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Thymidylate Synthase as a Determinant of 5-Fluoro-2′-deoxyuridine Response in Human Colonic Tumor Cell Lines

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SUMMARY

A panel of seven human colorectal cell lines of differing phenotype has been examined to elucidate the role of thymidylate synthase (TS) in the response to 5-fluoro-2′-deoxyuridine (FdUrd). Although TS is a major target of FdUrd, no consistent relationship was observed between the intracellular levels of TS and the response to FdUrd among the cell lines. Levels of thymidine kinase and dihydrofolate reductase, enzymes that are involved in generation of ligands that form the inhibitory ternary complex with TS, do not correlate with FdUrd response. Two cell lines that exhibit innate resistance to FdUrd, relative to the other cell lines, have variations in TS enzyme structure or gene structure. Cell line HCT 116 contains two forms of TS, as defined by isoelectric focusing. One form, which is unique to HCT 116, is more basic than the common form, which is present in all the cell lines. Cell line RCA contains a variation in the TS structural gene, as defined by restriction fragment-length analysis. These structural variations, which are associated with reduced response to FdUrd, may serve as markers for reduced clinical response to TS-directed chemotherapy.

The 5-fluoropyrimidines FUra and FdUrd are cytotoxic in numerous biological systems (1). A major mechanism of action of these agents is inhibition of the enzyme TS (EC 2.1.1.45) by the fluoropyrimidine metabolite FdUMP. FdUMP forms a complex with TS, which is essentially irreversible in the presence of the folate $CH_2H_4PteGlu$ (1). By inhibiting TS, cellular thymidylate is reduced, which, in turn, inhibits DNA biosynthesis; thus, cells with higher growth rates and, hence, higher DNA biosynthetic rates are expected to be more sensitive to TS-directed inhibition.

FUra has been utilized as a single agent in the therapy of carcinomas of the gastrointestinal tract, breast, ovary, and pancreas (2–4); 15–20% of patients bearing these tumors respond to this agent (5). Recent clinical studies have indicated that the administration of CF with FUra increases the objective response to 45% (5). Because CF is thought to serve as a precursor of intracellular $CH_2H_4PteGlu$, the combination of FUra and CF is designed to enhance the action of FUra at TS. These results indicate that therapy directed at TS is of clinical benefit and that TS plays a major role in the response to fluoropyrimidine agents.

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In mammalian cells in culture, the relationship between TS and response to fluoropyrimidines has been frequently assessed with FdUrd, because TS is a primary target of this agent (6–13). In many of these studies, a diminished response to FdUrd is associated with a reduction in the cellular levels of either FdUMP or $CH_2H_4PteGlu$. Phenotypic alterations resulting in decreased FdUMP include decreased influx or increased efflux of FdUrd (6, 7); decreased levels of TK (EC 2.7.1.21) activity, which converts FdUrd to FdUMP (8); or increased FdUMP phosphatase activity (9). Depletion of $CH_2H_4PteGlu$ has been induced in cells by growth in folate-depleted medium or by exposure to the DHFR (EC 1.5.1.3) inhibitor methotrexate (10). DHFR converts folic acid, present in cell growth medium, to $H_4PteGlu$, which is the precursor of $CH_2H_4PteGlu$. In other studies, a reduction in FdUrd response has been associated with an increase in the levels of TS (11) or with a decrease in the affinity of TS for either FdUMP or $CH_2H_4PteGlu$ (12, 13).

Studies of the role of TS in fluoropyrimidine action have been conducted in a variety of mammalian cell lines representing diverse tissue types (1). Because FUra and FdUrd have been used in the chemotherapy of gastrointestinal carcinoma (1), it is of potential clinical relevance to focus upon cells of this histiotype to elucidate the relationship between TS and FdUrd response. Moreover, it is important to examine cell lines

ABBREVIATIONS: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; CF, folinic acid, 5-formyltetrahydrofolic acid, calcium leucovorin; TS, thymidylate synthase; FdUMP, 5-fluoro-2′-deoxyuridylate; $CH_2H_4PteGlu$, 5,10-methylenetetrahydrofolic acid; TK, thymidine kinase; DHFR, dihydrofolate reductase; $H_4PteGlu$, tetrahydrofolic acid.

that may represent the spectrum of tumor response phenotypes encountered clinically. For these reasons, a panel of human colorectal tumor cell lines of varying differentiation status, growth rate, drug sensitivity, and tumorigenicity (14) has been used in this study. Three cell lines, HCT 116, HCT 116a, and HCT 116b were derived from the same tumor (14); thus, differences among these cell lines reflect intratumoral heterogeneity. The other cell lines, MOSER, C, RCA, and CBS, were established from four separate tumors (14). Among these cell lines, variation in TS has been identified that is associated with reduced innate response to FdUrd.

Experimental Procedures

Materials. [6-^{3}H]FdUMP (20 Ci/mmol), [5-^{3}H]thymidine (70 Ci/mmol) and [3′,5′,7-^{3}H]methotrexate (20 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). FdUrd, phosphocreatine, creatine phosphokinase, and Sephadex G-50-80 were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum and Dulbecco's modified Eagle medium were purchased from GIBCO (Grand Island, NY). $CH_2H_4PteGlu$ was prepared from $H_4PteGlu$ as described by Dunlap *et al.* (15); $H_4PteGlu$ was prepared from folic acid by the procedure of Zakrzewski and Sansone (16). [^{32}P]FdUMP was prepared by incubating [γ-^{32}P]ATP (New England Nuclear, Boston, MA) with FdUrd in the presence of partially purified *Escherichia coli* TK (17); the labeled nucleotide was purified by DEAE-cellulose chromatography (18).

Cell culture and growth studies. The human colorectal cell lines were obtained from M. G. Brattain (Baylor College of Medicine, Houston, TX). They were maintained as monolayers in Dulbecco's modified Eagle medium (10 μM folic acid, final concentration) supplemented with 10% fetal bovine serum. The cells were routinely monitored for the absence of mycoplasma by the Mycotrim detection kit (New England Nuclear). All growth studies were carried out in T-25 flasks with 100,000 cells as inoculum. FdUrd was added 24 hr after inoculation and the cells were exposed to drug for five to six cell generations (19). Cell growth was measured by protein determination by the method of Lowry *et al.* (20).

TS level determination. TS enzyme levels were determined in 100,000 × *g* extracts of subconfluent cells as described previously (21) except that all extracts were preincubated with 100 mM NaF and 15 mM CMP before enzyme analysis (22). TS levels were determined by incubating cell extracts with [6-^{3}H]FdUMP and $CH_2H_4PteGlu$ as described previously (18). In several studies, the standard TS assay was modified by increasing the final concentration of [6-^{3}H]FdUMP by 10-fold (0.31 μM). The increased FdUMP resulted in nonspecific background in some cell extracts. To eliminate the background, control assays were carried out in the presence of a 500-fold excess of unlabeled FdUMP. In all assays, free FdUMP was separated from enzyme-bound FdUMP after heating the samples to 90° in 1% SDS by centrifugal elutriation on Sephadex G-50 columns.

Isolation and analysis of nucleic acids. The isolation of total RNA and DNA was carried as described previously (23). For Northern blotting (24), 10–15 μg of RNA was denatured, fractionated on 1.5% agarose-formaldehyde gels, transferred to nylon membranes, and hybridized to a nick-translated [^{32}P]TS probe; the TS mRNA was observed by autoradiography. For Southern blot analysis (25), 10 μg of DNA was digested with the appropriate restriction endonuclease and the resulting DNA fragments were separated on 1.0% agarose gels, transferred to a nylon membrane, and hybridized to the ^{32}P-labelled probe; TS gene fragments were observed by autoradiography. The TS probe was a gel-purified 750-base pair fragment of plasmid pMTS-3, which is a cDNA clone corresponding to mouse TS mRNA (26).

TS structure determination. Extracts of tumor cell lines were prepared as described previously (21). TS was labeled by ternary complex formation with [^{32}P]FdUMP and $CH_2H_4PteGlu$ as described previously (18), except that the FdUMP was increased by 10-fold. The ternary complexes were denatured in 6 M urea and subjected to isoelectric focusing in 9 M urea/4% acrylamide gels containing 0.67% (v/v) each of ampholines pH 4–6, pH 6–8, and pH 3.5–10 (LKB, Piscataway, NJ) (27). After electrophoresis, the gels were fixed, dried, and subjected to autoradiography at −70°.

TK activity. TK activity was determined in subconfluent cells by a modification of the procedure of Johnson *et al.* (28). An ATP-regenerating system, consisting of 5 mM phosphocreatine and 6.6 units of creatine phosphokinase, was included in the assay (29).

DHFR level determination. The levels of DHFR were determined in subconfluent cells by the procedure of Johnson *et al.* (30).

Radioactivity determination. Aqueous samples were counted in 10 ml of Liquiscint-2 scintillation solution (National Diagnostics, Inc., Somerville, NJ) using a Packard liquid scintillation counter.

Results

Growth sensitivity to FdUrd. The concentrations of FdUrd required for 50% inhibition of cell growth (ID_{50} values) are presented in Table 1. The cell lines vary 150-fold in sensitivity to FdUrd. There was no relationship between FdUrd response and tumor of origin inasmuch as three cell lines derived from a single colorectal tumor, namely HCT 116, HCT 116a, and HCT 116b (14), differ 3-fold in sensitivity. Likewise, no relationship was observed between FdUrd sensitivity and state of differentiation or growth rate. For example, HCT 116 and C are undifferentiated cell lines with similar growth rates (14), yet they differ nearly 15-fold in sensitivity to FdUrd; RCA and MOSER form differentiated tumors *in vivo* and exhibit similar growth rates *in vitro* (14), yet they have a 2-fold difference in FdUrd response. Finally, no correlation was observed between response to mitomycin C (14) and to FdUrd among the cell lines.

TS enzyme levels. The levels of TS enzyme in cell extracts are shown in Table 1. The cell lines vary 11-fold in TS enzyme concentration. The enzyme concentration was determined by the standard FdUMP-binding assay and by a modification of the assay, in which a 10-fold higher concentration of FdUMP was used. In all cell lines except HCT 116, the quantitation of TS was unaffected by the higher level of FdUMP; enzyme levels in HCT 116 doubled under these conditions. Higher concentrations of FdUMP are required to titrate the HCT 116 enzyme completely because these cells express two forms of TS (see Fig. 3). The unique, more basic, form has a reduced affinity for both FdUMP and $CH_2H_4PteGlu$ (31); thus, the presence of the

TABLE 1

TS levels and FdUrd sensitivity in human colorectal cell lines

To measure TS levels, TS in cell extracts was labeled with [6-^{3}H]FdUMP in the presence of excess $CH_2H_4PteGlu$, as described in Materials and Methods. The values for TS concentration are the mean ± standard deviation. The number of separate determinations, each carried out in duplicate, is shown in parentheses. To measure FdUrd sensitivity, cells in monolayer were exposed to FdUrd continuously for five to six cell generations. The ID_{50} is the concentration of FdUrd required to inhibit cell growth by 50% with respect to untreated controls. The number of separate determinations, each carried out in triplicate, is shown in parentheses.

Cell line	TS concentration	ID_{50} (FdUrd)
	pmol of TS/mg of protein	*nM*
HCT 116	5.8 ± 0.9 (4)	45 (3)
HCT 116a	1.7 ± 0.2 (5)	14 (2)
HCT 116b	5.3 ± 0.9 (8)	13 (2)
MOSER	3.8 ± 0.7 (5)	20 (2)
C	2.5 ± 0.7 (6)	3 (2)
RCA	1.0 ± 0.3 (7)	34 (2)
CBS	0.5 ± 0.2 (4)	0.3 (2)

unique TS polypeptide in HCT 116 underlies the requirement for increased FdUMP for complete titration of TS.

Relationship between FdUrd sensitivity and TS enzyme levels. The relationship between the ID_{50} for FdUrd and the TS concentration in cell extracts is shown in Fig. 1. It is immediately apparent that no consistent relationship exists between FdUrd response and TS levels; this observation is in contrast to that of Washtien (11), who reported a direct correlation between TS levels and sensitivity to FdUrd among five human gastrointestinal tumor cell lines. Although CBS, with the lowest TS concentration, is the most sensitive to FdUrd and HCT 116, with the highest TS levels, is the least sensitive, little correlation exists among the remaining cell lines. For example, cell line C, with 2.5-fold higher TS levels, is 10-fold more sensitive to FdUrd than is cell line RCA. In addition, the three cell lines isolated from the same tumor, namely HCT 116, HCT 116a, and HCT 116b, show no correlation between TS and FdUrd sensitivity; the lack of correlation among these cells reveals the extent of phenotypic heterogeneity that may exist within tumors. TS levels are similar in HCT 116 and HCT 116b, yet the sensitivity differs by 3-fold; conversely, TS concentration differs 3-fold in HCT 116a and HCT 116b, yet sensitivity is similar. Of all the cell lines examined, HCT 116 and RCA exhibit the highest innate resistance to FdUrd, relative to TS concentration.

TS mRNA levels and structure. TS-specific sequences in total RNA isolated from the cell lines are shown in Fig. 2. TS mRNA levels, in general, parallel the TS enzyme levels measured in cell extracts. The exception is CBS, in which TS mRNA levels are reduced, relative to TS enzyme levels determined in cell extracts. For the other six cell lines, these data suggest that the steady state level of TS enzyme is controlled primarily by the concentration of TS mRNA; moreover, these results indicate that the levels of TS determined *in vitro* are representative of the *in situ* enzyme concentration. The size of the TS mRNA in all the cell lines is the same, namely 1.7 kilobases. This is identical in length to TS mRNA present in human cells of other histiotypes (23).

TS enzyme structure. The structure of TS in cell extracts was analyzed by isoelectric focusing gel electrophoresis after incubating the enzyme with [^{32}P]FdUMP and $CH_2H_4PteGlu$; the resulting complex contains two covalent linkages and is stable to denaturing conditions (1, 23). A common charge form is observed in all cell extracts, as shown in Fig. 3. In HCT 116 extracts, a novel, more basic, charge form is present in addition to the common form. No differences were observed among the cell lines in TS size, as determined by electrophoresis of ^{32}P-labeled ternary complexes on SDS-polyacrylamide gels.[1]

TS gene structure. The TS gene in the colorectal cell lines was analyzed after digestion of the DNA with the restriction endonucleases *Eco*RI, *Bcl*I, *Hind*III, *Bgl*II, and *Pvu*II. A representative restriction fragment pattern after *Eco*RI digestion is shown in Fig. 4. The TS gene from RCA contains a slightly larger *Eco*RI restriction fragment than the TS genes from the other cell lines; in addition, a slightly larger *Bcl*I restriction fragment is present in the TS gene from RCA.[2] No alterations in TS gene structure were observed among the cell lines after digestion with the other enzymes.[3] This indicates that differences that occur among the TS genes are likely to be the result of point mutations, rather than insertional or deletional mutations.

Levels of TK activity. The presence of TK activity is required for the anabolism of FdUrd to FdUMP. The activity

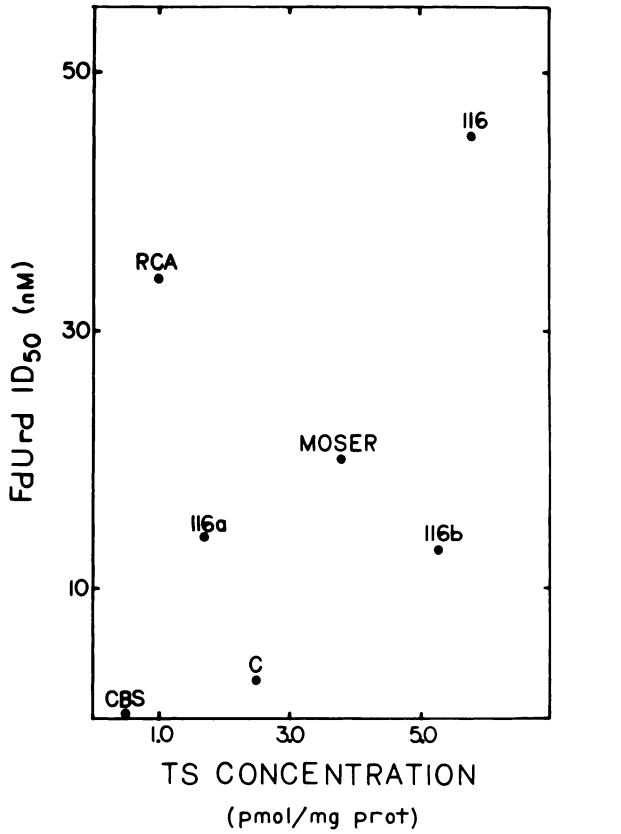

Fig. 1. Relationship between TS enzyme levels and FdUrd sensitivity. The graphical relationship between TS levels and the growth sensitivity to FdUrd among human colorectal cell lines is shown. The methods for analysis of TS concentration in cell extracts and for determination of ID_{50} values for FdUrd are presented in the legend to Table 1.

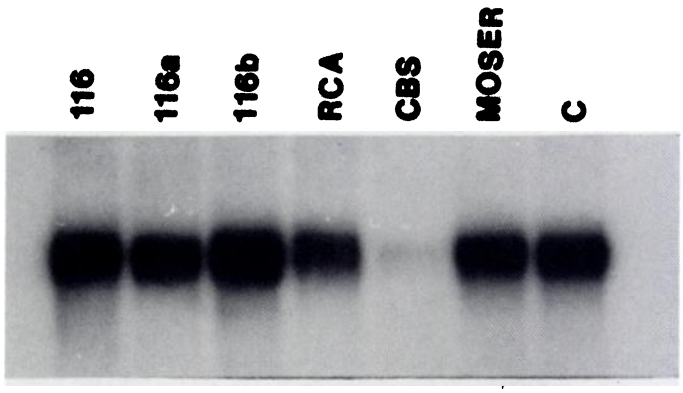

Fig. 2. Northern blot analysis of TS mRNA in human colorectal cell lines. Total cellular RNAs were separated on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized to a ^{32}P-labeled TS cDNA-containing probe as described in Materials and Methods. TS-specific sequences were detected by autoradiography. Ribosomal RNAs were used as size markers.

[1] S. Davis and S. Berger, unpublished results.
[2] S. Berger, unpublished results.
[3] S. Berger and F. Berger, unpublished results.

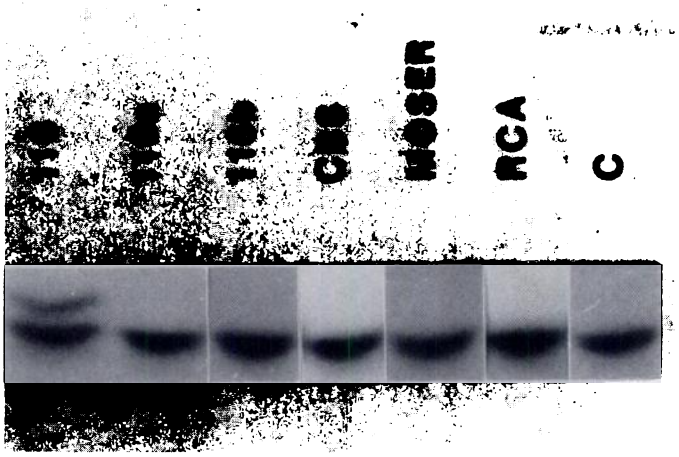

Fig. 3. Isoelectric focusing gel electrophoresis of the [^{32}P]FdUMP-TS-CH_2H_4PteGlu complex from human colorectal cell lines. Extracts of human colorectal cells were incubated with [^{32}P]-FdUMP and CH_2H_4PteGlu under conditions in which TS enzyme is limiting; the resulting ternary complexes were denatured and separated by isoelectric focusing on urea-polyacrylamide gels as described in Materials and Methods. The labeled complexes were detected by autoradiography. The amount of extract protein was adjusted so that the intensity of labeled compexes is similar; thus, the intensity does not reflect the intracellular concentration of TS enzyme.

of TK in cell extracts is shown in Table 2. The cell lines vary 18-fold in enzyme activity, with C having the lowest levels of activity. All the cells exhibited normal growth rates in folate-free medium supplemented with thymidine, hypoxanthine, and glycine,[2] indicating that the TK activity is sufficient for thymidine utilization for cell survival under these growth conditions.

DHFR levels. The enzyme DHFR converts medium folic acid to H_4PteGlu, which is the intracellular precursor to CH_2H_4PteGlu; thus, it plays a role in regulating the availability of folate cosubstrate both for TS catalytic activity and for the binding of FdUMP to TS. The levels of DHFR in cell extracts are shown in Table 2. The cell lines vary 6-fold in DHFR concentration. The levels of this enzyme vary less among the cell lines than either TS levels or TK activities. No relationship, in general, was apparent between DHFR levels and sensitivity to FdUrd.

Discussion

No consistent relationship between growth response to FdUrd and the levels of TS was observed among the human colorectal cell lines (Fig. 1). Linear regression analysis of the data revealed a correlation coefficient (r) of 0.45. This is in contrast to the studies of Washtien (11), in which a direct correlation ($r = 0.98$) between FdUrd response and TS enzyme levels among human gastrointestinal tumor cell lines was observed. The basis for the discrepancy is unknown; unfortunately, no cell lines were used in common in these two studies. In the present study, enzyme levels were quantitated by a standard assay (18) and by a modified assay in which the FdUMP concentration was increased by 10-fold to ensure complete titration of TS. Furthermore, TS mRNA levels were analyzed and observed to parallel the enzyme levels determined in cell extracts (Fig. 2). These results indicate that the TS

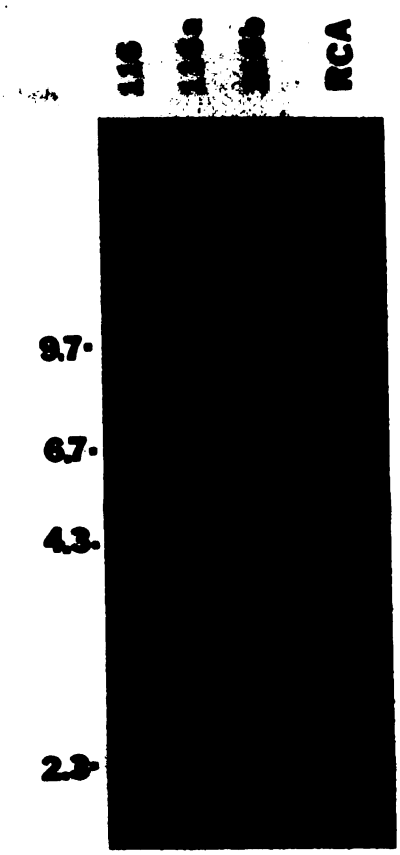

Fig. 4. Southern blot analysis of TS genes in human colorectal cell lines. DNAs were digested with *Eco*RI and the resulting fragments were separated by agarose gel electrophoresis, transferred to nylon, and hybridized to a ^{32}P-labeled TS cDNA-containing probe as described in Materials and Methods. TS-specific sequences were detected by autoradiography. Fragments of λ DNA, digested with *Hind*III, are size markers. The 2.3-kilobase fragment corresponds to a putative TS pseudogene, unlinked to the TS structural gene (23).

TABLE 2

TK activities and DHFR levels in human colorectal cell lines

The levels of TK activity in cell extracts were determined as described in Materials and Methods. The values for enzyme activity are the mean ± standard deviation. The number of separate determinations, each carried out in duplicate at three time points, is given in parentheses. The levels of DHFR in cell extracts were determined as described in Materials and Methods. The values are the mean ± standard deviation. The number of separate determinations, each carried out in duplicate, is given in parentheses.

Cell line	TK activity	DHFR levels
	nmol/min/mg of protein	*pmol/mg of protein*
HCT 116	0.55 ± 0.14 (5)	1.3 ± 0.1 (3)
HCT 116a	0.94 (2)	4.3 ± 0.5 (3)
HCT 116b	1.25 ± 0.24 (3)	1.8 ± 0.5 (3)
MOSER	0.46 (2)	2.3 ± 0.5 (3)
C	0.07 (2)	0.9 ± 0.1 (3)
RCA	0.35 (2)	0.7 ± 0.1 (3)
CBS	0.26 (2)	1.3 (2)

enzyme levels determined *in vitro* accurately reflect the *in situ* situation. A linear relationship between FdUrd response and TS may exist within the following subgroups of the cell lines: a sensitive group, CBS, C, and HCT 116b (r = 0.98); an intermediate group, HCT 116a and MOSER; and a resistant group, RCA and HCT 116. This would indicate that mechanisms other than TS are responsible for the deviation of the intermediate and resistant subgroups from the sensitive group.

Analysis of additional cell lines is required to establish whether such a trend exists. Regardless of whether subgroups exist, the data suggest that mechanisms other than TS levels govern the response to FdUrd.

The molecular basis for the relative resistance of cell lines HCT 116 and RCA to FdUrd (Table 1) has been investigated. In both cell lines, alterations in TS that are associated with reduced response to FdUrd have been observed. HCT 116 cells contain two forms of TS encoded by different genes; the polypeptide encoded by one of the genes has a reduced affinity for both FdUMP and $CH_2H_4PteGlu$ (31). This polypeptide is the unique, more basic, form shown in Fig. 2. The presence of TS with reduced affinity for ligands underlies the requirement of a 10-fold excess of FdUMP in the TS binding assay to completely titrate the enzyme in HCT 116 cell extracts (Table 1). Thus, the unique TS form may confer resistance to FdUrd in HCT 116 cells. In the case of RCA, recent results have demonstrated that the TS enzyme purified from RCA has a reduced affinity for $CH_2H_4PteGlu$, relative to at least two other human tumor cell enzymes.[1] It is interesting that the TS gene in RCA is variant (Fig. 4); whether this variation underlies the functional alteration is under investigation.

In addition to alterations in TS, other cellular parameters may influence the response to FdUrd and, hence, affect the relationship between FdUrd response and TS enzyme levels. The levels of TK activity could affect the anabolism of FdUrd to FdUMP; thus, cells with lower enzyme levels may be less sensitive to FdUrd. The data in Table 2 indicate that TK is not limiting the response to FdUrd, inasmuch as the cell line with the lowest enzyme activity, namely C, is quite sensitive to FdUrd (Table 1; Fig. 1). The levels of DHFR were examined, because the levels of intracellular folates influence the response to FdUrd (10). DHFR catalyzes the conversion of folic acid, present in cell growth medium, to $H_4PteGlu$, the intracellular precursor of $CH_2P_4PteGlu$; thus, reduced levels of this enzyme may reduce intracellular $CH_2H_4PteGlu$, which is essential for tight binding of FdUMP to TS. The levels of DHFR are lowest in RCA, which is relatively resistant to FdUrd (Table 1). Because low levels of DHFR may reduce the availability of $CH_2H_4PteGlu$, this could contribute to the resistance of RCA, which contains a TS with reduced affinity for $CH_2H_4PteGlu$.

The cellular mechanisms underlying the apparent intermediate sensitivity of HCT 116a and MOSER (Fig. 1) are under investigation. Neither TS molecular parameters, TK enzyme activities, nor DHFR enzyme levels appear to account for the relative resistance of these cell lines. It is important to determine whether the mechanisms associated with the apparent resistance to FdUrd are similar in these cells. If so, it may be possible to predict the cell phenotype, with regard to TS-directed cytotoxicity, by examining the relationship between FdUrd response and TS enzyme levels.

Of potential importance to the effect clinical utilization of TS-directed chemotherapy is the identification in the present study of two markers associated with diminished response, the unique enzyme structural form in HCT 116 and the variant TS structural gene in RCA. Future efforts are aimed at determining whether these structural alterations underlie TS functional alterations and, if so, whether similar structural changes exist in the human population.

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