucleotide and antifolate resistance that was equal to resistance conferred by vectors encoding the two enzymes separately. Our results show that TS can serve as a chemoprotecting agent and as a selectable marker for gene therapy.

MATERIALS AND METHODS

Retroviral vector cloning. The cDNA that encodes human TS and the Y33H human TS variant was cloned into the *Sac* II/*Xho* I sites of the Harvey murine sarcoma-based retroviral vector (HaMSV), described previously (5,28), generating hTS/Neo and Y33HhTS/Neo. The Y33HhTS cDNA and *E. coli* TS genes were cloned downstream of the murine CMV promoter (a gift from Franklin Berger, University of South Carolina, SC) and the β-actin promoter (a gift from Paul Ney, St. Jude Children's Research Hospital, TN). These constructs were inserted into the *Stu* I/*Age* I sites HaMSV generating L22YhDHFR-CMV/Y33HhTS, L22YhDHFR-βact/Y33HhTS and L22YhDHFR-CMV/ecTS. The Y33HhTS cDNA was inserted into the MDR-1 site of the previously described DHFRiresMDR-1 bicistronic retroviral vector generating DHFRiresY33HhTS. A chimeric DHFR-TS protein was designed by joining the cDNAs for L22YhDHFR and Y33HhTS with a 75 bp linker sequence. This construct was made by PCR amplification of the L22YhDHFR cDNA introducing a 5′ *Cla* I site and removing the termination sequence by introducing a 3′ *Eco* RV site. The PCR product was ligated in frame between the CMV promoter and Y33HhTS into a Bluescript construct containing CMV-Y33HhTS. The chimeric construct was shuttled into the cloning vector pLITMUS (New England Biolab, Beverly, MA) and inserted into the *Sac* II/*Age* I sites of the HaMSV.

Cell lines and culture. All cells were grown in DMEM (Sigma, St. Louis, MO) containing L-glutamine (4 mmol/L), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (Gibco Co., Gaithersburg, MD). Plasmids containing retroviral vectors were introduced into the murine fibroblast amphotropic packaging cell line, PA317 (29), using a commercially available kit for calcium phosphate transfections (Stratagene, La Jolla, CA). Forty eight hours after transfection, cells were selected with 300 nM trimetrexate (TMTX) (Spencer *et al.*, 1996 a,b) and 5μM dipyridamole (Sigma, St. Louis, MO). Ten days after applying selection, viable colonies were replated at a density of 1×10^6 cells/100 mm culture dish. Near confluent dishes were frozen as primary stocks.

Ecotropic producer cells were generated by transducing, in the presence of 6 μg/ml polybrene (Sigma, St. Louis MO), the packaging cell line GP+E86 with viral containing supernatant from transfected and TMTX selected PA317 cells (30). Transduced cells were selected in 300 nM TMTX and 5 μM dipyridamole and primary polyclonal cell populations were stored as primary stocks.

Survival curves. Drug containing medium was added at varying concentrations twenty-four hours after plating 1.5×10^4 cells in a 6-well culture dish. Six days post-plating, the cells were stained for 3 hours with 1 mg/mL XTT (Sigma, St. Louis, MO) and 16 μM phenazine methyl sulfate (Sigma, St. Louis, MO) in serum free DMEM. The reaction was terminated by addition of SDS to 1% final concentration. The plates were read at 450 nm on a Dynatech Laboratories, Inc. MR650 plate reader. Cell survival was calculated relative to the growth of cells in the absence of drug (31).

Proton NMR spectral analysis. Transduced and untransduced GP+E86 cells were grown as described above but in the presence and absence of 1.5 μM AG337 and 5 μM dipyridamole. NMR spectra were obtained as described by Blankenburg *et al.*, 1996 (32). After four days 5×10^7 cells were washed 3 times in deuterated phosphate buffered saline (PBS), resuspended in 600 μL deuterated PBS and placed on ice. NMR spectroscopy was performed at 18°C on a Varian Mercury 400 MHz spectrometer equipped with a 5 mm probe using parameters adapted from Blankenburg *et al.* 1996 (90° tip angle, 10 second recycle time, 64 scans, 8k points, 5 kHz spectral window, presaturation of residual water protons, 20 Hz spin). A concentrically mounted capillary filled with 0.1% 3-(trimethysilyl)propionic acid served as the zero ppm reference for each experiment. The methylene and methyl peaks at 1.3 and 0.9 ppm, respectively, were deconvoluted and integrated using programs supplied with the Varian spectrometer.

Southern and Western analysis. Southern analysis was performed by trypsinizing a near-confluent 100 mm culture dish, pelleting the cells by centrifugation, and incubating the cells overnight at 37°C in 100 mM NaCl, 10 mM EDTA, 50 mM Tris (pH 8.0), 1% SDS, and 0.6 mg/mL proteinase K. DNA was extracted with phenol and chloroform, precipitated in ethanol and resuspended in 10 mM Tris (pH 8.0), 1 mM EDTA and the concentration was determined spectrophotometrically. Genomic DNA (10 μg) was digested for 17 hours with *Nhe* I, separated on an 0.8% agarose gel, transferred to a nitrocellouse membrane and probed with full length L22Y hDHFR cDNA.

Western analysis was performed on protein extracts by trypsinizing a near-confluent 100 mm culture dish, pelleting the cells by centrifugation, resuspending the cells in 50 mM (Tris pH 8.0), 10 mM 2-mercaptoethanol and performing 3 freeze/thaw cycles. Cell debris was removed by centrifugation, the extract was collected, and stored at −70°C. Protein concentrations were determined using the Biorad protein assay with bovine serum albumin as the standard. Protein (10 μg) was separated on a 10% SDS-PAGE gel and transferred electrophoretically to a nylon membrane (Hybond, Amersham). The membrane was blocked for 1 hr at 37°C in 5% evaporated milk and incubated with the primary monoclonal antibody specific for human thymidylate synthase at 37°C for 2 hrs. The membrane was washed, incubated with a 1:1000 dilution of goat anti-mouse secondary antibody (Bio Rad, Hercules, CA) for 1 hr at 37°C, washed with PBS, 0.1% Tween 20 and incubated with ECL solutions according to the manufacturer's protocol (Amersham).

RESULTS

Retroviral Vectors

The cDNA that encodes wild-type human TS (hTS) or a Y33H human TS variant (Y33HhTS) was inserted into the Harvey murine sarcoma virus (HaMSV) vector with TS expression under the control of the HaMSV promoter, generating the constructs hTS/Neo and Y33HhTS/Neo (see Figure 1 for schematic depicting all retroviral constructs). The Y33HhTS variant was previously shown to have a 4-fold lower affinity for FdUMP compared to that of the wild-type enzyme (22,23). Expression of Neo by a downstream thymidine kinase promoter allows G418 selection of transduced cells. Constructs that express both TS and the L22Y

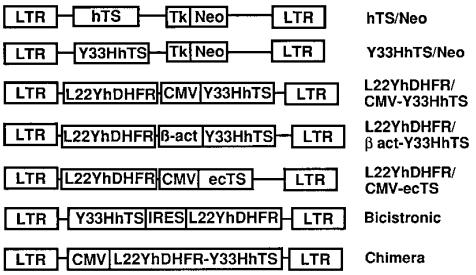

FIG. 1. Structures of retroviral vectors that encode variants of TS. Each vector shown was transfected into the amphotropic packaging cell line PA317 and the ability of these vectors to confer drug resistance to TS or DHFR targeted inhibitors was determined. L22YhDHFR is a variant of human DHFR previously shown to confer antifolate resistance and Y33HhTS is a variant of human TS previously shown to confer FdUrd resistance.

human DHFR variant (L22YhDHFR) utilize the viral promoter for L22YhDHFR expression and internal promoters (CMV or β-actin) to drive either TS or L22YhDHFR-Y33HhTS chimera expression. L22YhDHFR is a human DHFR variant that has been well characterized with respect to its kinetic properties and ability to confer TMTX and methotrexate (MTX) resistance (5,33). Expression of the bicistronic vector is under the control of the HaMSV promoter.

Transfection of Murine Fibroblast Cells

To determine if enforced expression of TS confers resistance to TS targeted chemotherapeutic agents, cell culture techniques employed previously to investigate the degree of drug resistance conferred by transfection of chemoprotectant genes were used (5,28). Plasmid DNA constructs were transfected into PA317 murine fibroblast packaging cells and, depending on the transfected construct, selected with either G418 or TMTX. Polyclonal cell populations that survived selection were assayed for their ability to confer resistance to fluorodeoxyuridine (FdUrd) or TMTX. Cells transfected with hTS/Neo or Y33HhTS/Neo conferred approximately 4-fold resistance to FdUrd (Figure 2, Table 1). The retroviral constructs L22YhDHFR/CMV-Y33HhTS, L22YhDHFR/β-act-Y33HhTS and L22YhDHFR/CMV-ecTS were used to compare the effectiveness of two internal promoters and to determine if TS enzymes derived from different species confer differing degrees of resistance. Expression of Y33HhTS from the CMV promoter showed greater FdUrd resistance compared with expression from the HaMSV or β-actin promoters (Figure 2, Table 1). Similar resistance to FdUrd and AG337 (approximately 4-fold for both inhibitors, AG337 data not shown) was conferred by CMV promoted expression of Y33HhTS and wild-type ecTS, indicating neither construct is substantially more effective. Cells transfected with vectors that drive L22YhDHFR expression from the HaMSV promoter showed comparable resistance to TMTX, and the degree of resistance was similar to that previously reported (5). As controls, transfected cells that overexpress L22YhDHFR did not provide protection against TS directed inhibitors (FdUrd or AG337) and cells transfected with either hTS/Neo or Y33HhTS/Neo were not protected against DHFR directed inhibitors (MTX or TMTX, data not shown).

Utilization of an internal ribosomal entry sequence for the expression of both Y33HhTS and L22YhDHFR driven by the HaMSV promoter conferred resistance to FdUrd that is comparable to the degree of resistance conferred by Y33HhTS/Neo, which also expresses Y33HhTS by the HaMSV promoter. However, resistance to TMTX is 3-4 fold lower compared to HaMSV promoted L22YhDHFR constructs (Figure 2, Table 1). Transfection of cells with the L22YhDHFR-Y33HhTS chimera vector also conferred resistance to FdUrd and TMTX (Figure 2, Table 1). This vector consists of a 1575 bp reading frame that encodes a 525 amino acid chimeric protein with 25 amino acid residues linking the carboxy terminus of L22YhDHFR and the amino terminus of Y33HhTS. An internal CMV promoter was utilized to drive the expression of this construct. Resistance conferred by this vector is comparable to resistance conferred by vectors expressing the two enzymes separately (Figure 2, Table 1).

Transduction of Murine Fibroblast Cells

Murine fibroblast cells were transduced with recombinant retroviruses that encode L22YhDHFR and Y33HhTS separately (L22YhDHFR/CMV-Y33HhTS) or as the chimeric enzyme. Polyclonal populations of cells selected with 300 nm TMTX and expressing the enzyme activities separately or as the chimeric protein

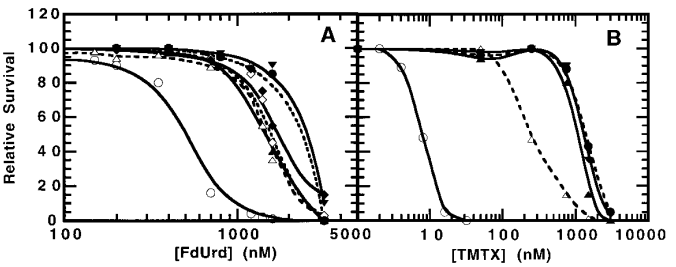

FIG. 2. Protection of murine fibroblast cells. Retroviral vectors shown in Figure 1 were transfected into PA317 cells and selected with either G418 or TMTX. After selection, cells were plated in various concentrations of FdUrd (A) or TMTX (B) and the relative survival at each drug concentration was determined based on cells grown in the absence of drug. Each line represents the average of 3 experiments. Error bars are omitted for clarity (see Table 1 for the standard error of the mean of the IC_{50} value for each construct). In each panel survival of untransfected PA317 cells, and cells transfected with Y33HhTS/Neo, L22YhDHFR/CMV-Y33HhTS, L22YhDHFR/β-act Y33HhTS, L22YhDHFR/CMV-ecTS, bicistronic or CMV-chimera is represented by ○, ♦, ●, ▲, ▼, △, ◇, respectively.

TABLE 1

Resistance Conferred to Transfected Fibroblast Cells by Retroviral Constructs Encoding Variants of Human TS and DHFR

Construct	IC_{50} FdUrd/FA[a] (nM)	Fold increase[b]	TMTX[c] (nM)	Fold increase[b]
PA317 cells	450 ± 30	—	8 ± 2	—
hTS/Neo	1580 ± 90	3.5	—	—
H33YhTS/Neo	1710 ± 120	3.8	—	—
L22YhDHFR CMV-Y33HhTS	2430 ± 190	5.4	1150 ± 20	140
L22YDHFR βact-Y33HhTS	1350 ± 160	3.0	870 ± 25	110
L22YhDHFR CMV-ecTS	2450 ± 180	5.4	1000 ± 20	120
Bicistronic	1400 ± 80	3.1	240 ± 10	30
Chimera	1800 ± 250	4.0	N.D.	N.D.

[a] Growth inhibition in the presence of FdUrd and 10 μM folinic acid (FA).
[b] Fold increase is relative to the IC_{50} of untransfected PA317 cells.
[c] Growth inhibition in the presence of antifolate and 5 μM dipyridamole. N.D. Not determined.

were equally resistant to the TS targeted inhibitors FdUrd and AG337 and to the DHFR targeted inhibitors MTX and TMTX (Figure 3, Table 2). To further confirm that protection against TS targeted inhibitors is conferred by retroviral gene transfer of the chimeric construct, we used a technique developed by Blankenburg *et al.* (32) that utilizes proton NMR analysis of untreated and drug treated cells. Comparison of NMR data collected for untransduced and transduced cells grown in approximately 4 times the AG337 IC_{50} for untransduced cells is shown in Figure 4. The ratio of the methylene peak at 1.3 ppm to the methyl peak at 0.9 ppm was previously shown to be a quantitative measure of apoptotic cell death. Ratios of the methylene peak areas to methyl peak areas were calculated from NMR spectra as described in the *Materials and Methods* section. In the absence of AG337 untransduced and transduced cells have similar methylene to methyl peak ratios of 0.8:1.0 and 1.0:1.0, respectively. When cells are challenged with AG337, this ratio increases to 2.6:1.0 for untransduced cells whereas the ratio for transduced cells remains virtually unchanged at 1.2:1.0, indicating protection against mechanisms leading to apoptotic cell death.

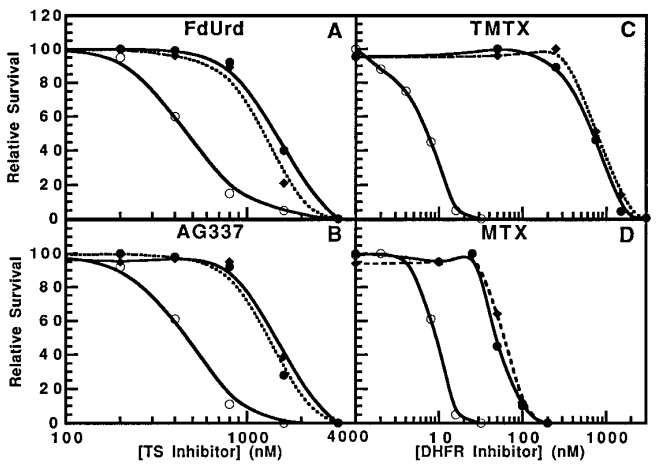

FIG. 3. Retroviral co-expression of Y33HhTS and L22YhDHFR enzyme activities confers resistance to TS and DHFR targeted inhibitors. Polyclonal populations of transduced GP+E86 cells were grown in various concentrations of the TS inhibitors FdUrd (A) and AG337 (B) or the DHFR inhibitors TMTX (C) and MTX (D). Open circles represent data from nontransduced cells, solid circles represent chimera transduced cells and solid diamonds represent L22YhDHFR/CMV-Y33HhTS transduced cells. The percent of surviving cells is relative to the number of cells growing in the absence of drug.

Proviral Integrity and Viral Derived Protein

It is essential to confirm the integrity of the proviral sequence of transduced cells because the cDNA region that encodes the amino terminal residues of human TS contains a GC rich region and several tandem repeats. Southern analysis of genomic DNA digested with *Nhe* I, which cleaves once within each LTR, showed that a single band is released for L22YhDHFR/CMV-Y33HhTS (6.0 kb) and for chimera transduced cells (5.7 kb), verifying that the proviral sequence integrated without evidence of rearragements (Figure 5 A). The expression of TS and the chimeric construct was demonstrated by Western analysis using a monoclonal antibody to human TS (Figure 5B). The predicted molecular weight of the L22YhDHFR-Y33HhTS chimera is 58 kDa (20 kDa from L22YhDHFR, 3 kDa from the linker sequence, and 35 kDa from Y33HhTS) and a band migrating at this approximate size is observed for cells transduced with retroviruses encoding the chimeric enzyme. A 35 kDa band is observed in cells transduced with the L22YhDHFR/CMV-Y33HhTS construct.

DISCUSSION

Retroviral transfer and expression of chemoprotecting genes have the potential to provide significant ad-

TABLE 2

Resistance of Murine Fibroblast Cells Transduced with Retroviruses That Encode Both DHFR and TS Enzyme Activities

Construct	IC_{50}							
	FdUrd/FA[a] (nM)	Fold[b] increase	AG337[c] (nM)	Fold[b] increase	MTX[c] (nM)	Fold[b] increase	TMTX[c] (nM)	Fold[b] increase
GP+E86	400 ± 20	—	450 ± 40	—	9 ± 0.8	—	7 ± 0.5	—
L22YhDHFR CMV-Y33HhTS	1280 ± 80	3.2	1400 ± 60	3.1	65 ± 7	7	760 ± 20	110
Chimera	1440 ± 50	3.6	1370 ± 50	3.0	45 ± 3	5	700 ± 15	100

[a,b,c] See Table 1 legend.

vancements in gene therapy for the treatment of hematopoietic disorders. For example, calculations by Blau *et al.*, 1996 (34), demonstrate that if bone marrow can be transduced with a gene that confers resistance to 5-fluorouracil (5-FU), a single cycle of 5-FU administered after cytokine stimulation can theoretically increase the number of transduced clonogenic bone marrow cells from 0.1%, which is a readily achievable retroviral transduction frequency, to over 43%. And Bordignon *et al.*, 1995 (11), concluded that although natural selection is eventually achieved in gene therapy for inherited adenosine deaminase deficiency, selection for gene modified cells by the introduction of drug resistant genes may be necessary to decrease the time lag required for sufficient numbers of gene modified cells to appear in the blood. For the successful gene therapy treatment of other hematopoietic disorders *in vivo* selection of gene modified cells may be critical.

It has been shown that decreasing thymidine salvage with nucleoside transport inhibitors such as dipyridamole or NBMPR-P sensitizes clonogenic progenitor cells *in vivo* to TMTX, and likely to other antifolates (35). We showned that resistance to TS and DHFR antifolate inhibitors and FdUrd can be conferred by retroviral gene transfer of the cDNA that encodes TS and DHFR. It can be envisioned that dual resistance can

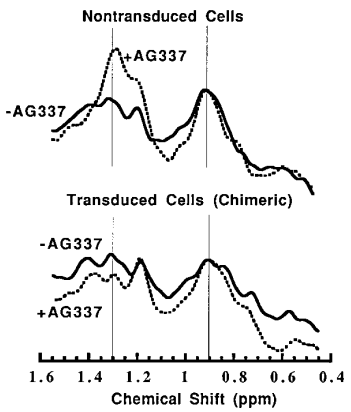

FIG. 4. Chemoprotection confirmed by ^{1}H NMR. Spectra collected from nontransduced and CMV-chimera transduced GP+E86 cells are represented in the upper and lower panels, respectively. The spectral resonances of methylene protons (CH_2) at 1.3 ppm and methyl protons (CH_3) at 0.9 ppm are indicated by vertical lines within the figure. Solid lines represent spectra collected from cells grown in the absence of AG337 and dashed lines represent spectra collected from cells grown in the presence of 1.5 μM AG337.

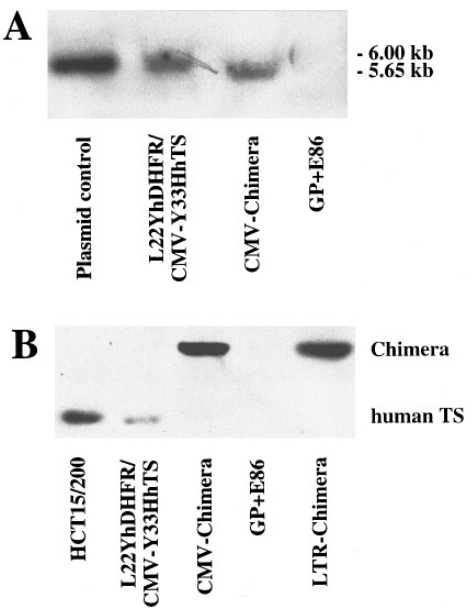

FIG. 5. Southern and Western analysis of transduced GP+E86 cells. (A) Southern analysis of genomic DNA prepared from polyclonal cell populations transduced with L22YhDHFR/CMV-Y33HhTS or CMV-chimera, digested with *Nhe* I, separated on an 0.8% agarose gel and probed with full length L22YhDHFR. The expected fragment sizes for L22YhDHFR/CMV-Y33HhTS and CMV-chimera are 6,000 and 5653 bp, respectively. Plasmid DNA containing the L22YhDHFR/CMV-Y33HhTS sequence was used as a positive control and genomic DNA isolated from nontransduced GP+E86 cells was used as a negative control. (B) Vector derived protein was determined by Western analysis using a monoclonal human TS antibody. Protein was isolated from murine fibroblast cells transduced with L22YhDHFR/CMV-Y33HhTS, CMV-chimera, or a construct that expresses the chimeric protein utilizing the viral LTR promoter (LTR-Chimera). HCT 15/200 cells were used as a positive control and non-transduced GP+E86 cells were used as a negative control.

provide *in vivo* protection against antifolates when nucleoside transport inhibitors are used to sensitize primitive hematopoietic cells, and selection of gene modified cells *in vivo* may be possible. Because the chimeric construct that we described encodes both enzymatic activities from a single polypeptide, complications associated with epigenic suppression due to multiple promoters within a single retroviral construct are alleviated. In addition, the chimeric protein confers dual resistance while maintaining a reasonable gene size, which allows for incorporation of therapeutic genes within the retroviral construct.

Enforced expression of chemoprotecting genes in hematopoietic cells can also attenuate deleterious side effects of chemotherapeutic agents. Several studies have shown that overexpression of drug resistant DHFR variants or MDR-1 in hematopoietic cells confer substantial *in vivo* drug resistance and transplant of drug resistant bone marrow cells has allowed the administration of increased doses of chemotherapy. Interestingly, McIvor and coworkers (36) showed that overexpression of variant DHFR in hematopietic cells confers protection not only against myelosuppression but against mucositis as well. Newly developed TS inhibitors have shown promising results in early phases of clinical trials but transient myelosuppression and mucositis are limitations to the use of these drugs. We show that the cDNA which encodes human TS can be transferred using recombinant retroviruses and that the integrity of the proviral sequence is maintained. Retroviral expression of TS confers greater than 3-fold resistance against AG337 and FdUrd, indicating *in vivo* protection against TS targeted inhibitors is feasible.

Although DHFR and TS exist as separate, monofunctional enzymes in bacteria, yeast, and vertebrates many protozoans such as *Leishmania major, Crithidia fasciculata* and *Plasmodium berghie* contain DHFR and TS on a single polypeptide, producing a bifunctional enzyme (37). *E. coli* DHFR and TS have been linked using gene fusion and the product was a bifunctional enzyme of approximately 50 kDa possessing V_{max} and K_m values for both DHFR and TS similar to that of the separate enzymes (38). The DHFR-TS chimera that we developed confers resistance to both TS and DHFR inhibitors and the degree of resistance is comparable to the degree of resistance achieved when the two enzymes are expressed separately.

Overexpression of TS confers substantial resistance to FdUrd and AG337 but, based on investigations using human DHFR, it is anticipated that greater resistance can be achieved by developing drug resistant variants of TS. Bertino and coworkers (23) recently identified TS variants (D49G and G52S hTS) that conferred up to 40-fold resistance against AG337, substantiating the possibility of genetically engineering TS variants that confer resistance to TS inhibitors. In this study we used a natually occurring TS variant (Y33HhTS) isolated from a colonic cell line that was shown to confer fluoropyrimidine resistance due to a 4-fold increase in the thermodynamic FdUMP dissociation constant. Although this variant consistently conferred greater resistance compared to the wild-type enzyme, variants that confer even greater resistance would be more advantageous for *in vivo* applications of TS gene therapy. Because the structure of FdUMP is very similar to the natural substrate dUMP it is unlikely that substantial resistance to FdUMP can be genetically engineered without drastically decreasing enzymatic activity. In addition, the mode of cytotoxicity of the FdUMP precursor 5-FU is complicated by the fact that its active metabolites not only inhibit TS but also incorporate into DNA and RNA, which mediates the cytotoxic effects of 5-FU. Although possible, it is unlikely that increased TS levels alone are sufficient to confer 5-FU resistance *in vivo.* TS inhibitors have been developed that are less similar to the natural substrates and these inhibitors provide a more rational approach for the design of TS variants. For example, AG337 is among the first anticancer agents developed based upon the molecular structure of a substrate binding site and it comprises a new class of TS inhibitors that is structurally distinct from the natural TS substrates. In hopes that the affinity of inhibitor binding can be decreased without drastically altering catalytic activity, we are focussing mutagenic investigations on residues involved in the binding of newly developed TS inhibitors but which are not involved in substrate binding.

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