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QUANTITATION OF THYMIDYLATE SYNTHASE IN HUMAN TUMORS USING AN ULTRASENSITIVE ENZYME-LINKED IMMUNOASSAY

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Abstract—Thymidylate synthase (TS; EC 2.1.1.45) is an important therapeutic target for fluoropyrimidine cytotoxic drugs that are widely used for the treatment of solid tumors. Using the monoclonal antibody TS 106, we have developed an ultrasensitive enzyme-linked immunoassay (ELISA) for the detection and quantitation of TS. Using a chemiluminescent ELISA technique, TS was detectable in serially diluted lysates from NCI H630 and HCT 116 human colon carcinoma cell lines. The ELISA assay was reliably able to detect activity down to a level of 30 attamol of TS protein above background ($P_2 = 0.016$). The usable range of detection was from 0.03 to 500 fmol of enzyme. There was a close correlation between the optical density signal and the total TS enzyme between both cell lines ($r^2 = 0.96$). The ELISA was used to measure TS in cytosolic extracts from human tumor samples, and it was able to quantitate TS levels using as little as 1-mg tumor biopsy samples. The mean total TS measured by ELISA in seven tumor samples from patients with breast cancer and sarcomas was 131 fmol/mg cytosolic protein (range 60–240) compared with a mean TS of 85 fmol/mg cytosolic protein (range 35–163) using the fluorodeoxyuridine monophosphate binding assay. While the TS levels were uniformly higher when measured by ELISA, there was close proportional agreement between both assays ($r^2 = 0.84$). Thus, the chemiluminescent TS ELISA would appear to be an extremely sensitive and specific assay that may be used to quantitate TS in tumor tissue specimens.

Thymidylate synthase (TS§; EC 2.1.1.45) catalyzes the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), an essential step in DNA biosynthesis [1, 2]. TS is also a critical target for the fluoropyrimidine drugs that are widely used for the treatment of gastrointestinal, breast, and epithelial cancers of the upper aerodigestive tract [3–6]. Both 5-fluorouracil (5-FU) and fluorodeoxyuridine (FUdR) are converted in tumor cells to fluorodeoxyuridine monophosphate (FdUMP), which forms a tight-binding covalent complex with TS in the presence of the folate cofactor 5,10-methylenetetrahydrofolate ($CH_2H_4PteGlu$).

The conventional methods of quantitation and detection of TS have been by enzymatic biochemical assays that measure the amount of radiolabeled FdUMP bound to TS following extraction of the enzyme from cells and tissues or the catalytic activity of the enzyme in intact cells or cell-free systems [7–9]. These assays have several limitations, particularly when applied to the measurement of TS activity in human tissue samples and cell lines. Both the catalytic and FdUMP binding assays require that the enzyme is active; therefore, they are limited to prospective studies using fresh or fresh–frozen tissues with the caveat that no enzyme degradation has occurred during tissue harvesting and preparation. The lower range of sensitivity of the TS FdUMP binding assay is 5 fmol of TS enzyme [9]. While previous studies have demonstrated a large variation of TS levels in tumor tissue, 10–500 fmol/mg tumor cytosol, the majority of tumors have TS levels at the lower range of sensitivity of the FdUMP assay given the small amount of tumor available in patient biopsies [10, 11].

We recently developed several monoclonal antibodies to human TS that are highly specific and detect TS in the cytoplasm of human cells and tissues [12, 13]. A TS enzyme-linked immunoassay (ELISA) using these antibodies would have the advantage of being a convenient method to measure TS in small samples of cell lysate or tumor tissue. It would facilitate the study of primary and metastatic tumor samples and allow detailed correlations to be made between the level of TS and various clinical and morphological parameters. Thus, the development of a quantitative TS ELISA would help determine whether the quantity of TS within a given tumor or cell provided information that is valuable in patient selection for therapy and prognosis.

In this study we describe the development of an

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§ Abbreviations: TS, thymidylate synthase; ELISA, enzyme-linked immunoassay; 5-FU, 5-fluorouracil; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; FUdR, fluorodeoxyuridine; $CH_2H_4PteGlu$, 5,10-methylenetetrahydrofolate; FdUMP, fluorodeoxyuridine monophosphate; PMSF, phenylmethylsulfonyl fluoride; KH_2PO_4, potassium phosphate; and PBS, phosphate-buffered saline.

ultrasensitive ELISA for the quantitation of TS in human tumors using the monoclonal antibody TS 106. The utility and reliability of this assay for measuring TS levels are evaluated by comparison to a standard biochemical assay.

MATERIALS AND METHODS

Cell culture. The characteristics of the human colon cancer cell lines HCT 116 and NCI H630 have been described previously [14, 15]. The cells were maintained in RPMI-1640 (Biofluids Inc., Rockville, MD) with dialyzed fetal bovine serum (Gibco Inc., Grand Island, NY) plus 2 mM glutamine and grown in 75 cm^2 plastic culture flasks (Falcon Labware, Oxnard, CA). Dextran (clinical grade), bovine albumin fraction V, and acid-washed activated charcoal were purchased from the Sigma Chemical Co. (St. Louis, MO). [6-^{3}H]5-FdUMP (sp. act. 18 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Chemiluminescent ELISA kits were obtained from Tropix (Bedford, MA). Microlite II ELISA plates were purchased from Dynatech (Chantilly, VA). All other chemicals were obtained from Sigma or the National Institutes of Health (Bethesda, MD) stores.

Preparation of tumor and cell lysates. Tumor biopsies were obtained from patients with breast cancer at the time of surgery and immediately frozen on dry ice. The tumor tissue was weighed and cut into small fragments using a scalpel. Fat and fibrous tissue were removed. Tumor fragments were incubated in 500 μL of 0.1 M potassium phosphate (KH_2PO_4), pH 7.4, containing protease inhibitors [0.1 mM phenylmethylsulfonyl fluoride (PMSF), 3.3 μg/mL aprotinin, 10 μg/mL leupeptin]. Tumor samples were subsequently homogenized at 4° using a ground-glass tissue homogenizer followed by sonication using a Branson sonicator equipped with a microtip. The lysates were centrifuged at 5000 *g* for 30 min at 4° and the supernatants collected.

The human cell lines in the log phase of growth were washed with phosphate-buffered saline (PBS) twice, harvested, and resuspended in 1 mL KH_2PO_4, pH 7.4. Cell lysis was accomplished using three 3-sec bursts from a Branson sonicator. The cell lysates were processed in a fashion similar to the tumor lysates.

TS chemiluminescent ELISA. A Microlite II 96-well plate (Dynatech) was coated with 50 μL of serially diluted cell lysate in coating buffer (0.015 M $NaHCO_3$, pH 9.0). Each well was subsequently dried in an oven at 60°. One row of each ELISA plate contained 1 μg/mL casein as an internal protein control. The wells were washed two times with wash buffer (0.2% I-block reagent, 0.1% Tween 20, in PBS) and incubated with 100 μL of blocking buffer (0.5% I-block reagent, 0.1% Tween, in PBS) for 2 hr. After washing the wells in 50 μL wash buffer, 10 μg/mL mouse monoclonal antibody TS 106 was added and incubated at room temperature for 2 hr; 10 μg/mL control mouse IgG_1 was also added to separate wells as a control. The unbound antibody was removed with four additional washes using wash buffer, then incubated with an alkaline phosphatase-conjugated secondary antibody (1/10,000 dilution in 0.2% I-block reagent, 0.1% Tween, in PBS) for 30 min. The unbound secondary antibody was removed with four further washes in wash buffer and two washes in assay buffer (0.1 M diethanolamine, 1 mM $MgCl_2$, 0.02% sodium azide). Substrate solution containing Sapphire Enhancer (10%), adamantyl-1,2-dioxetane phosphate (AMPPD 0.24 mM) in assay buffer was added and incubated at room temperature for 20 min. The chemiluminescent signal was measured after 20-, 30- and 40-min incubations using a Chemiluminescent ELISA reader (Dynatech).

TS FdUMP binding assay. The assay was performed according to previously published methods [9] in a total volume of 200 μL containing 50 μL tumor cytosol or cell lysate, 75 μM CH_2H_4Pte-Glu, 3 pmol [^{3}H]FdUMP, 100 mM 2-mercapto-ethanol, and 50 mM KH_2PO_4, pH 7.4. Samples were incubated at 37° for 30 min, and subsequently 1 mL of albumin-coated charcoal slurry (prepared by mixing 10 g acid-washed activated charcoal with 2.5 g bovine albumin, 0.25 g dextran, and 100 mL ice-cold water), pH 7.2, was added. The mixture was vortexed, allowed to stand at room temperature for 10 min, and then centrifuged for 30 min at 5000 *g*. The residual radioactivity representing enzyme-bound FdUMP in the supernatant was counted by liquid scintography. The results were standardized for cytosolic protein measured by the method of Bradford [16] and expressed in femtomoles of enzyme per milligram of cytosolic protein.

RESULTS

We initially determined the total TS activity in NCI H630 and HCT 116 cells using the FdUMP binding assay. The total TS levels in NCI H630 and HCT 116 cells were 0.31 ± 0.05 and 0.22 ± 0.03 pmol/mg cytosolic protein, respectively. Serial dilutions were then made from each cellular lysate into Microlite II 96-well plates. The total amount of TS present in each well was calculated using the dilutional factor. Lysates from each cell line were run in triplicate at each dilution. The chemiluminescent light-emission signal for each TS concentration was calculated by subtracting the background signal (signal generated by the non-specific mouse IgG_1 antibody) from the mean TS signal.

To determine the lower level of sensitivity of the assay, replicate experiments were compared using the signed rank test to determine whether the observed TS signal (observed value − background value) was significantly different from the background value for low TS concentrations (Fig. 1). Based on this analysis, TS protein of 30 attamol or above gave signals that were reliably above background in both cell lines ($P_2 = 0.016$).

The range of TS that could be quantitated using the chemiluminescent ELISA in NCI H630 and HCT 116 cells was from 30 attamol to 500 fmol of TS enzyme. There was close agreement between the optical density measurements and the total TS enzyme and between both cell lines for individual ELISA assays ($r^2 = 0.96$) (Fig. 1).

Measurement of TS levels in tumor cytosols. Tumor

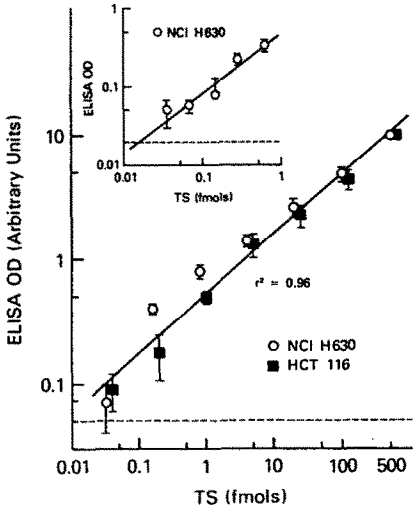

Fig. 1. Correlation of TS activity as measured by the radiolabeled FdUMP binding assay versus the ELISA assay in NCI H630 (○) and HCT 116 (■) cells. The inset represents the lower range of sensitivity of the chemiluminescent ELISA assay in NCI H630 cells. The dotted lines represent the average background optical density signal. These values are means ± SD of six separate experiments.

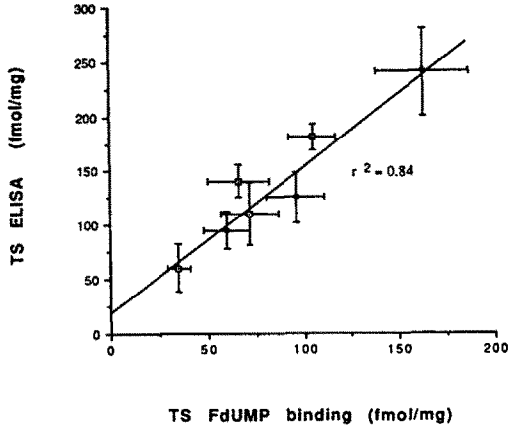

Fig. 2. Correlation of TS activity as measured by radiolabeled FdUMP binding assay versus ELISA in four breast tumor (⊡) and three sarcoma (◆) specimens. Each cytosol was assayed for TS using the FdUMP binding and ELISA assay on three separate occasions, and the values are means ± SD.

cytosols were prepared from four breast tumor and three sarcoma specimens obtained at surgery. Each cytosol was assayed for TS using the FdUMP binding assay on three separate occasions. By FdUMP analysis the TS level in these tumors ranged from 35 to 163 fmol/mg cytosolic protein, with a mean TS of 85 fmol/mg cytosolic protein (breast = 69.5 fmol/mg, sarcoma = 106 fmol/mg) (Fig. 2). The tumor cytosols used in the biochemical assay were also analysed using the chemiluminescent assay. The NCI H630 cell cytosol was used to determine the standard curve for each Microlite II plate. The chemiluminescent ELISA assay was able to detect TS with optical density readings that were at least an order of magnitude greater than background. By ELISA analysis the TS levels ranged from 60 to 240 fmol/mg cytosolic protein, with a mean TS concentration of 131 fmol/mg cytosolic protein (breast = 123 fmol/mg, sarcoma = 140 fmol/mg). A comparison of both assays revealed that the TS levels in tumors were uniformly higher (36%) when measured by ELISA assay compared with the FdUMP binding assay; however, there was a close linear agreement between both assays ($r^2 = 0.84$) (Fig. 2).

We also performed the ELISA on several small pieces of tumor tissue (25, 10 and 1 mg in weight) to determine the sensitivity of the assay using tumor tissue. TS enzyme was detectable in all three specimens assayed by the ELISA method. Fifty microliters of supernatant from a 1-mg sample of tumor tissue that had been homogenized in 200 μL lysate buffer (0.25 mg protein) gave signals that were 12-fold background activity. Using the entire tissue sample for the FdUMP binding assay, only background counts could be obtained from each of these small samples (1–25 mg). Thus, the TS chemiluminescent ELISA assay is a specific and sensitive technique and can be reliably applied to the measurement of TS in tumor cytosols.

DISCUSSION

TS is an important cellular enzyme that plays a role in DNA nucleotide synthesis and represents a therapeutic target for the fluoropyrimidine antineoplastic group of drugs. In the present study, we have described the development of an ultrasensitive ELISA using the monoclonal antibody TS 106 to quantitate TS protein in human tumor cytosols.

Our results have demonstrated that the ELISA assay is a reliable and sensitive technique for the quantitation of TS enzyme in human tumor cytosols. We have shown that the values obtained by the ELISA assay are directly proportional to the amount of TS measured by the FdUMP binding assay in human cancer cell lines ($r^2 = 0.96$). Moreover, we have demonstrated previously that the chemiluminescent signal generated by the TS antibody–TS protein interaction is highly specific as demonstrated by the presence of a single 36 kDa band on chemiluminescent western immunoblotting [13]. The ELISA technique is capable of detecting as little as 30 attamoles of TS protein and represents a 200-fold increase in sensitivity compared with the published sensitivity of the conventional FdUMP binding assay (5 fmol) [9]. Despite the apparent sensitivity of the FdUMP binding assay using tissue culture cells, previous investigations have found the sensitivity of this assay to be more limited in tissue samples (10–20 fmol) [11, 17].

Close agreement was also noted between both assays when applied to the measurement of TS in

tumor tissue ($r^2 = 0.84$). The enhanced sensitivity of the ELISA permitted the quantitation of TS using as little as one quarter of the cytosol obtained from 1 mg of tumor tissue. In cytosolic extracts from tumor tissue, the mean TS value was significantly higher when measured by ELISA (131 fmol/mg cytosolic protein) compared with the FdUMP binding assay (85 fmol/mg cytosolic protein). The ELISA is standardized to TS extracted from cell lines and measured by the FdUMP binding assay. The discrepancy between the two assays may represent degradation of TS resulting from tissue proteolytic activity during procurement and processing. Previous studies from this laboratory have shown that the addition of soybean trypsin inhibitor leupeptin, chymostatin, aprotinin and EDTA is important for maintaining the functional activity of the enzyme [11]. Thus, tissue-associated proteolysis would result in underestimation of TS levels by the FdUMP binding assay. Another reason for this discrepancy would include the presence of an inactive form of TS within the cell or the presence of high concentrations of dUMP within the cytosol that could potentially inhibit [^{3}H]FdUMP binding. In contrast, the ELISA assay is able to detect all immunoreactive enzyme regardless of its functional integrity.

The clinical importance of TS in the development of tumor resistance has been suggested by studies that have demonstrated an acute induction of TS protein as well as stable gene amplification of TS [18–21]. While the underlying mechanisms involved in enhanced TS expression have not been fully defined, these *in vivo* and *in vitro* studies suggest that the ability of a tumor to overexpress TS in response to cytotoxic agents may play a role in the development of tumor resistance. Thus, the measurement of TS enzyme may be critical to understanding the biochemical and molecular mechanisms involved in antimetabolite resistance and as a potential prognosticator of survival and/or response to fluoropyrimidine-based regimens.

In summary, this study demonstrates that the chemiluminescent TS ELISA is a reliable ultrasensitive test that may be applied to the quantitation of TS in human tumor specimens. The availability of sensitive quantitative ELISA for detecting total TS will permit a determination of the prognostic importance of TS levels in patient tumor samples.

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