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Modulation of the Cytotoxicity of 3′-Azido-3′-deoxythymidine and Methotrexate after Transduction of Folate Receptor cDNA into Human Cervical Carcinoma: Identification of a Correlation between Folate Receptor Expression and Thymidine Kinase Activity [1]

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ABSTRACT

Cervical carcinoma is an AIDS-defining illness. The expression of folate receptors (FRs) in cervical carcinoma (HeLa-IU$_1$) cells was modulated by stable transduction of FR cDNA encapsidated in recombinant adeno-associated virus-2 in the sense and antisense orientation (sense and antisense cells, respectively). Although sense cells proliferated slower than antisense or untransduced cells *in vivo* and *in vitro* in 2% (but not 10%) FCS, [*methyl*-^{3}H]thymidine incorporation into DNA was significantly increased in sense cells in 10% serum; therefore, the basis for this discrepancy was investigated. The activity of thymidine kinase (TK) was subsequently directly correlated with the extent of FR expression in single cell-derived clones of transduced cells. This elevated TK activity was not a result of recruitment of the salvage pathway based on the presence of adequate dTTP pools, normal thymidylate synthase (TS) activity, persistence of increased thymidine incorporation despite the exogenous provision of excess 5,10-methylene-tetrahydrofolate, and documentation of adequate folates in sense cells.

The increase in TK activity conferred significant biological properties to sense cells (but not antisense or untransduced cells) as demonstrated by augmented phosphorylation of 3′-azido-3′-deoxythymidine (AZT) and concomitantly greater sensitivity to the cytotoxic effects of AZT. Conversely, sense cells were highly resistant to methotrexate, but this was reversed by the addition of AZT. The direct correlation of FR expression and TK activity indicates a previously unrecognized consequence of FR overexpression.

INTRODUCTION

Cell surface FRs[3] mediate the transport of physiological folates and antifolates into normal and several malignant cell lines (reviewed in Ref. 1). It is now well established that transfection of human FR cDNA can enhance the capacity of cells to survive growth in low concentrations of folate (2–4) and mediate the cellular uptake and susceptibility to antifolates (5). Thus, understanding the mechanisms and consequences of modulation of FR content is of critical importance for chemotherapy with antifolates.

The first indication that FRs may have additional functions came from studies that demonstrated that the interaction of specific anti-FR antiserum with intact hematopoietic progenitor cells led to profound effects on the proliferation of these cells independent of the folate concentration (6, 7). When recombinant adeno-associated virus 2-based virions containing the full-length FR cDNA in either the sense or antisense orientation were transduced into cervical cancer (HeLa-IU$_1$) cells, those cells transduced with sense FR cDNA (sense cells) proliferated slower *in vitro* (in the presence of 2% FCS) and *in vivo* (in athymic mice) when compared with antisense FR cDNA-transduced cells (antisense cells) and untransduced cells (8). However, preliminary studies in 10% FCS, where there was no difference in proliferation of sense, antisense, and untransduced cells, also revealed that [*methyl*-^{3}H]thymidine incorporation into DNA was unexpectedly greater in sense cells when compared with antisense and untransduced cells. Therefore, the basis for this discrepancy warranted further investigation.

Thymidylate utilization by cells is dependent on the activity of the enzyme TS (EC 2.1.1.45), which is dependent on the availability of 5,10-methylene-tetrahydrofolate from one-carbon metabolism (9, 10). In addition, TK (EC 2.7.1.21) is an important enzyme of the pyrimidine salvage pathway that catalyzes the phosphorylation of deoxythymidine or deoxyuridine to form dTMP or dUMP in the presence of ATP (11). Because cells depend on FR-mediated uptake of folate to perpetuate one-carbon metabolism, gene transfer of FRs offered the potential for further study of the interrelationship between FR gene dose and enzymes related to one-carbon metabolism. Accordingly, we determined the basis for increase in thymidine incorporation into DNA and whether this had selective implications for chemotherapy of patients with cervical cancer with antinucleosides. This latter issue is of clinical significance because antinucleosides such as AZT are used in patients infected with HIV; and in this setting, the development of cervical cancer is recognized as an AIDS-defining illness.

MATERIALS AND METHODS

Materials. All materials for cell culture were from Life Technologies, Inc. (Gaithersburg, MD). Except otherwise noted, all chemicals were from Sigma Chemical Co. (St. Louis, MO). [*methyl*-^{3}H]Thymidine (specific activity, 6.7 Ci/mmol, 1 mCi/ml) was from New England Nuclear (Boston, MA), and [5-^{3}H]-2′-deoxyuridine, (specific activity, 22 Ci/mmol) and [*methyl*-^{3}H]AZT (specific activity, 14 Ci/mmol) were from Moravek Biochemicals (Brea, CA). Purified AZT-5′-MP, AZT-5′-DP, and AZT-5′-TP were kindly provided by Dr. David A. Cooney (National Cancer Institute, Bethesda, MD). pc32, a pUC 8 plasmid containing full-length human FR-α cDNA, was a generous gift from Dr. P. C. Elwood (Medicine Branch, National Cancer Institute, NIH, Bethesda, MD; Ref. 12).

Cell Lines and Growth Conditions. Transduction of recombinant adeno-associated virus 2 containing FR-α cDNA in either the sense or antisense orientation into HeLa-IU$_1$ cells was accomplished as described (8). Briefly, sense or antisense FR cDNA was driven by a TK promoter that was placed proximal to a SV40 promoter-driven neomycin resistance (*neo*^{R}) gene. After encapsidation into recombinant AAV, transduction and selection of geneticin-resistant cells, the proliferative characteristics and FR expression of mixtures (pooled) and single cell-derived clones of sense and antisense cell lines were documented (8), and the cells were frozen at −80°C. Before use in the present studies, cells were thawed and propagated for no more than five passages in

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[3] The abbreviations used are: FR, folate receptor; AZT, 3′-azido-3′-deoxythymidine; TK, thymidine kinase; TS, thymidylate synthase; -MP, monophosphate; -DP, diphosphate; -TP, triphosphate; MTX, methotrexate.

minimum essential media containing suprapharmacological concentrations (2.3 μM) of folic acid and 10% FCS at 37°C in 5% CO_2 (8).

[*methyl*-^{3}H]Thymidine Incorporation into DNA. For each pooled cell line of sense, antisense, and untransduced cells, 2×10^4 cells were plated in 96-well plates. At various times later (24-, 48-, 72-, 96-, and 120-h), 1 μCi [*methyl*-^{3}H]thymidine was added. After incubation for 17 h, cells were trypsinized and collected on glass fiber filters using a Multiple Automated Sample Harvester (MASH II; MA Bioproducts, Walkersville, MD). After adding 10 ml of Bio-Safe II Counting Cocktail (Research Products International, Mount Prospect, IL), the radioactivity in the filters was determined using a Beckman LS 6800 β-scintillation counter at ~50% efficiency.

To determine whether radioactivity retained on glass fiber filters represented [*methyl*-^{3}H]thymidine incorporation into DNA, 6×10^4 cells of each pooled cell line were plated in 60 × 15-mm dishes with 3 ml of medium. Five days later, the medium was changed (when the density was 3–4 × 10^5 cells), and 15 μCi of [*methyl*-^{3}H]thymidine were added. After 17 h, the cells were harvested by EDTA-trypsin (8) and washed once with 10 ml of PBS (at 1000 × *g* for 10 min/centrifuge wash cycle); then cells were solubilized in 3 ml of 10 mM Tris-HCl (pH 8.0), containing 100 mM EDTA and 0.5% SDS. An aliquot of each sample was analyzed for protein (13). The sample was then treated with 40 μg/ml DNase-free RNase A at 37°C for 1 h and digested with 100 μg/ml proteinase K at 37°C overnight, and DNA was extracted (14). DNA was dissolved in 400 μl of 10 mM Tris-HCl (pH 8), containing 1 mM EDTA, and stored at 4°C. Five-μl aliquots of each sample were used for the determination of absorbance at 260 and 280 nm and for radioactivity.

Assay for TK Activity. Quadruplicate samples of 1×10^8 cells from pooled sense, antisense, or untransduced cells were cultured in 500 cm^2 capacity three-tier Nunclon flasks (A/S Nunc, Roskilde, Denmark) and harvested at ~70–80% confluency, and TK activity was assayed as described (15). Briefly, cells were extracted with 0.15 M KCl (pH 7.4), and the supernatant was used as a source of the enzyme. For the assay, the reaction mixture in a total volume of 20 μl contained 50 mM Tris-HCl (pH 7.4), 5 mM ATP, 2.5 mM $MgCl_2$ and 1 mM [2-^{14}C]thymidine (specific activity, 4.6 mCi/mmol) and enzyme extract. The reactants were incubated at 37°C for 8 min, heated at 95°C for 1 min to stop the reaction, and then centrifuged at 18,000 × *g* for 5 min. An aliquot of the reaction mixture was spotted on 3M chromatography paper, overspotted with a mixture of thymidine and dTMP (used as markers), and subjected to ascending chromatography using 1 M ammonium acetate (pH 5.0) and ethanol (30:70 v/v) as the solvent system. The separated markers were then visualized under UV light, and the [2-^{14}C]dTMP formed was cut out and counted. TK activity was defined as nmol of dTMP formed/h/mg protein.

Assay for TS. Cytosolic TS activity was assayed in pooled sense, antisense, or untransduced cell lines as described (16). Briefly, the reaction product of the dTMP synthase activity assay, tritiated water (generated after release of tritium from carbon-5 of [5-^{3}H]dUMP) was selectively absorbed into 100% KOH, and the radioactivity was determined.

Assay of TS *in situ*. To determine whether reduced availability of 5,10-methylene-tetrahydrofolate led to a reduction in TS, the enzyme activity was determined in intact pooled sense, antisense, or untransduced cells as described (17). Briefly, [5-^{3}H]deoxyuridine (1 μM; 5 μCi/flask) was incubated with ~1–2 × 10^6 cells for various time points. Aliquots of cell suspension were transferred into Eppendorf tubes, and the reaction was quenched by charcoal in trichloroacetic acid to remove excess substrate. After centrifugation, the supernatant containing (enzyme-catalyzed and released) tritium was quantitated.

Measurement of 2′-Deoxyribonucleotides. Cells (1×10^8) were used to determine 2′-deoxynucleotides by the technique cited (18), with minor modifications. Briefly, pooled sense, antisense, or untransduced cells were washed with 20 ml of PBS at 4°C, extracted with 300 μl of cold 10% trichloroacetic acid, and centrifuged for 30 s. The trichloroacetic acid extracts were immediately neutralized with 0.5 ml of 0.5 M tri-*n*-octylamine in freon and subjected to periodate oxidation, and 2′-deoxyribonucleotides were separated on a Whatman Partisil PXS 10 SAX-25 column that was equilibrated with 5 mM ammonium phosphate (pH 2.8) and eluted with a linear gradient to 0.75 M ammonium phosphate (pH 3.7), over 38 min at 2 ml/min, and maintained at 0.75 M ammonium phosphate (pH 3.7), over 7 min. Under conditions of the assay, >95% of dCTP, dTTP, and dATP and >90% of dGTP were recovered; dCTP, dTTP, dATP, and dGTP eluted at 29.9, 31.1, 34.4, and 40.3 min, respectively.

Metabolism of [*methyl*-^{3}H]AZT. Pooled cells from each cell line (1×10^7) were incubated in medium containing 50 μM [*methyl*-^{3}H]AZT at 37°C for 3 h, centrifuged, washed once with 10 ml PBS at 4°C, harvested with a rubber policeman, and then extracted with 10% trichloroacetic acid and neutralized, as described above. An aliquot of the neutralized extract was analyzed on high performance liquid chromatography using a Partisil 10-SAX column, as described (19). One-ml fractions were collected, and radioactivity in the samples was determined. Under the conditions of analysis, AZT, AZT-5′-MP, AZT-5′-DP, and AZT-5′-TP eluted at 6, 16, 32, and 46 min, respectively.

Studies on AZT Transport *in Vitro*. The techniques used for influx and efflux studies were as cited (20). Briefly, for influx studies, aliquots of pooled sense, antisense, or untransduced cells in logarithmic growth phase (2×10^6 cells/ml) were incubated at 37°C (in triplicate) with [*methyl*-^{3}H]AZT to a final concentration of 6.25, 12.5, 25.0, or 50.0 μM. At various times, 0.5-ml aliquots of the mixture were layered over 0.5 ml of Versilube F 50 silicone fluid (General Electric Company, Waterford, NY) in 1.5-ml Eppendorf tubes that were centrifuged at 12,000 × *g* for 1 min. After the apex of each tube containing the cell pellet was excised, the cells were solubilized in 1 N NaOH, and radioactivity was determined. The radioactivity contributed by the entrapment of [*methyl*-^{3}H]AZT in the extracellular space was corrected using radiolabeled inulin as a marker (20).

For efflux studies, similar cell suspensions were incubated at 37°C for 10 min with 6.25 or 25.0 μM [*methyl*-^{3}H]AZT, as described above, and the mixture was centrifuged at 400 × *g* for 1 min. The supernatant was aspirated and replaced with fresh medium lacking AZT. Aliquots of the cell suspension were sampled at timed intervals and then processed, as described above.

Colorimetric Assays for Cell Proliferation. The proliferation of pooled sense, antisense, or untransduced cells in the absence and presence of various chemotherapeutic agents was assessed by the use of the cellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay (Promega Corp., Madison, WI). In data not shown, we validated this method for our cells by demonstrating that a various number of cells (1×10^3 to 6.4×10^4) added to 96-well plates (Falcon, flat-bottomed tissue culture plates; Microtest) yielded a direct correlation between the absorbance and known numbers of cells. In addition, there was increased absorbance that coincided with increasing cell numbers with progressive duration (in days) of culture.

To determine drug sensitivity, 1×10^3 of pooled cells from each cell line in culture medium were added to 96-well plates, and 24-h later, increasing concentrations of the following drugs were added: AZT (5–100 μM), and MTX (5–10,000 nM) alone or in combination with AZT (20 μM). After 3 days of continuous exposure to drugs, cell numbers were determined by the cell proliferation assay.

Colony (Clonogenic) Assays. To determine the inhibitory capacity of AZT or MTX on colony formation, 500 pooled sense, antisense, or untransduced cells in 3 ml of media were added to 60 × 15-mm culture dishes and incubated in the absence or presence of increasing concentrations of AZT or MTX. After 14 days, plates were washed, fixed, and stained, and colony numbers were enumerated (8).

Folate Determination. Cellular folates were assayed by the Quantaphase II B_{12}/Folate Radioassay commercial kit (Bio-Rad, Hercules, CA). Briefly, 1.5×10^6 of pooled cells from either untransduced, sense, or antisense cohorts were harvested with 2 ml of trypsin-EDTA and resuspended in 10 ml of media. After centrifugation at 500 × *g* for 10 min at 22°C, cell pellets (in <20 μl of media) were lysed with 1 ml of 0.4% ascorbic acid, and aliquots were analyzed for folate. The data reported is the average of two independent experiments carried out in duplicate as recommended by Bio-Rad.

Statistical Analysis. Unless stated otherwise, all studies were conducted in triplicate on three independent occasions; the data were averaged, and SEs were determined. The statistical significance of the data was determined by using Student's *t* test, and a probability of <5% was considered significant.

RESULTS

FR Expression, Thymidine Uptake, and TK Activity

Preliminary studies suggested that despite a reduced proliferation of sense cells observed *in vitro* and *in vivo* (8), this was not accompanied by a proportionately reduced amount of [*methyl*-^{3}H]thymidine incorporation into DNA. When this analysis was extended to pooled cells from each cell line under conditions where there was no difference in

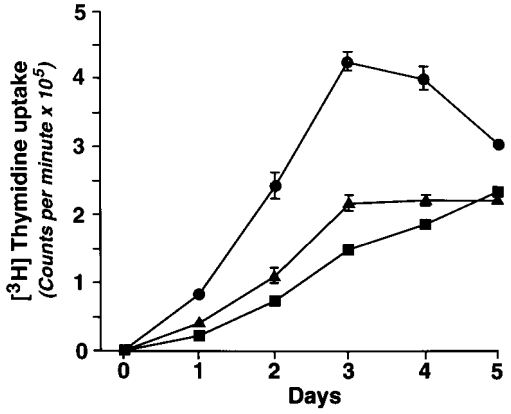

Fig. 1. [*methyl*-^{3}H]Thymidine incorporation into DNA for sense cells (●), antisense cells (▲), and untransduced cells (■) as a function of days in culture. Equal numbers of pooled cells (2×10^4) from each cell line was cultured in 96-well plates in the presence of minimum essential media containing 2.3 μM folic acid and 10% FCS (final 5-methyltetrahydrofolate concentration was 9 nM). At various times indicated, [*methyl*-^{3}H]thymidine (1 μCi) was added, and 17 h later, the DNA retained on filters was counted for radioactivity. There was no difference in cell proliferation among the three cell lines during the duration of the study (8). *Bars*, SE.

Table 1 *Incorporation of [methyl-^{3}H]thymidine into DNA and TK activity of nonclonal, unsynchronized mixtures of untransduced, sense, and antisense cells*

	Incorporation into DNA (cpm $\times 10^5$)			TK activity
Cell lines	Per 10^6 cells	Per mg protein	Per μg DNA	nmol/h/mg protein
Untransduced cells	3.7 ± 0.5	19.9 ± 4.1	0.8 ± 0.1	12.3 ± 0.3
Sense cells	11.0 ± 1.2[a]	40.3 ± 3.9[a]	2.2 ± 0.3[a]	14.9 ± 1.5
Antisense cells	5.7 ± 0.2	27.9 ± 4.4	1.1 ± 0.2	11.2 ± 0.6

[a] Sense cells incorporated thymidine into DNA significantly different compared with untransduced cells ($P < 0.05$).

cell proliferation among the three cell lines during the duration of the study (8), sense cells had a much greater amount of radiolabel incorporation when compared with the antisense and untransduced cells (Fig. 1); and the difference between sense and antisense cells was maintained throughout the duration of study.

When [*methyl*-^{3}H]thymidine incorporated was determined as a function of either cell number, mg protein, or μg DNA (Table 1), a consistent and correlated significant increase in [*methyl*-^{3}H]thymidine incorporation into pooled sense cells was obtained when compared with pooled untransduced cells. Based on these results, the activity of TK was determined in all three cell lines. The data (Table 1) indicated that sense cells also had slightly higher TK activity when compared with both untransduced and antisense cells. Because sense cells had significantly increased expression of FR mRNA and FR protein than antisense and untransduced cells (8), this suggested the possibility of a relationship between FR expression and TK activity. However, because the sense cells used were composed of mixtures of cells that had varying levels of FR expression, that correlated inversely with cell proliferation (8), these cells were heterogeneous with respect to cell proliferation. In addition, because cell proliferation is well known to be correlated with TK activity, it was evident that the relationship between FR expression and TK activity needed to be formally assessed in clonal populations of sense cells. Thus, when several single cell-derived clones of unsynchronized sense and antisense cells were randomly analyzed for TK activity, there was a statistically significant ($P < 0.005$) direct correlation between FR expression and TK activity (Fig. 2).

Further analysis revealed that deoxyribonucleotide triphosphate (dTTP, dATP, dGTP, and dCTP) levels were not statistically different from one another among pooled sense, antisense, and untransduced cells (Table 2), which suggested that the increase in TK activity was not a compensatory response to reduced availability of dTTP (*i.e.*, TK was not activated via the salvage pathway). This was confirmed by finding that TS activity was comparable among all three cell lines, the values (in nmol/mg protein/h) were as follows: pooled untransduced cells, 14.8 ± 7.2 ($n = 5$); pooled sense cells, 16.9 ± 6.3 ($n = 10$); and pooled antisense cells, 17.4 ± 6.1 ($n = 11$). Parenthetically, it should be clarified here that both *in vitro* assays for TK and TS are routinely performed with excess substrates. However, *in situ*, the activity of these enzymes is limited and depends on the availability of the substrates. Therefore the observations of an apparently "comparable" activity for TK and TS *in vitro* (on a nmol/mg protein/h basis) is unlikely to be representative of the true activity of these enzymes within the cell. Thus, although comparison of the activity of either TK or TS among the three cell lines can give valuable information, this cannot be extrapolated to the situation within cells.

Because 2 μM of 5,10-methylene-tetrahydrofolate is routinely added to the reaction mixture, this assay for TS activity (21, 22) is insensitive to small reductions in 5,10-methylene-tetrahydrofolate (as found in folate deficiency). To resolve this issue, an *in situ* assay for the enzyme was used where the endogenous 5,10-methylene-tetrahydrofolate was the sole source of folate for TS activity. The results of TS *in situ* for pooled untransduced, sense, and antisense cells were 1.22 ± 0.17, 1.67 ± 0.08, and 0.91 ± 0.04 pmol/10^6 cells/h, respectively ($P < 0.05$ for sense *versus* antisense cells). Moreover, untransduced, sense, and antisense cells had 170, 708, and 226 pmols of

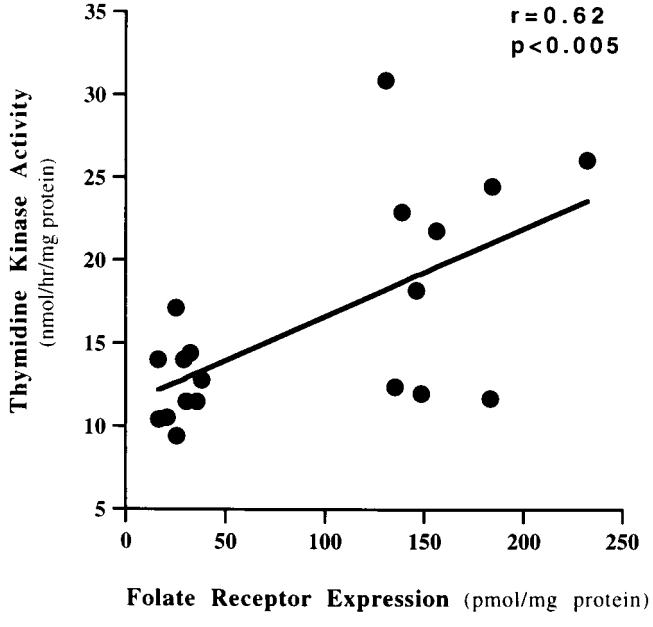

Fig. 2. The relationship between FR expression and TK activity in single cell-derived clones from sense and antisense cells. The cells transduced with sense and antisense FR cDNA according to Sun *et al.* (8) were analyzed for TK activity. Quadruplicate samples of 1×10^8 cells from each cell line were harvested during logarithmic growth phase at ~70–80% confluency, and TK activity was assayed (14). The cells with FR expression over 100 pmol/mg protein were from sense cells, whereas those below this cutoff point were from antisense cells.

Table 2 *Deoxyribonucleoside triphosphate levels in untransduced, sense, and antisense cells*[a]

	Nucleotide concentration (pmol/10^6 cells)			
Cell lines	dATP	dCTP	dGTP	dTTP
Untransduced cells	115.8 ± 18.9	6.0 ± 2.3	27.0 ± 2.3	38.2 ± 3.8
Sense cells	181.0 ± 106.0	4.4 ± 4.7	17.3 ± 10.6	49.4 ± 4.1
Antisense cells	131.5 ± 87.6	8.6 ± 3.9	43.2 ± 25.0	37.1 ± 5.4

[a] Mean and SD from two independent experiments carried out in triplicate.

Table 3 *Phosphorylation of AZT in untransduced, sense, and antisense cells*

Cell lines	Concentration of AZT phosphates (pmol/10^6 cells)		
	AZT	AZT-MP	AZT-DP
Untransduced cells	41.3 ± 6.6	194.7 ± 0.7	1.3 ± 0.3
Sense cells	55.2 ± 2.9[a]	311.0 ± 2.5[a]	2.7 ± 0.3[a]
Antisense cells	34.1 ± 7.3	180.0 ± 17.5	1.1 ± 0.1

[a] Significantly different compared with antisense cells ($P < 0.05$).

Table 4 *Effect of AZT and MTX on the clonogenic response of untransduced, sense, and antisense cells*[a]

Cell lines	IC_{50} (nM)	
	AZT	MTX
Untransduced cells	87,000	53
Sense cells	26,500[b]	>6,600[b]
Antisense cells	55,000	45

[a] Approximately 500 cells in 3 ml of media were added to 60 × 15-mm culture dishes and were exposed to increasing concentrations of buffer (controls), AZT, or MTX. After 14 days, plates were washed, fixed, and stained, and colony numbers were enumerated (8).
[b] Significantly different compared with untransduced and antisense cells ($P < 0.05$).

folate/10^6 cells, respectively. Furthermore, when those single cell-derived clones with a high expression of TK (25 nmol/h/mg protein) and FR (230 pmol/mg protein) were exposed to supraphysiological (2 μM) 5-formyl-tetrahydrofolate for 5 days, the preexisting increased [*methyl*-^{3}H]thymidine incorporation failed to return to the lower values found with control untransduced and antisense cells (data not shown). Together, these results strongly suggested that TS was not perturbed, and that the increase in TK activity in sense cells was not due to intracellular folate deficiency, or from triggering of the salvage pathway.

Functional Consequences of Increased TK Activity

Effect of AZT. Cellular TK converts AZT to AZT-MP before it is eventually converted to AZT-TP by other cellular enzymes (23). At therapeutic doses in humans, however, the concentration of AZT-TP achieved *in vivo* is not as toxic to human cells as it is to HIV. Because sense cells had higher TK activity, we tested the hypothesis that more AZT would be phosphorylated (23) and thereby exhibit a greater cell kill in sense cells. As shown in Table 3, after exposure to 50 μM [*methyl*-^{3}H]AZT for 3 h, the AZT-MP and AZT-DP were similar in pooled untransduced and antisense cells; however, pooled sense cells had a 1.6-fold and 2-fold significantly greater amount of AZT-MP and AZT-DP, respectively. Furthermore, although the colony-forming efficiency among pooled sense, antisense, and untransduced cells was similar (8), the concentration of AZT at which colony formation was reduced by 50% was lowest for sense cells compared with untransduced or antisense cells (Table 4). Thus, sense cells appeared to be selectively sensitive to the cytotoxic action of AZT.

Because increased transport of AZT by the sense cells could account for its greater sensitivity to the cytotoxic action of AZT, we determined the influx and efflux of radiolabeled AZT by pooled untransduced, sense, and antisense cells. The K_m of AZT transport (the concentration of AZT at half-maximal velocity) was similar in untransduced and sense cells but 2-fold lower in antisense cells (Table 5). In addition, the V_{max} (the maximum velocity of transport of AZT) was similar in untransduced and sense cells but 4-fold greater in antisense cells. The rate of influx of AZT (the time taken to reach half-maximal saturation) was faster in sense cells compared with untransduced and antisense cells, but the rate of efflux (the time to eliminate one-half of the cellular AZT) was similar in all three cell lines. Therefore, there was no set pattern to suggest that transport favored net accumulation of a greater amount of AZT in sense cells. Based on the data in Table 5, we also determined whether a concentration of AZT near the IC_{50} and another concentration far below this value would elucidate differences in AZT transport. The transport of AZT at 6.25 and 25.0 μM revealed essentially similar patterns (Fig. 3). These results concurred with the velocity of transport, influx, and efflux of AZT in the three cell lines. Thus, these results provided further evidence that differences in AZT transport could not explain the increased metabolism of AZT found in sense cells.

Effect of MTX and Its Relationship to AZT. MTX inhibits dihydrofolate reductase and leads to a reduction in functional intracellular folates that participate in one-carbon metabolism, causing inhibition of TS (24–27). Because sense cells had the highest levels of TS by the *in situ* assay and more TK (with equivalent amounts of dTTP and comparably high cellular folate concentrations) when compared with antisense and untransduced cells, these data predicted that sense cells would exhibit resistance to the cytotoxic effects of MTX (22). In fact, when the viability of pooled cells cultured in 9 nM of 5-methyl-tetrahydrofolate, 2.3 μM of folic acid, and increasing concentrations of MTX (5–10,000 nM) was determined, the IC_{50} for antisense and untransduced cells was virtually identical at 25 nM, whereas sense cells were markedly resistant to even 10,000 nM MTX (Table 6).

If MTX resistance of sense cells was primarily conferred by an increase in activity of TK, the addition of AZT could be predicted to alter such resistance. The steady-state concentrations of AZT achieved in humans taking therapeutic doses of AZT is ~5 μM. In contrast, the IC_{50} for most cells in culture is >180 μM (100 μM AZT was nontoxic to the three cell lines). But because a minority of human cells can be inhibited by ~18 μM AZT, a level of AZT of 20 μM was chosen (which was comparable with the more sensitive cultured cells, while being only 4-fold greater than levels achieved *in vivo*). As shown in Table 6, both pooled antisense and untransduced cells had an IC_{50} of 25 nM with MTX that was unaffected by the addition of 20 μM of AZT. In contrast, with the addition of 20 μM AZT, there was a dose-dependent reduction of cell proliferation in pooled sense cells, leading to an IC_{50} for MTX of 100 nM. Thus, the augmentation of MTX effect in the presence of AZT in sense cells supported the hypothesis that the resistance to MTX in sense cells was primarily mediated through an increase in TK activity.

DISCUSSION

Correlation between FR Expression and TK Activity

The increase in [*methyl*-^{3}H]thymidine uptake into DNA in sense FR cDNA-transduced cells (sense cells) compared with antisense and untransduced controls in 10% FCS suggested a discrepancy that warranted further study. Under normal conditions, TS generates the major portion of dTMP for DNA synthesis and a substantially smaller amount, believed to be <10% in hematopoietic bone marrow progenitor cells, of dTMP is generated via the TK-mediated salvage pathway. However, this salvage pathway can be activated to provide up to ~15% of the total thymidylate when there is a reduction of intracellular folate availability (*e.g.*, in folate deficiency or through use of antifolates) or by inactivation of TS (10). The present studies were not carried out at physiological folate concentrations primarily because

Table 5 *AZT transport by human cervical carcinoma HeLa-IU$_1$ cells in vitro*

Cell line	K_m (μM)	V_{max} (pmol/10^6 cells/min)	Influx at 25 μM AZT ($t_{0.5}$ in min)	Efflux at 25 μM AZT ($t_{0.5}$ in min)
Untransduced cells	200.0	11.5	3.5[a]	10.5
Sense cells	166.7	12.2	2.0	10.5
Antisense cells	83.3[a]	47.6[a]	4.5[a]	10.6

[a] Significantly different compared with sense cells ($P < 0.05$).

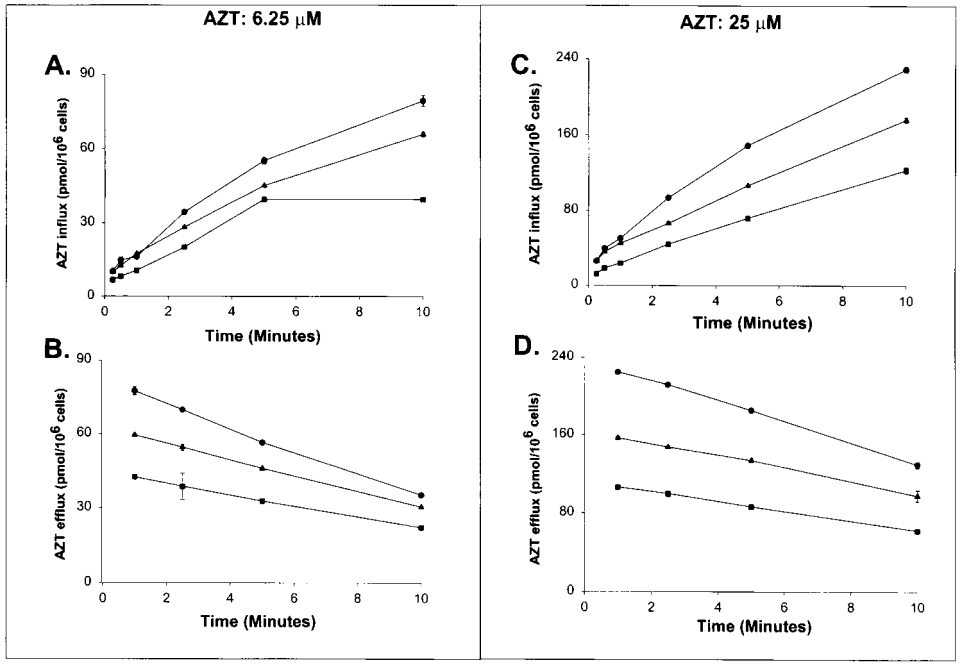

Fig. 3. Transport of [*methyl*-^{3}H]AZT into untransduced (▲), sense (●), and antisense cells (■). Pooled cells from each cell line were incubated with 6.25 μM (*A* and *B*) or 25.0 μM (*C* and *D*) of AZT, and at various times indicated samples were assayed to determine the rate of influx (*A* and *C*) and rate of efflux (*B* and *D*). *Bars*, SE.

the transfer of cells from a high extracellular folate milieu to physiological concentrations would have led to: (*a*) up-regulation of FR[4]; and (*b*) changes in cell cycle kinetics (these cells grow slower in lower folate containing media).[4] These new variables would have independent effects on TK and profoundly complicate our analysis. Therefore, to negate the additional effects of lower folate concentrations in modulating the activity of TK, these cells were cultured in high folate (2.3 μM) containing media. Under these conditions, any effects on TK after gene therapy could be directly related to the inducing variable.

To control for the potential for *trans*-activation of the endogenous *TK* gene by the TK promoter (which was used to drive expression of sense FR cDNA), an internal control was the use of the same TK promoter in both sense and antisense FR cDNA constructs (8). Because (any) *trans*-activation would be common to clonally derived sense and antisense FR cDNA-transduced cells, this potential mechanism cannot account for the observation that TK activity correlated with the extent of FR expression (Fig. 2). Thus, it is highly unlikely that *trans*-activation from the TK promoter accounted for the observed relationship.

Although varying the FR expression does not influence cell proliferation in the short term, TK activity is known to vary depending on the phase of the cell cycle. For example, TK activity is elevated in the S-phase of the cell cycle in cultured human lymphoblasts (28). Therefore, to reduce the influence of cell cycle-related variations in TK activity, we determined whether there was a relationship between TK and FR expression in unsynchronized subcloned cells. An expected trade-off is that some subcloned cell lines that coincidentally had a higher number of cells in the S-phase of the cell cycle would have higher TK activity. This can explain the lack of an even higher correlation between FR expression and TK activity in unsynchronized single cell-derived clones; but this should also not detract from the fact that this relationship is statistically significant at a level that clearly could not be explained on the basis of chance.

The critical demonstration that there was a direct correlation between the expression of FR and TK activation in several single cell-derived clones of sense and antisense cells suggested the need to investigate the basis for this linkage. Because of the established link between TK and folate metabolism, studies were designed to determine whether the overexpression of FR somehow negatively influenced either the intracellular folate availability or the major folate-dependent enzyme TS, which may have triggered activation of TK via the salvage pathway. However, our results from several different studies argued against the hypothesis that TK activity was increased as a salvage response to transduction of FR genes. In addition, the similar dTTP pool in sense, untransduced, and antisense cells was also incompatible with the likelihood that the salvage pathway was activated. Thus, although TK has been stimulated through obscure epigenetic mechanisms by a variety of stimuli such as progesterone (29), granulocyte/macrophage colony-stimulating factor (30), and even pH (31), we have now shown that stable transduction of FR cDNA also results in activation of TK through a mechanism that does not involve the thymidine salvage pathway.

Sensitivity and Resistance of Sense Cells to Chemotherapeutic Agents

An unusual feature of cervical carcinoma in patients who test positive for HIV (32–34) is the presentation in young women with far advanced disease. This limits the application of simple local surgery and/or radiation therapeutic modalities (35), and together with the low response rates with existing chemotherapeutic agents (36), warrants

Table 6 *Cytotoxicity of MTX and a combination of MTX plus AZT to untransduced, sense, and antisense cells, as analyzed by cell proliferation assays*[a]

	IC_{50} (nM)	
Cell lines	MTX	MTX plus 20 μM AZT
Untransduced cells	25	25
Sense cells	>10,000[b]	100[b]
Antisense cells	25	25

[a] Approximately 1×10^3 cells were cultured in 96-well plates; 24 h later, various concentrations of MTX (5–10,000 nM) alone or with AZT (20 μM) were added. After 3 days, cells were tested for proliferation using the colorimetric assay. Under conditions of the assay, 100 μM AZT was nontoxic to all three cell lines.

[b] Significantly different compared with untransduced and antisense cells ($P < 0.05$).

[4] A. C. Antony, Q-J. Li, X-L. Sun, X. Xiao, and Y-S. Tang, manuscript in preparation.

the development of novel and innovative forms of alternative therapy for locally advanced and metastatic disease for the ~4 million HIV-positive women at risk worldwide. Because patients with AIDS-defining cervical carcinoma are frequently on AZT, we determined the sensitivity of AZT and MTX.

Sensitivity to AZT. Cellular TK first converts AZT into AZT-MP, which is converted to AZT-DP and then into AZT-TP (23, 37). Therefore, we hypothesized that if TK activity was increased in sense cells, there would be greater phosphorylation of AZT, which would be expected to lead to greater toxicity in these cells. This was shown to be the case; thus, AZT-MP was significantly increased in sense compared with antisense or untransduced cells. Although an increased amount of AZT-TP could not be detected, the functional consequence of activation of AZT by TK was demonstrated by finding greater cytotoxicity of sense cells to AZT.

Resistance to MTX. When enough FRs are expressed, these proteins mediate the uptake of 5-methyl-tetrahydrofolate and antifolates (such as MTX) with comparable rates to cells expressing only the reduced-folate carrier (38); thus, FRs have both physiological and pharmacological importance. This has led to the hypothesis that overexpression of FRs can render cells more susceptible to antifolates. However, in contrast to data on *FR* gene transfer into MCF-7 cells where this was shown to be the case (5), transfected ZR-75-1 breast cancer cells did not exhibit an increased sensitivity to MTX (39). In contrast to both these studies, our results demonstrated that the transduction of FR cDNA clearly induced the resistance of cells to MTX. One possibility to explain these findings was that sense cells accumulated a significant amount of folate; therefore, cell kill would have eventually been evident if our studies were carried out for longer periods. Another possibility was that because the cells proliferated slower over time, they did not use folates as much as antisense and untransduced cells; therefore the MTX would have had less effect in sense cells. These valid arguments were, however, mitigated by the finding that MTX resistance of sense cells was reversed by the coadministration of AZT (*i.e.,* the IC_{50} was reduced 100-fold, from 10,000 to 100 nM) in a 3-day cell proliferation assay. The most likely explanation is that the increased TK activity of sense cells, which allowed survival in high MTX concentrations, also led to greater phosphorylation of AZT, which contaminated dTTP pools and precluded the continued growth of cells in MTX. This hypothesis is supported by the studies of Miyachi *et al.* (40), who showed a 2-fold increase in TK activity as one of the mechanisms for MTX resistance in CCRF-CEM cells (made MTX resistant by step-wise increases in extracellular MTX); and interestingly, these cells were collaterally sensitive to AZT. Thus, in addition to known causes of MTX resistance involving gene amplification or altered binding of MTX to dihydrofolate reductase, reduced polyglutamation of MTX, and altered membrane transport (41, 42), our results strongly suggest that increased activity of TK can also lead to MTX resistance. The mechanistic basis for the development of MTX resistance in relation to TK expression will be examined in detail in future studies.

Despite the fact that the correlation coefficient between FR expression and TK activity was not very high, this relationship was nevertheless highly statistically significant; therefore, the question of biological relevance was important. Our observations of increased TK activity correlating with increased sensitivity to AZT and resistance to MTX represent a validation of the biological significance of this relationship.

Conclusion

Our studies demonstrate a relationship between transduction of FR cDNA and resulting activation of TK, leading to induction of sensitivity of cervical carcinoma cells to AZT. This has serendipitously achieved similar goals set forth in recent clinical trials in pediatric brain tumors, which examine whether TK activity induced through gene transfer leads to enhanced tumor cell kill by ganciclovir and other drugs activated by TK (43, 44). More recently, this approach has also been exploited for several other solid tumors including breast (45), gastric (46), mesothelioma (47), and melanoma (48). An advantage of transduction of FR cDNA into cervical carcinoma is that the overexpressed FRs: (*a*) induce a significant reduction in cell proliferation *in vivo* (8); (*b*) increase the susceptibility of these cells to the cytotoxic effects of AZT, which HIV-positive patients are invariably on; and (*c*) can also bind and internalize newer folate-based cytotoxic agents and folate-tethered liposomes containing either chemotherapy or molecular medicine, such as antisense oligonucleotides (1, 49–51). Thus, this area warrants further study.

These studies also highlight the fact that an increase in [*methyl*-^{3}H]thymidine incorporation into DNA, as traditionally used as a surrogate marker of an increase in proliferation, is potentially fraught with error if concomitant studies on cell proliferation are not performed. As demonstrated for sense cells that had greater [*methyl*-^{3}H]thymidine incorporation into DNA, this was not due to greater cell proliferation but through an increase in TK activity.

Finally, because of cellular heterogeneity with respect to intrinsic proliferation rates, differences in tissue origin, different proportion of FR isoforms, number of passages since primary culture, and culture conditions, we do not know whether the correlation between FR expression and TK activity can be extrapolated to other cancer cells that constitutively overexpress FRs. Thus, although there appears to be a unique relationship between FRs and TK in HeLa-IU_1 cells, the hypothesis that a similar relationship may also be identified in other cells needs to be formally tested.

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