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A Naturally Occurring Variation in Thymidylate Synthase Structure Is Associated with a Reduced Response to 5-Fluoro-2′-deoxyuridine in a Human Colon Tumor Cell Line

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SUMMARY

Inhibition of thymidylate synthase (TS) is an important mechanism of action of fluoropyrimidine antimetabolites. Thus, TS structure and expression are expected to be determinants of response to these agents. The role of TS in fluoropyrimidine response has been analyzed in a panel of human colonic tumor cell lines. Previous work has demonstrated that there is little correlation between TS concentration and sensitivity to 5-fluoro-2′-deoxyuridine (FdUrd) among these cell lines, suggesting that parameters other than the TS levels are responsible for the variations in drug response. One such parameter has been identified in cell line HCT 116. This line, which is relatively resistant to FdUrd, produces two structural forms of TS, as determined by mobility of the enzyme in isoelectric focusing polyacrylamide gels. One form is common to all the cell lines, whereas a variant form, which is more basic and is encoded by a separate structural gene, is unique to HCT 116. Cells expressing one or the other TS form have been isolated and used to demonstrate that the variant form is associated with FdUrd resistance. Kinetic experiments indicate that the variant TS has reduced affinities for 5-fluoro-2′-deoxyuridylate and 5,10-methylenetetrahydrofolate, which are ligands involved in formation of a stable inhibitory complex with the enzyme. Thus, the innate resistance of cell line HCT 116 to FdUrd is derived, at least in part, from production of an altered structural form of TS having reduced affinity for ligands.

Fluoropyrimidine antimetabolites, particularly FUra and FdUrd, have been useful in the chemotherapy of a variety of solid tumors (1–3). It is generally considered that these agents exert their cytotoxic effects by two primary mechanisms. One involves the disruption of RNA structure and function through incorporation of 5-fluoro-UTP into RNA (4). The other mechanism is thymidylate deprivation, which is due to potent inhibition of TS by the metabolite FdUMP (5, 6). TS inhibition by FdUMP occurs through formation of a relatively stable covalent ternary complex involving the enzyme, FdUMP, and CH_2H_4PteGlu (5, 6). In cultured cells, the relative importance of these two mechanisms to cytotoxicity appears to be highly dependent upon the phenotype of the cells as well as the growth conditions.

Resistance to fluoropyrimidines occurs through a variety of mechanisms. In addition to changes in TS structure, which can perturb the interaction between enzyme and FdUMP or CH_2H_4PteGlu (7, 8), fluctuations in TS concentration have profound effects upon drug response. Washtien (9) found a strong correlation between the TS level and FdUrd response among human gastrointestinal tumor cell lines. Furthermore, several laboratories have isolated FdUrd-resistant mutants that overproduce TS (10–13); the high enzyme concentration in several of these mutants is due to amplification of the TS structural gene (12, 13).

We have embarked upon the analysis of TS expression as a determinant of innate variation in FdUrd sensitivity among human tumor cell lines of colonic origin. These cell lines, which have been isolated in a number of laboratories (14–17), represent a tumor type for which fluoropyrimidines have been therapeutically useful (1–3). In a companion paper to the present one (18), FdUrd response was found to vary extensively among seven such cell lines; in contrast to the findings of Washtien (9), the variations in drug response did not correlate closely with the TS level, indicating that parameters other than the concentration of TS are important determinants of heterogeneity in FdUrd sensitivity. In the present study, we have identified one such parameter. We show that cell line HCT 116 produces an altered structural form of TS that contributes, at

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ABBREVIATIONS: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridylate; CH_2H_4PteGlu, 5,10-methylenetetrahydrofolate; TS, thymidylate synthase.

least in part, to its relative FdUrd resistance; the variant TS has a reduced affinity for both FdUMP and $CH_2H_4PteGlu$.

Experimental Procedures

Materials. [6-^{3}H]FdUMP (20 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). [^{32}P]FdUMP was prepared as described by Berger and Berger (18).

Cell culture. The maintenance of human colonic tumor cell line HCT 116 has been described (18). Cells were subcloned in 100-mm tissue culture dishes (100 cells per dish) in maintenance medium containing 33% conditioned medium and 15% fetal bovine serum. The cloning efficiency was 70%. FdUrd-resistant HCT 116 cells were selected by step-wise adaptation to increasing FdUrd concentrations between 10 and 200 nM. At each step, cells were maintained for at least 2–4 weeks before doubling the drug concentration. The population resistant to 200 nM FdUrd was subcloned as described for parental HCT 116 cells (see above).

FdUrd response curves were generated by inoculating 100,000 cells in T-25 flasks in drug-free RPMI 1640 medium supplemented with 5% fetal bovine serum (FdUrd-resistant cells were grown for 2 weeks in this medium before analysis of FdUrd response). After overnight incubation to allow the cells to attach to the plastic surface, medium containing 10 μM folinic acid, 100 μM deoxyinosine, and various concentrations of FdUrd was added. After five to six cell generations, the relative growth was determined by measuring total cellular protein in the cultures (19).

TS protein analysis. Cell extracts were prepared as described previously (13). FdUrd-resistant cells were grown for 2 weeks in drug-free medium before preparation of extracts. TS concentrations were determined by the FdUMP binding method, which measures covalent ternary complex formation among TS, [^{3}H]FdUMP, and $CH_2H_4PteGlu$ (3, 4). The assays were performed according to the methods of Moran *et al.* (20), except that the [^{3}H]FdUMP concentration was increased by 10-fold to 0.31 μM.

Analysis of ternary complexes by isoelectric focusing gel electrophoresis (21) was in the presence of urea, and was performed according to procedures that have been described (18). Under the conditions of these experiments, the complexes exist as monomers.

Extraction and analysis of nucleic acids. DNA and RNA were isolated from the cultured cells according to the methods of Bowman *et al.* (22). Analyses of the TS gene and mRNA by Northern and Southern blotting (23, 24) have been described (18).

Results

The two TS forms in HCT 116 are encoded by separate structural genes. In previous work (18), it was shown by isoelectric focusing gel electrophoresis that HCT 116 is different from other human colonic cell lines in that it produces two structural forms of TS; one form is common to all cell lines, whereas a more basic form is unique to HCT 116. Because HCT 116 exhibits the highest level of FdUrd resistance among the colonic cell lines tested (18), further analyses were undertaken to determine the origin of the variant TS species and its role in FdUrd response.

The possibility that HCT 116 is heterogeneous and that the two TS charge forms are present in different cells within the population was tested by analysis of subclones. Each of 13 clones was found to contain the same two charge forms in exactly the same ratio as in the uncloned population (data not shown). Thus, both the common and the variant forms of TS are found in most, if not all, HCT 116 cells.

Several mechanisms could explain the origin of the variant form of TS. The fact that the two forms co-exist within cells (see preceding paragraph) makes it unlikely that the two TS species derive from some sort of posttranslational processing, because processing variants are generally either dominant or recessive. The novel TS may have a different primary structure that is encoded by a variant structural gene. This gene could either be allelic with the normal TS gene or represent an additional gene within the HCT 116 genome. Alternatively, the two TS forms might be encoded by the same structural gene and arise as a consequence of differential transcription and/or processing of TS mRNA.

To distinguish among these alternatives, we adopted a strategy based upon the observation that during selection for gene amplification, only one allele of a heterozygous gene becomes amplified within a cell (25, 26). Thus, if the two TS species are encoded by separate structural genes, then one or the other, but not both, should be overproduced in clonal populations of cells that have undergone TS gene amplification; in contrast, if the two species are encoded by a single structural gene, then both should be overproduced in such cells.

To select for TS gene amplification, a population of HCT 116 cells resistant to FdUrd was developed by adaptation to stepwise increases in the drug concentration between 10 and 200 nM. The drug-resistant population, denoted 116/200, as well as several subclones from this population, overproduce TS by a factor of 10–20-fold, relative to the parental HCT 116 cells (data not shown). This overproduction is a consequence of high mRNA levels that derive from TS gene amplification (Fig. 1). The results of electrophoretic analysis of the TS produced by the 116/200 subclones are shown in Fig. 2. Clones 1, 9, and 10 express predominantly the common structural form of TS, whereas clones 11 and 15 express predominantly the variant form. Thus, differential expression of the two TS species occurs in HCT 116 cells undergoing gene amplification. This suggests that the two TS forms are products of separate structural genes, with the gene encoding the common form of TS being amplified in clones 1, 9, and 10, and that encoding the novel form being amplified in clones 11 and 15.

The variant TS species confers a FdUrd resistant phenotype. FdUrd sensitivities for several of the 116/200 subclones were measured, to determine whether the drug response in cells producing the novel form of TS differs from that in cells producing the common form. To ensure that the growth studies reflected TS inhibition, they were conducted in the presence of folinic acid and deoxyinosine, which potentiate fluoropyrimidine toxicity and direct its action to TS (27, 28). This was necessary because it was observed that, during the later stages of development of the 116/200 population, phenotypes other than TS overproduction were selected.[1] Thus, TS may not be the sole drug target at high FdUrd doses.

Folinic acid and deoxyinosine caused the ID_{50} for FdUrd inhibition of parental HCT 116 cells to decrease from 45 nM (18) to 9 nM (data not shown). Fig. 3 shows the results of analyses of the 116/200 subclones. The ID_{50} for clones 11 and 15, both of which produce mainly the novel form of TS, was about 100 nM, whereas that for class 9 and 10, which produce predominantly the common form, was about 25 nM. Thus, cells expressing the novel TS are 4-fold more drug resistant than are cells expressing the common form. Inasmuch as all clones had approximately the same TS level, this correlation between the presence of a particular TS structural form and the FdUrd

[1] S. Berger, unpublished results.

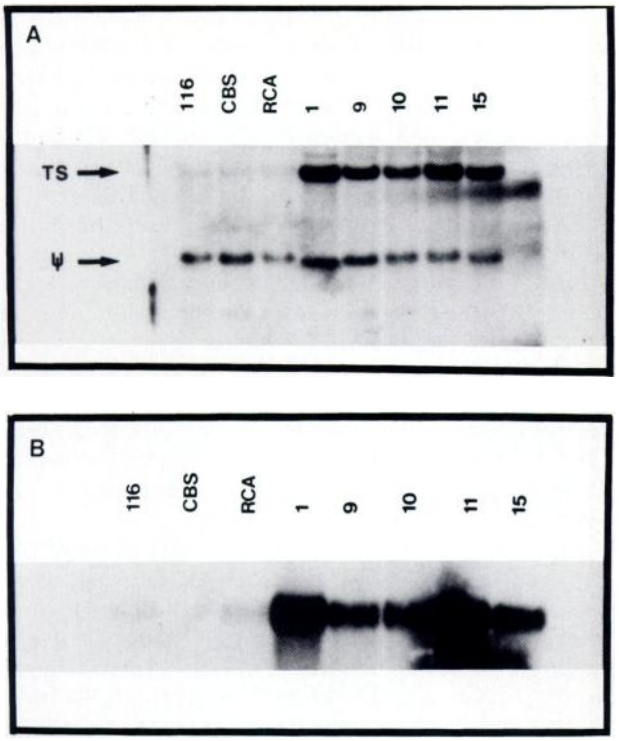

Fig. 1. TS gene amplification and mRNA overproduction in FdUrd-resistant derivatives of HCT 116. DNA and RNA were purified from several cell lines and from TS-overproducing clones 1, 9, 10, 11, and 15. A, DNA was digested with *Eco*RI, fractionated by agarose gel electrophoresis, transferred to a nylon membrane, hybridized to a TS-specific cDNA probe, and observed by autoradiography. Fragments of the expressed gene are denoted *TS*, whereas a putative pseudo-gene fragment (13) is indicated by ψ. B, RNA was fractionated by electrophoresis through formaldehyde-containing agarose gels, transferred to a nylon membrane, hybridized to the TS probe, and observed by autoradiography.

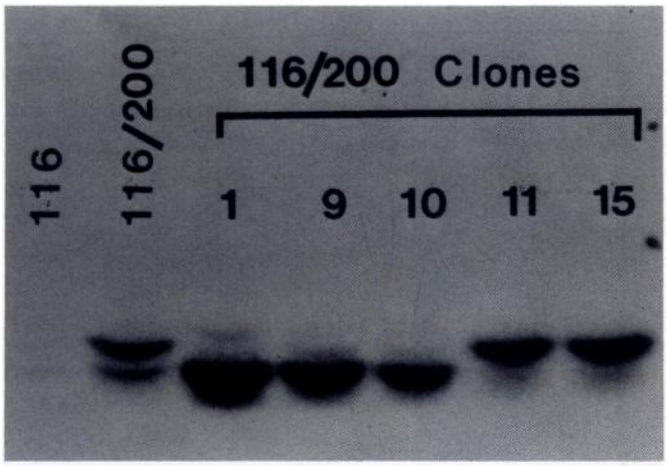

Fig. 2. Expression of TS forms in overproducing clones. Extracts were incubated in the presence of [^{32}P]FdUMP under conditions favorable for formation of ternary complexes, subjected to isoelectric focusing in an acrylamide slab gel, and observed by autoradiography after fixation and drying of the gel. The intensity of the label on the autoradiogram does not reflect relative TS concentrations in the extracts.

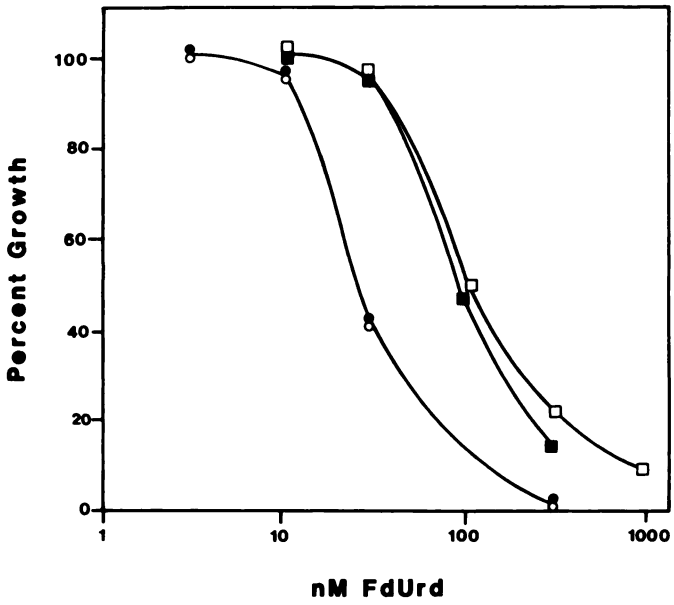

Fig. 3. FdUrd dose-response curves for TS-overproducing clones. Cells in monolayer culture were exposed to various concentrations of FdUrd in the presence of 10 μM folinic acid and 100 μM deoxyinosine. Growth inhibition was measured as described in Materials and Methods. ○, Clone 9; ●, clone 10; □, clone 11; ■, clone 15.

response indicates that the two TS species confer a differential drug response to cells, with the novel species generating a more resistant phenotype.

The novel TS species has a reduced affinity for ligands. The results described above indicate that the two forms of TS generate different FdUrd sensitivities. The relative resistance of cells producing the novel TS could be due to an impaired interaction between this form of TS and ligands, thereby reducing formation of a stable inhibitory ternary complex (7, 8).

The differential mobility of the two TS forms on isoelectric focusing gels allows determination of their reactive affinities for ligands involved in ternary complex formation. Ligand affinities can be compared by measuring the ratio of [^{32}P]FdUMP binding to the two forms as a function of the concentration of nucleotide or of cofactor. To determine FdUMP affinities, extracts of 116/200 cells were incubated with varying concentrations of [^{32}P]FdUMP in the presence of excess cofactor and applied to isoelectric focusing gels. Fig. 4A shows that the ratio of FdUMP binding to the two TS forms was altered at lower FdUMP concentrations, predominantly as a consequence of decreased binding to the novel form. Thus, the novel species requires a higher FdUMP concentration for maximal complex formation, indicating that it has a reduced affinity for the nucleotide analog.

To measure $CH_2H_4PteGlu$ affinities, 116/200 extracts were incubated with varying concentrations of $CH_2H_4PteGlu$ in the presence of excess [^{32}P]FdUMP and applied to isoelectric focusing gels. As seen in Fig. 4B, there was a small difference between the two TS forms in affinity for $CH_2H_4PteGlu$, as indicated by the altered ratio of FdUMP binding at low cofactor levels. Though this difference in cofactor affinity was not as pronounced as the difference in FdUMP affinity, it was reproducible in several experiments.

Identical results were obtained with extracts of parental HCT 116 cells, indicating that the observed affinity differences are

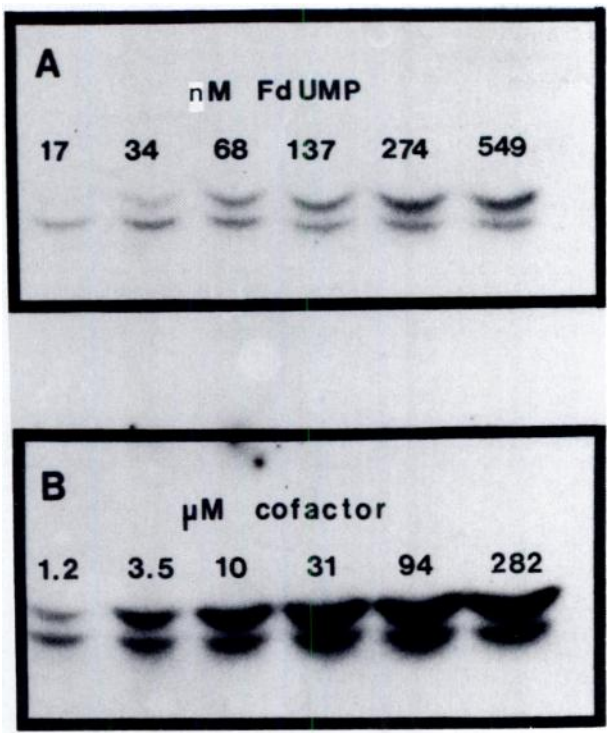

Fig. 4. Kinetics of FdUMP and CH_2H_4PteGlu binding to the two forms of TS. Extracts of uncloned 116/200 cells were incubated with varying concentrations of [^{32}P]FdUMP and excess CH_2H_4PteGlu (A) or varying concentrations of CH_2H_4PteGlu and excess [^{32}P]FdUMP (B). Ternary complexes corresponding to the two TS forms were separated by isoelectric focusing in polyacrylamide slab gels and observed by autoradiography after fixation and drying of the gels.

not a consequence of some unusual property of 116/200 cells. Thus, the novel TS species appears to have reduced affinities for both FdUMP and CH_2H_4PteGlu. Because these experiments were performed with crude extracts, they must be regarded as preliminary, pending more detailed analyses of purified enzymes.

Discussion

Although a considerable amount of information is available on the biochemical and molecular determinants of fluoropyrimidine response in a variety of cell lines of wide histiotypic origin (1–3), relatively little is known about the nature and mechanisms of differential drug response among cells of the same tissue type. We have therefore chosen to study colonic tumor cell lines, focusing on variations among the lines in drug sensitivity and the role of TS as a determinant of such variations. The seven colonic cell lines that we have used should be particularly valuable, because they serve as models for the interas well as intratumoral heterogeneity that characterizes neoplastic tissues (16, 17).

The results presented in this report indicate that human colonic tumor cell line HCT 116 produces a variant form of TS, in addition to a form that is common to all the cell lines examined. The novel TS, which has a more basic pI, is encoded by a structural gene separate from that encoding the common TS. These two structural genes could be allelic, because the karyotype of cell line HCT 116 is near-diploid (16, 17); however, it is possible that the novel TS is encoded by a nonallelic structural gene. Although Southern blot experiments (18) indicate no gross changes in TS gene structure or copy number in HCT 116, we cannot rule out the presence of additional copies of the TS gene within the genome.

The variant TS species generates resistance to FdUrd, inasmuch as cells expressing predominantly the novel form are more resistant to FdUrd, compared with cells expressing predominantly the common form (Fig. 3). It is, therefore, likely that the relatively high ID_{50} for FdUrd exhibited by HCT 116 (18) is due, at least in part, to the novel TS. However, other factors may contribute to the variations in drug response among the colonic cell lines examined in this study. For example, the low TS concentration in CBS is most likely responsible for its sensitivity to FdUrd (18). Among the other cell lines, the lack of correlation between TS and FdUrd response indicates that parameters other than enzyme concentration are important (18).

Experiments shown in Fig. 4 suggest that the novel TS has a reduced affinity for both FdUMP and CH_2H_4PteGlu. Verification of these findings by more detailed kinetic analysis of purified enzymes is underway. It is of interest that other workers have identified a variant form of TS with properties similar to the novel form produced by HCT 116 cells. Bapat *et al.* (7) isolated a FdUrd-resistant derivative of the human leukemia cell line CCRF-CEM and demonstrated that it contains an altered TS with decreased affinity for FdUMP. Their kinetic studies indicated that the rate constant for association between the variant enzyme and FdUMP is reduced by about 14-fold (7). Inasmuch as both the CCRF-CEM and the HCT 116 TS variants exhibit altered FdUMP binding and a more basic pI, it is possible that they represent identical mutations in the TS polypeptide. However, in contrast to the CCRF-CEM variant (7), the HCT 116 variant does show a reduced affinity for CH_2H_4PteGlu (Fig. 4), indicating that the two may in fact be different mutations.

An important question that arises from these studies is whether the TS variant identified in HCT 116 represents a polymorphism that is segregating within the human population or a spontaneous mutation that occurred during establishment and/or maintenance of the cell line. The individual from which HCT 116 was established may have been heterozygous for two alleles of the TS structural gene; such heterozygosity could have been maintained in HCT 116 but lost from HCT 116a and HCT 116b, which were established from the same tumor as HCT 116 (16, 17) but express only the common form of TS (18). Thus, loss of alleles and/or chromosomes may have occurred in the progenitors to these cell lines within the tumor from which they were isolated. This phenomenon may not be uncommon in colorectal carcinoma (29–31).

Most likely, the variant TS form is generated by one or more changes in the amino acid sequence. It will be important to determine the nature of such changes. The availability of cells overproducing either the novel or the common form of TS (Fig. 2) will facilitate purification of the two forms for detailed structural and kinetic studies. Furthermore, they provide a source for cloning cDNA sequences corresponding to the mRNAs that encode the two TS species. The purified proteins, as well as the cDNA clones, will be valuable in identifying the amino acid differences between the two TS forms and should lead to the development of molecular probes, such as monoclonal antibodies and oligonucleotides, that are specific to one or the other form; such probes could have utility in predicting

clinical response in patients being considered for fluoropyrimidine chemotherapy. Finally, the X-ray crystallographic analysis of variant forms of the enzyme, such as that found in cell line HCT 116, should be useful in high resolution studies of TS structure and its interaction with ligands, leading to improvements in the design of TS-targeted drugs (32).

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