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5-Fluorouracil-mediated Thymidylate Synthase Induction in Malignant and Nonmalignant Human Cells

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ABSTRACT. Thymidylate synthase (TS, EC 2.1.1.45) is an important target enzyme for the fluoropyrimidines used in cancer chemotherapy. Studies have documented a 2- to 4-fold induction of TS protein following 5-fluorouracil (5-FU) treatment of malignant cells. We measured the effect that 5-FU exposure had on TS protein expression in nonmalignant human breast (MCF-10 and HBL-100), colorectal (ATCC Co18, Co112, and Co33), and bone marrow cells along with malignant breast (MCF-7) and colon (NCI-H630) cells. Twenty-four hours after plating, cells were treated with 0.01 to 10 μM of 5-FU for a period of 24 hr. TS was quantitated by Western immunoblot using monoclonal antibody TS106. Absolute levels of TS in nonmalignant cells were substantially lower than in the malignant lines, ranging from approximately 40% in HBL-100 cells to less than 10% in the colon lines. An approximately two-fold induction in the level of TS was found for all cell lines examined, and there was a strong dependence on 5-FU exposure concentration in free TS levels of MCF-WT, and total TS levels of H630-WT, normal bone marrow, and MCF-10 cells. The induction of TS following 5-FU exposure is a generally observed phenomenon in both malignant and nonmalignant cells, suggesting that a selective means for inhibiting this induction may be critical for the development of alternative therapeutic strategies using 5-FU and the antifolate TS inhibitors. BIOCHEM PHARMACOL **56**;2:231–235, 1998.

KEY WORDS. TS; 5-FU; chemotherapeutic drugs; enzyme induction; translational autoregulation

TS‖ is the enzyme responsible for catalyzing the methylation of dUMP to dTMP. Thymidylate is a required precursor for DNA synthesis and repair. Because the TS-catalyzed reaction provides the sole *de novo* source of dTMP, TS is considered a critical enzyme target for cancer therapy. The fluoropyrimidines and certain antifolates specifically target TS. Several lines of evidence including the correlation between TS levels and chemosensitivity [1–4], and the relative clinical successes of leucovorin modulation of 5-FU and the specific antifolate TS inhibitors [5–7], suggest that TS is a critical clinical target. Detailed studies have revealed that TS protein binds its own mRNA as a unique mechanism of autoregulatory translational control [8, 9]. Several investigators using both *in vitro* and *in vivo* systems have observed that upon exposure to 5-FU, TS protein levels increase several fold [1–3]. Studies in tumor samples taken from patients following treatment with 5-FU also demonstrate TS induction by 2- to 3-fold within 24 hr after drug exposure [3]. This induction may be explained by the proposed model wherein unliganded TS binds to its own mRNA and decreases translational efficiency. When TS is complexed with FdUMP, the active metabolite of 5-FU, it releases or fails to bind to its own mRNA, which in turn causes a cessation of translational repression with resultant TS protein induction [9]. The accumulation of TS protein in response to 5-FU and antifolate inhibitors of TS represents one of the methods by which malignant cells gain resistance to these chemotherapeutic agents [10]. Considering the importance of TS as a determinant of sensitivity to 5-FU therapy, a possible clinical strategy to enhance the efficacy of TS inhibitors would be to interdict TS protein induction. Given that the translational regulation of TS is presumed to also occur in nonmalignant cells, we undertook these studies to define potential clinically exploitable differences in TS induction between malignant and nonmalignant cells.

MATERIALS AND METHODS

Cell Culture

The human colon cancer cell line NCI H630 and the human breast cancer line MCF-7 were maintained in RPMI

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‖ *Abbreviations:* 5-FU, 5-fluorouracil; and TS, thymidylate synthase.

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1640 medium supplemented with 10% dialyzed fetal bovine serum (dFBS) and 2 mM of glutamine (Life Technologies). HBL-100, a nonmalignant human breast epithelial cell line, was grown in McCoy's medium (Life Technologies) with 10% dFBS and 2 mM of glutamine, while a second human breast epithelial cell line, MCF-10 (obtained from ATCC), was maintained as previously described by Soule *et al.* [11]. Nonmalignant colon epithelial cell lines obtained from the ATCC [Co112 (CRL 1541), Co18 (CRL 1459), and Co33 (CRL 1539)] were grown in Eagle's Minimum Essential Medium (Gibco) with Earle's salts, 10% dFBS, and 0.1 mM of non-essential amino acids. Co112 cells also required 1 mM of sodium pyruvate. All cells were maintained at 37° in a 5% humidified CO_2 incubator in 75-cm^2 tissue culture flasks (Falcon Labware). Human bone marrow cells were obtained from human normal volunteers after informed consent following an NCI-approved protocol. Bone marrow aspirate (10–15 mL) was drawn into a syringe containing preservative-free heparin. The cells were diluted 1:4 in Hanks' Balanced Salt Solution (HBSS) and separated on a Ficoll-sodium diatrizoate gradient (lymphocyte separation medium). The cells were collected and washed twice with HBSS, and then were resuspended in enriched Iscove's Modified Dulbecco's Medium (IMDM) with 10% heat-inactivated FBS.

Treatment of Cells with 5-FU

5-FU at various concentrations was added to cells in log growth phase. Following a 24-hr drug exposure, cells were washed twice with PBS, pH 7.4 (1.2 mM of potassium phosphate monobasic, 154 mM of sodium chloride, and 5 mM of sodium phosphate dibasic) and harvested from the flasks following a 10-min incubation in 3 mL of 0.05% trypsin in 0.53 mM of EDTA (Life Technologies). Cell pellets were collected in 15-mL tubes by centrifugation for 10 min at 1000 *g*. The PBS was aspirated and replaced with 100 μL of 0.05 KH_2PO_4 buffer, pH 7.2. Cell lysates were prepared by sonication, followed by centrifugation in an Eppendorf refrigerated microfuge at 15,000 *g* for 15 min at 4°. Protein concentrations were determined by the Bio-Rad method (Bio-Rad Laboratories) [12].

Western Blot Analysis

Cytosols containing equal amounts of total protein (50 μg protein for malignant and 200 μg of protein for nonmalignant lines) were resolved on a 12.5% polyacrylamide gel by electrophoresis according to the method of Laemmli [13]. Gels were then electrotransferred onto nitrocellulose membranes (Schleicher & Schull). Nonspecific sites were blocked by a 30-min incubation in Blotto (5% instant nonfat milk, 10 mM of Tris, 0.01% thimerosal), after which the membranes were washed with PBS/0.1% Tween 20, and incubated in TS106 antibody, diluted 1:200 in Blotto. After thorough washing and another 5-min incubation in Blotto, the blots were overlaid with anti-mouse secondary antibody conjugated with horseradish peroxidase (Bio-Rad). Protein bands representing complexed and free TS were detected using the ECL method (Amersham). TS protein detected on the blots was quantitated by scanning densitometry using a Microtech Scanmaker III and data analysis with National Institutes of Health Image version 1.6 (Wayne Rasband, NIMH). Western blot analysis revealed TS protein in both the free form and complexed with FdUMP/5, 10-methylenetetrahydrofolate in the bound form [14]. Together, free and bound TS comprised the total absolute amount present. Quantitation of protein induction was accomplished by comparison of amounts detected in treated and untreated (control) cells, and expressed as fold increase over the control cells.

Statistical Methods

Because the free and total TS changes were not normally distributed, comparisons between malignant and nonmalignant cell lines were performed using the Wilcoxon rank sum test. Jonckheere's test for trend [15] was used to examine the association between increasing 5-FU concentration and TS changes. In view of the six equivalent statistical tests performed, it is warranted to interpret the *P* values obtained in the context of a Bonferroni correction for multiple tests. This means that *P* values of less than 0.008 = 0.05/6 would be interpreted as indicating statistical significance, while *P* values between 0.008 and 0.05 would reflect strong trends. All *P* values are two-sided and denoted by *P*2.

RESULTS

TS Protein Induction

We investigated the extent of total and free TS protein induction following a 24-hr exposure to 5-FU in eight cell lines. Figures 1 and 2 show the effect of 5-FU on TS expression at four concentrations (0.01 to 10 μM). Levels of TS induction were calculated as fold increase over TS expressed in untreated control cells. Although the nonmalignant cell lines typically express substantially lower levels of TS than the malignant lines (ranging from 40% in HBL-100 to 10% in the colon lines), all lines responded to 5-FU treatment with a similar relative extent of TS induction. The median induction in total TS for all cell lines was approximately two-fold. Accordingly, total TS induction between the malignant and nonmalignant lines was not significantly different, as shown in Table 1. However, the comparison between malignant versus nonmalignant free TS levels yielded a statistically greater level of induction in the nonmalignant lines ($P2 = 0.0033$) as tabulated in Table 1. In addition to comparing nonmalignant with malignant cell lines, we also investigated the effect of drug concentration on the level of TS induction. As summarized in Table 2, total TS levels increased in a statistically significant fashion in proportion to drug exposure in the H630 and the MCF-10 cell lines,

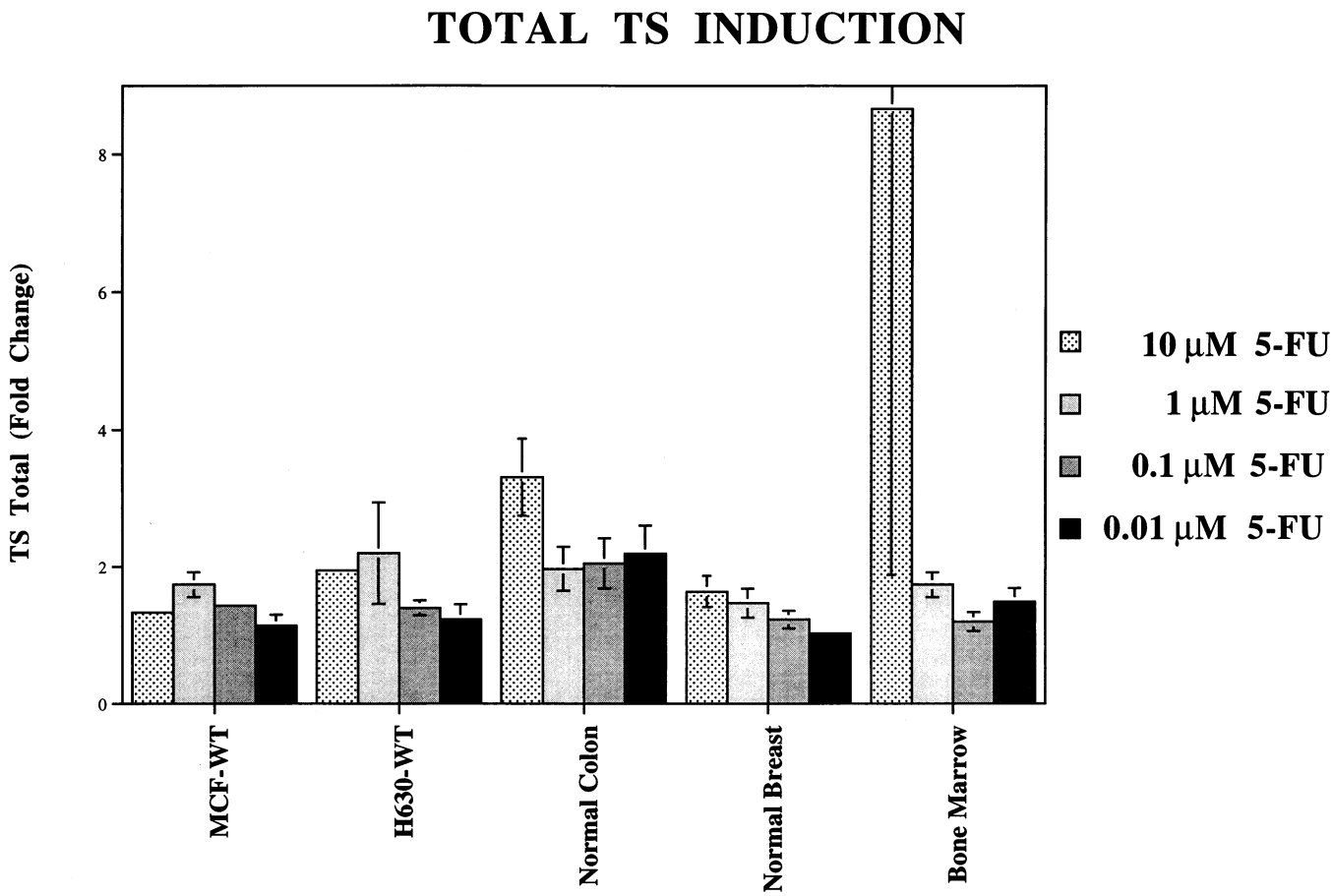

FIG. 1. Extent of induction of total TS protein following 24-hr treatment with 5-FU at the indicated concentrations, as quantitated by scanning densitometry of Western blots of resultant cell lysates. TS induction is expressed as fold increase over TS in control cells left untreated. Values are means ± SEM, N = 3–12.

and exhibited a strong trend toward significance in normal bone marrow cells, while the total TS levels of nonmalignant colon, MCF-WT, and HBL-100 lines did not exhibit a significant dependence upon 5-FU concentration. Free TS increased in proportion to 5-FU exposure only in the MCF-7 breast cancer cell line. In no other case was the degree of free TS induction related to exposure concentration.

DISCUSSION

This study demonstrates that acute induction in cellular levels of TS occurred in both malignant and nonmalignant cell lines exposed to 5-FU. The degree of induction was similar across all cell lines tested, and in some cases there appeared to be a dependence upon exposure concentration. Other investigators (including prior studies from our laboratory) have demonstrated a similar inductive process in a variety of *in vivo* and *in vitro* model systems and in patients undergoing therapy with the fluoropyrimidines [1–3]. The underlying cellular mechanism of TS induction has been investigated, and it has been postulated to result from enhanced translational efficiency resulting from release of the negative factor of TS binding to its own mRNA [8, 9]. This autoregulatory process of translational control is unique in eukaryotic cells, but similar autoregulatory mechanisms have been described in prokaryotes. The most extensively investigated prokaryotic system involves the translational autoregulation of the bacteriophage R17 replicase gene by its own coat protein [16].

It has been demonstrated that the cellular level of TS is a critical determinant of sensitivity to the TS inhibitors [1, 4]. Given our understanding of the TS autoregulatory system, it seems reasonable to expect that therapeutic strategies can be developed to inhibit the TS induction, thus sensitizing cells to the cytotoxic effects of TS inhibitors of both the antifolate and fluoropyrimidine classes. One obvious question focuses on the degree of enzyme induction in malignant versus nonmalignant cells with the concept that selectivity in addition to sensitivity of TS inhibitors may be favorably manipulated. While previous work has suggested that inhibition of TS induction results in enhanced sensitivity of cells to 5-FU [2], these studies did not address the issue of selectivity of the effect on malignant versus nonmalignant cells. The present investigation demonstrates that both malignant and nonmalignant cells undergo a similar degree of TS induction and, therefore, suggests that a selective advantage may not be gained by manipulating the inductive process with a strategy that

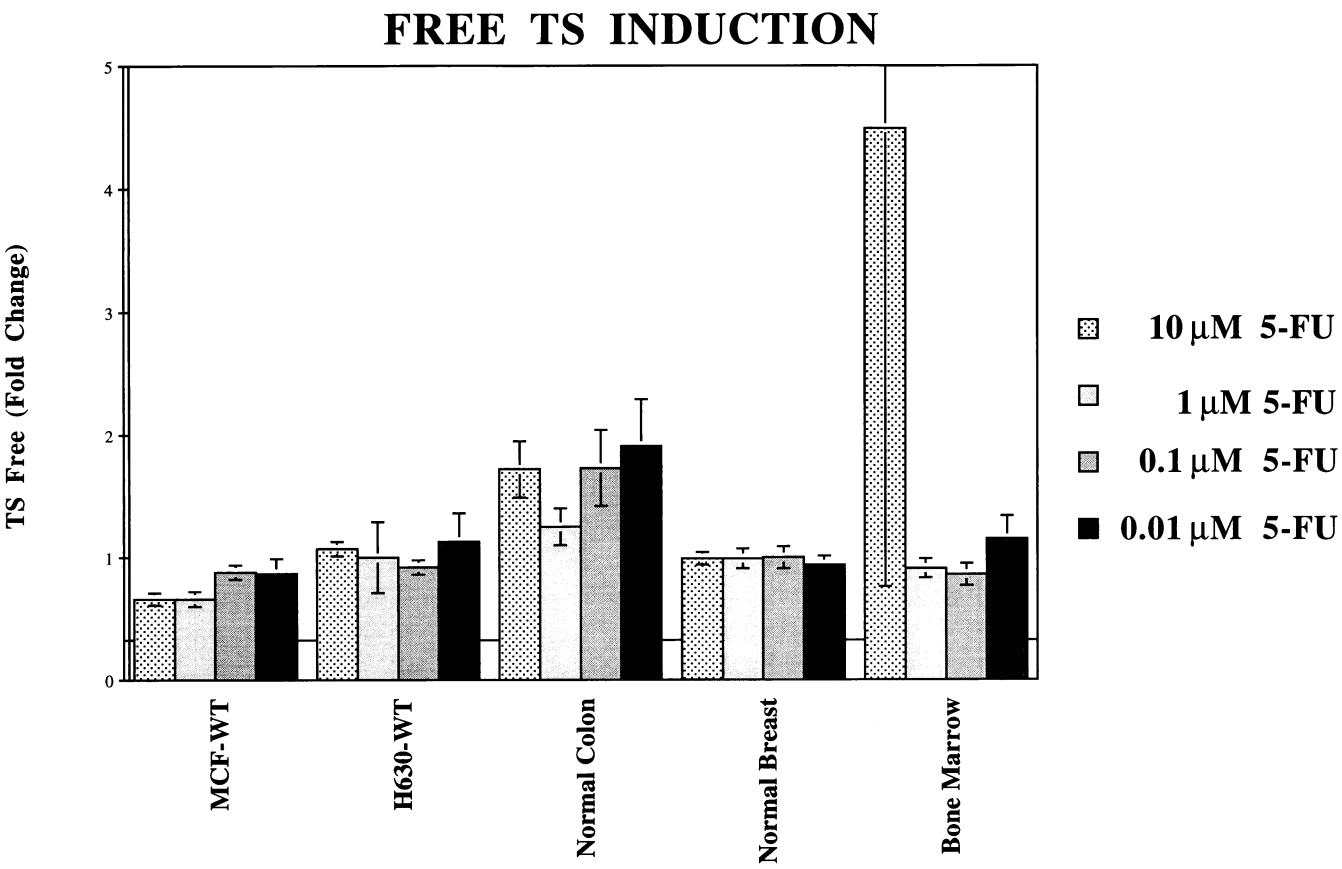

FIG. 2. Extent of induction of free TS protein following 24-hr treatment with 5-FU at the indicated concentrations, as quantitated by scanning densitometry of Western blots of resultant cell lysates. TS induction is expressed as fold increase over TS in control cells left untreated. Values are means ± SEM, N = 3–12.

itself is not selective. These studies provide evidence that the induction of TS following exposure to TS inhibitors is a general phenomenon not restricted to malignant cells and suggest that a selective means for inhibiting the induction of TS will need to be designed if such inhibition is to serve as the basis for the development of more effective alternative clinical strategies. However, it is clear that despite the 2- to 10-fold lower absolute levels of TS in nonmalignant cells, therapy with TS inhibitors is relatively selective in favor of more cytotoxicity in the malignant and higher expressing cells, a result not intuitively obvious given the general observation that sensitivity to TS inhibitors is inversely related to TS levels [1–3]. Thus, even a nonselective means for inhibiting TS induction may have clinical utility due to the multitude of other differences between malignant and nonmalignant cells.

In summary, these studies demonstrated that the induction of TS following 5-FU exposure is a general phenomenon observed in both malignant and nonmalignant cell lines, and the degree of induction was similar among the lines studied. These data suggest that the focus of future studies should be on the development of selective strategies for the inhibition of TS induction rather than a general, nonselective approach which, while it may sensitize malignant cells to the effects of TS inhibitors, may not enhance the therapeutic index of these inhibitors.

TABLE 1. TS induction (fold increase) following exposure to 5-FU

Cell type	$\bar{x}$	N	SEM
		Total TS*	
Breast cancer (MCF-7)	1.40	18	0.07
Colon cancer (H630-WT)	1.81	24	0.17
Cancer lines combined	1.64	42	0.10
Normal bone marrow	2.56	13	1.08
Non-malignant breast cells (MCF-10)	2.06	16	0.23
Non-malignant breast cells (HBL-100)	1.10	30	0.24
Non-malignant colon epithelium (Co18, Co33, Co112)	2.46	31	0.24
All non-malignant lines combined	1.95	90	0.19
		Free TS†	
Breast cancer (MCF-7)	0.76	18	0.04
Colon cancer (H630-WT)	1.05	24	0.07
Cancer lines combined	0.92	42	0.05
Normal bone marrow	1.48	13	0.56
Non-malignant breast cells (MCF-10)	1.11	16	0.06
Non-malignant breast cells (HBL-100)	0.91	30	0.03
Non-malignant colon epithelium (Co18, Co33, Co112)	1.62	31	0.13
All non-malignant lines combined	1.27	90	0.10

Statistical analysis of pooled data from the indicated number of experiments in which total and free TS induction were measured following a 24-hr exposure to 0.01 to 10 μM of 5-FU.

*†Comparison of malignant cells versus nonmalignant cells: *$P2 = 0.68$ for total TS changes; and †$P2 = 0.0033$ for free TS changes.

TABLE 2. Tests for the trend of free or total TS with increasing concentration of 5-FU

	Two-tailed *P* values from Jonckheere's test for trend*	
Cell line (group)	**Free**	**Total**
MCF-WT (breast cancer)	0.024	0.50
H630-WT (colon cancer)	0.20	0.0078
Normal bone marrow	0.95	0.028
Nonmalignant colon epithelium	0.70	0.12
MCF-10 (nonmalignant breast cells)	0.74	0.0026
HBL-100 (nonmalignant breast cells)	0.44	0.20

Statistical analysis of the extent of both free and total TS induction in the indicated cell lines following a 24-hr 5-FU exposure to determine whether 5-FU concentration is significant in TS induction.

*In view of the six equivalent statistical tests performed, it is warranted to interpret the *P* values presented in the context of a Bonferroni adjustment for multiple tests. This means that *P* values of less than 0.008 = 0.05/6 would be considered significant, whereas *P* values between 0.008 and 0.05 would indicate strong trends.

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