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Immunological quantitation of thymidylate synthase–FdUMP–5,10-methylenetetrahydrofolate ternary complex with the monoclonal antibody TS 106

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Thymidylate synthase (TS) is responsible for the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate. One of the principal mechanisms of action of 5-fluorouracil (5-FU) is the inhibition of TS by formation of a ternary covalent complex consisting of TS–5-fluorodeoxyuridylate–5,10-methylenetetrahydrofolate. We have developed a Western immunoblot assay using the monoclonal antibody TS 106 to measure ternary complex and free TS in intact human carcinoma cells following exposure to either 5-FU alone or 5-FU plus leucovorin. Lysates from cells treated with either 5-FU or 5-FU/leucovorin were resolved in 15% polyacrylamide gel, transferred onto nitrocellulose and immunoblotted using TS 106 antibody. Detection of positive bands was by a chromogenic substrate strain. Immunoblotting detected free TS at 36 kDa and TS in ternary complex at 38.5 kDa which were quantitated by densitometric scanning. This assay was able to detect a ternary complex from intact cells treated with 5-FU or 5-FU/leucovorin up to 96 h after drug removal. The ratio of complex to free TS was up to 2-fold greater in 5-FU/leucovorin-treated cells compared to those treated with 5-FU alone. This assay may be applied to measuring the formation and stability of ternary complex and free TS in patient tissue samples.

Key words: 5-Fluorouracil, monoclonal antibody, thymidylate synthase.

Introduction

Thymidylate synthase (TS) catalyzes the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (TMP). This reaction is critical in the *de novo* synthesis of thymidylate, a requirement for DNA biosynthesis. Thymidylate synthase is the target enzyme for the fluoropyrimidines, 5-fluorouracil (5-FU) and fluorodeoxyuridine (FUdR), which are used in the treatment of gastrointestinal, breast, and head and neck cancers.[1,2] One of the principal mechanisms of action of the fluoropyrimidines is inhibition of TS by the formation of a tight-binding covalent ternary complex consisting of TS, the active anabolite fluorodeoxyuridine monophosphate (FdUMP) and the folate cofactor 5,10-methylenetetrahydrofolate (5,10-methylene-H_4PteGlu).[3]

The modulation of 5-FU by reduced folates has attracted intense interest in both the laboratory and the clinic. Numerous studies have documented the enhanced cytotoxicity of 5-FU by the co-administration of leucovorin (5-formyltetrahydrofolate, citrovorum factor; 5-formyl-H_4PteGlu).[4] The biochemical rationale for this combination relates to the fact that high levels of intracellular-reduced folates are necessary for the optimal binding and inhibition of TS by FdUMP. 5-Formyl-H_4PteGlu is converted intracellularly to 5,10-methylene-H_4PteGlu, which promotes the formation and stabilization of ternary complex.[5]

One of the most commonly used conventional methods of quantitating free TS has been by a biochemical assay that measures the amount of radiolabeled FdUMP bound to the TS enzyme.[6,7] The assay is accomplished by allowing the formation of a covalent complex consisting of [^{3}H]FdUMP–TS–5,10-methylene-H_4PteGlu. Free [^{3}H]FdUMP not bound in this complex is then separated either by adsorption to activated charcoal or filtration through G-25 Sephadex. A modification of this method was devised to quantitate TS ternary complex.[8] Free TS is measured as described above and total enzyme is quantitated by dissociating the TS ternary complex in the presence of excess radiolabeled FdUMP in 0.6 M ammonium bicarbonate buffer, pH 8. Unlabeled FdUMP bound to TS is allowed to exchange with labeled FdUMP and serves as a measure of the total enzyme. These assays have several limitations when applied to the measurement of TS in human tissue specimens and cell lines. The TS enzyme must be enzymatically

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active and able to bind FdUMP; thus, enzyme activity may be underestimated in samples containing proteolytic enzymes. Moreover, the majority of human tumor specimens have TS enzyme levels at the lower range of sensitivity of the biochemical assay.[2] Our laboratory has recently developed a panel of monoclonal antibodies to human TS which are highly specific in their ability to detect TS in human cells and tissues.[9] In this study, we describe the development of an immunological method using the TS 106 antibody for the detection and quantitation of TS as free enzyme and as ternary complex in human tumor cell lines treated with 5-FU.

Materials and methods

Cell culture

The characteristics of the human colon cancer cell lines NCI $H630_{wt}$ and NCI $H630_{R10}$ have been previously described.[10] The NCI $H630_{R10}$ cell line was selected for resistance *in vitro* by exposure to step-wise increases in 5-FU and is 35-fold resistant to 5-FU when compared with the NCI $H630_{wt}$ cell line. The cells were maintained in RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% dialyzed fetal calf serum (Gibco, Grand Island, NY) and 2 mM glutamine (Gibco), and grown in 75 cm^2 plastic culture flasks (Falcon Labware, Oxnard, CA) at 37°C in a humidified, 5% CO_2 incubator.

An equal number of cells (1×10^5/ml) from each line were plated onto 75 cm^2 flasks. Twenty-four hours following plating, the cells were exposed to 5-FU (10 μM) in the presence or absence of leucovorin (10 μM). After 24 h, the cells were washed with two 10 ml aliquots of phosphate buffered saline (PBS) and placed in fresh media. Cells were subsequently harvested at various times up to 96 h after the removal of drugs. Cells were harvested from the plates using a 10 min incubation in 5 ml of 0.05% trypsin in 0.05 mM EDTA. Cell pellets were collected in 15 ml tubes (Falcon) by 10 min centrifugation at 500 *g*. Pellets were resuspended in 1 ml of PBS, transferred to Eppendorf tubes and centrifuged for 10 min at 500 *g*. PBS was aspirated and replaced with 100 μl of 0.1 M KH_2PO_4 buffer, pH 7.2. Cell lysates were prepared by sonication of cells using three 10 s bursts from a Branson sonifier equipped with a microtip, followed by centrifugation at 15 800 *g* in an Eppendorf refrigerated microfuge for 15 min. Proteins were determined by the BioRad method.[11]

Western blot analysis

Equal amounts of cell lysate (100 μg) were resolved by polyacrylamide gel electrophoresis using 15% acrylamide gels under denaturing conditions according to the method of Laemmli.[12] The gels were electroblotted onto a nitrocellulose membrane (Schleicher and Schüll, Keene, NH), immersed in transfer buffer (48 mM Tris, 39 mM glycine, 0.5 M EDTA in 20% methanol) for 2 h using 150 mA.

Immunoblotting was accomplished using the monoclonal antibody TS 106 followed by colorometric detection using 4-chloro-1-napthol (Sigma, St Louis, MO). Following electrotransfer, blots were washed twice with 50 ml of PBS and placed in blocking solution (5% non-fat milk, 10 mM Tris, 0.01% thimersol) for 1 h. Blots were washed with 50 ml PBS and incubated with mouse monoclonal antibody TS 106 (20 μg/ml) in blocking solution for 2 h, rinsed twice with 50 ml of PBS (5 min each), then washed three times for 5 min each with 50 ml of blocking solution.

Blots were next incubated for 1 h with goat anti-mouse secondary antibody conjugated with horseradish peroxidase (BioRad) diluted 1:1000 in 50 ml of blocking solution. They were subsequently washed four times in 50 ml of PBS for 5 min each wash, then placed in 50 ml of development solution, 2.8 mM 4-chloro-1-naptho (Sigma), to which 25 μl of 30% H_2O_2 was added just prior to use. All reactions and incubations were performed at room temperature.

Quantitation of TS protein from autoradiographs

TS protein visualized on the blots was quantitated by densitometric scanning using a HP Scan Jet digital imager and an image analysis software (NIH IMAGE v. 1.38; Wayne Rasband, National Institute of Mental Health).

Results

To determine whether the immunoblotting technique could distinguish ternary complex from free unbound TS enzyme, we prepared cytosolic extracts from the NCI $H630_{wt}$ cell line. Parallel incubations were then performed with one fraction containing excess 5,10-methylene-H_4PteGlu (300 μM) and FdUMP (10 μM), allowed to incubate at room temperature for 30 min and a second used as a

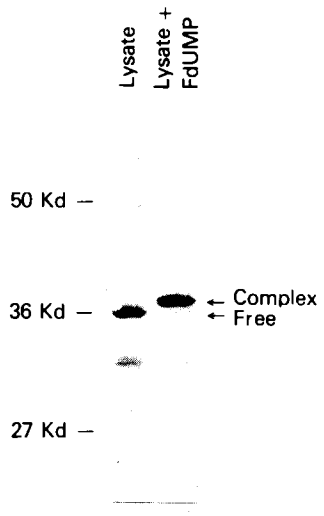

Figure 1. Western blot analysis of cell lysate from human colon carcinoma cell line NCI $H630_{wt}$ either untreated or exposed to FdUMP (10 μM) and 5,10-methylene-H_4PteGlu (300 μM) for 30 min. Lysates (200 μg) were resolved on 15% SDS–polyacrylamide gels transferred to nitrocellulose and immunoblotted with TS 106 monoclonal antibody.

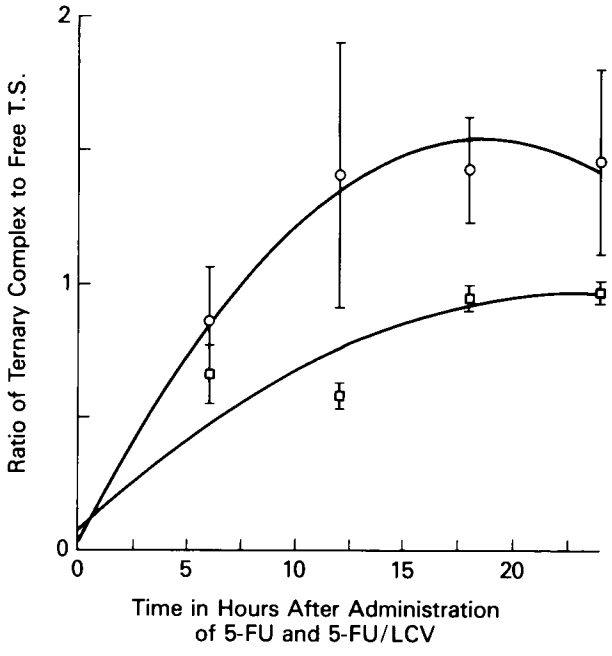

Figure 2. Ratio of bound:free TS complex versus time for NCI $H630_{R10}$ cell lysates treated with either 10 μM 5-FU (□) or 10 μM 5-FU plus 10 μM leucovorin (○).

control to which no folate or FdUMP was added. Both cytosolic fractions were resolved on a 15% polyacrylamide gel under denaturing conditions, transferred to nitrocellulose and immunoblotted using the TS 106 antibody. In the control fraction, only the unbound native TS could be detected (36 kDa); however, in the cytosol exposed to excess folate and FdUMP, a 38.5 kDa band representing ternary complex was visualized above the native TS band (Figure 1).

The immunoblotting technique was next applied to measure the ratio of ternary complex to free TS in NCI $H630_{wt}$ and NCI $H630_{R10}$ cells exposed to either 5-FU (10 μM) alone or 5-FU (10 μM) plus leucovorin (10 μM) for various time intervals up to 24 h. In cytosol preparations from untreated control cells only free TS was apparent, while in 5-FU- and 5-FU/leucovorin-treated cells two protein bands, one at 36 kDa representing free TS and the other at 38.5 kDa representing ternary complex, were detected. Within 6 h of drug exposure, ternary complex was apparent in both NCI $H630_{wt}$ and NCI $H630_{R10}$ cells following either 5-FU or 5-FU/leucovorin treatment. The intensity of the ternary complex band continued to increase, reaching a maximum after 18–24 h of drug exposure (Figure 2). Free and complexed enzymes were also examined in both cell lines following 24 h exposure to either 5-FU or 5-FU leucovorin. Complex was detectable in cells up to 96 h after they had been removed from either 5-FU- or 5-FU/leucovorin-containing media (Figures 3 and 4). In both NCI $H630_{wt}$ and NCI $H630_{R10}$ cells the ratio of ternary complex to free TS was maximal between 18 and 24 h after the start of exposures to 5-FU or

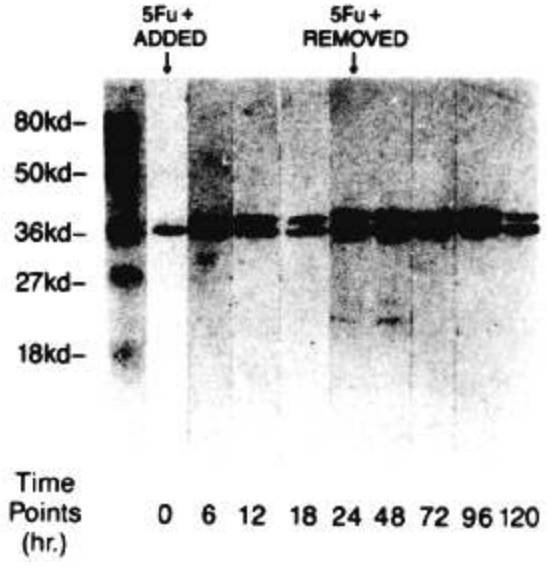

Figure 3. Western blot analysis of cell lysates from human colon carcinoma cell line NCI $H630_{wt}$ exposed to 10 μM 5-FU for 24 h. Drug was removed and sequential lysates prepared up to 96 C. Lysates (200 μg) were resolved on 15% SDS–polyacrylamide gels, transferred to nitrocellulose and immunoblotted with TS 106 monoclonal antibody.

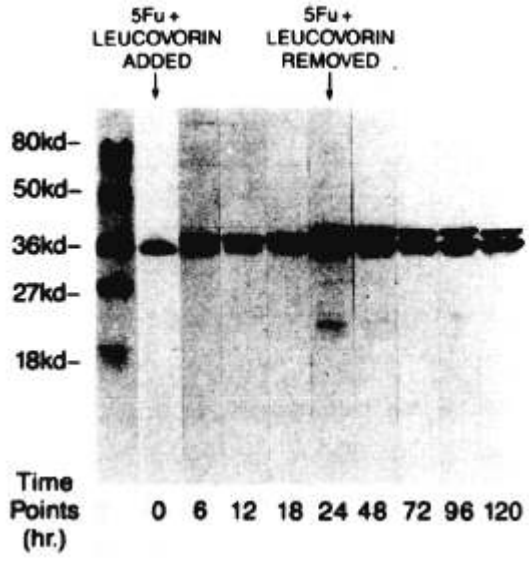

Figure 4. Western blot analysis of cell lysates from human colon carcinoma cell line NCI $H630_{wt}$ exposed to 10 μM 5-FU and 10 μM leucovorin for 24 h. Following drug removal, sequential lysates were prepared and resolved by SDS–polyacrylamide gel electrophoresis.

5-FU/leucovorin. However, the ratio of ternary complex to free TS in both cell lines was consistently higher (up to 2-fold) in the 5-FU/leucovorin-treated cells compared to those treated with 5-FU alone (Table 1).

Discussion

TS is an important therapeutic target for the fluoropyrimidines. In the present study, we have described the development and application of an immunological method using the TS 106 antibody for the detection and quantitation of free and complexed TS in 5-FU- and 5-FU/leucovorin-treated cells. We have demonstrated that this technique can distinguish complexed from free TS enzyme within the same cell lysate and, using densitometry scanning, we have been able to quantitate the relative amount of each form of the enzyme. We have shown that the ratio of ternary complex to free TS is greater in 5-FU/leucovorin-treated cells than in cells treated with 5-FU alone in both wild-type and TS-overproducing cell lines. The amount of ternary complex formed is greatest 18–24 h after cells have been exposed to drug. Moreover, while the complexed TS decreases with time, it remains detectable for up to 96 h after cells have been removed from drug-containing medium.

Previous methods of quantitating ternary complex have relied on the binding of radiolabeled FdUMP to the intact TS enzyme and the subsequent removal of the free unbound FdUMP by adsorption to either activated charcoal or a G25 Sephadex column.[7] Electrophoretic methods have relied on the separation of bound complex labeled with tritiated FdUMP using a 9% non-denaturing polyacrylamide gel.[13,14] These assays are tedious and subject to enzyme loss through proteolysis and, therefore, may underestimate the true amount of enzyme. Furthermore, the assays lack adequate sensitivity to measure low levels of ternary complex, particularly those found in human tumor samples.[2] In contrast, the immunological assay does not depend on the functional state of the TS enzyme and free, bound and total TS may be measured simultaneously in a single sample. Furthermore, the immunologic assay has been shown to have

Table 1. The ratio of ternary complex (FdUMP–TS–5,10-methylene-H_4PteGlu) to free TS (C/F) at various time points after treatment with 5-FU or 5-FU/leucovorin

Time points (h)	NCI $H630_{wt}$		NCI $H630_{R10}$	
	FU (10 μM)	FU (10 μM)/ leucovorin (10 μM)	FU (10 μM)	FU (10 μM)/ leucovorin (10 μM)
0	0	0	0	0
6	0.71 ± 0.12	1.31 ± 0.09	0.66 ± 0.11	0.86 ± 0.2
12	0.73 ± 0.24	1.46 ± 0.14	0.58 ± 0.05	1.41 ± 0.5
18	0.84 ± 0.14	1.24 ± 0.12	0.95 ± 0.05	1.43 ± 0.2
24	0.88 ± 0.11	1.75 ± 0.3	0.97 ± 0.04	1.46 ± 0.35
48	0.62 ± 0.02	2.01 ± 0.02	0.76 ± 0.19	1.04 ± 0.25
72	0.5 ± 0.03	0.66 ± 0.05	0.68 ± 0.18	1.02 ± 0.4
96	0.5 ± 0.06	0.6 ± 0.07	0.62 ± 0.15	0.63 ± 0.02
Overall p-value of FU versus FU/leucovorin	$p = 0.001$		$p = 0.03$	

approximately 10-fold greater sensitivity (0.3 fmol) when compared with the radiolabeled FdUMP binding assay (5.0 fmol).[10]

In conclusion, we have developed an immunological method for the detection and quantitation of bound, free and total TS in human cancer cells. This technique may be applicable to studies measuring ternary complex formation and response to fluoropyrimidine-based regimens in human tumor samples.

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