Acta Oncologica Vol. 35, No. 4, pp. 469–472, 1996

THYMIDYLATE SYNTHASE IN ADVANCED GASTROINTESTINAL AND BREAST CANCERS

PER-ANDERS LARSSON, GÖRAN CARLSSON, BENGT GUSTAVSSON and C. PAUL SPEARS

Knowledge of population thymidylate synthase (TS) levels in malignant tumors and normal tissues is essential for the use of TS as a predictor for 5-fluorouracil treatment. Tumor tissue TS levels in fresh frozen surgical biopsies from 136 patients with gastrointestinal or breast cancer, not previously subjected to chemotherapy, were analysed by [^{3}H]FdUMP radioligand binding assay. TS levels were 2.4 ± 0.31 pmol/g in liver metastases of colorectal cancer (n = 87), 4.2 ± 1.0 pmol/g in primary colorectal cancer (n = 13), 2.7 ± 0.93 pmol/g in gastric cancer (n = 13), 3.1 ± 1.7 pmol/g in pancreatic cancer (n = 10), 3.4 ± 1.4 pmol/g in breast cancer (n = 13) and 0.58 ± 0.075 pmol/g in normal liver parenchyma (n = 24). TS levels were significantly higher in malignant tumor tissues compared to normal liver parenchyma.

For patients with advanced local growth or metastases of gastrointestinal or breast cancer the prognosis is poor. Surgery and radiotherapy may be beneficial for localized problems, such as intestinal, biliary, or urinary obstruction, painful osseal metastases, or neural compression, but generally these measures do not affect survival. Palliative chemotherapy, based on fluorinated pyrimidines, can prolong survival with an improved quality of life for a significant number of patients (1, 2). As it is difficult to predict the individual response to chemotherapy in these patients (3), an understanding of the biochemical strategy of cancer cells will be of importance for the design of enzyme pattern-directed anticancer chemotherapy (4). Inhibition of thymidylate synthase (TS, EC. 2.1.1.45) has been proposed as the major mechanism for the effect of 5-fluorouracil (5-FU) against tumor cells (5, 6).

TS catalyses de novo thymidine synthesis in eukaryotic cells, i.e. methylation of deoxyuridylate (dUMP) to thymidylate (dTMP). The source of the methyl one-carbon group transferred by TS is polyglutamated 5,10-methylenetetrahydrofolate (CH_2FH_4). The expression of the TS gene is cell cycle associated and high in the S-phase. High TS-levels are found in tissues with high cell turnover such as tumors. Surveys of normal tissues, recently reviewed by Spears (7), have shown relatively high levels in thymus, spleen, bone marrow, and testis, and low levels in visceral hollow organs. It has also been suggested that the expression of the TS gene is inhibited by its own end product and increased by dUMP in an autoregulatory manner (8–10). High TS enzyme levels prior to treatment, possibly due to gene amplification in tumors, may be responsible for innate 5-FU resistance (11). However, data on baseline levels of TS in normal human tissues and malignant tumors are scarce (12, 13).

The aim of the present study was to measure TS-levels in previously untreated human gastrointestinal cancer, female breast cancer and normal liver parenchyma, and to establish baseline levels of TS activity when measured with the ligand binding (ternary complex formation) assay technique.

Received 12 July 1995.
Accepted 11 December 1995.
From the Department of Surgery, Östra Sjukhuset, Göteborg, Sweden (P.-A. Larsson, G. Carlsson, B. Gustavsson), and Comprehensive Cancer Center, University of Southern California, Los Angeles, California, USA (C.P. Spears).
Correspondence to: Dr P.-A. Larsson, Department of Surgery, Östra Sjukhuset, S-416 85 Göteborg, Sweden.

Material and Methods

Patients

In 160 patients operated on at the Department of Surgery, Östra Sjukhuset, Göteborg, tissue specimens were

 ISSN 0284-186X

analysed for TS binding activity. The patients were operated on due to clinical routine indications, and all tissue specimens for TS analysis were taken from fresh tumor specimens or tumor biopsies for routine histological examinations. Normal liver biopsies were taken from 24 patients operated on for cholecystolithiasis. Histological examination of these showed normal liver parenchyma and these patients had no previous history of malignant disease. Eighty-seven patients had liver metastases of colorectal cancer, 13 had primary colorectal cancer, 13 gastric cancer, 10 advanced pancreatic cancer, and 13 female patients had breast cancer. The patients were followed postoperatively according to clinical routines, but there was no follow-up study protocol. Patient characteristics, i.e., age and sex, and tumor characteristics such as intratumor TS, degree of differentiation, and Dukes' classification of primary colorectal cancer were registered. Tissue CH_2FH_4-content was also measured in 38 tumor specimens and in liver parenchyma from 11 patients. Informed consent was obtained from all patients before the biopsy procedure.

Tissue preparation

The tumor biopsies were immediately frozen in liquid nitrogen and stored at −70° C until the analyses of free TS and reduced folates were performed. Portions of frozen tumor tissue were then weighed and minced by means of a pair of scissors in a 10-fold excess of homogenisation buffer at 4°C. Homogenates were prepared by the action of rotating shearing blades (Turrax) for 30 s at 4°C.

Analysis of TS and methylentetrahydrofolate

The [^{3}H]FdUMP ligand-binding assay was used for the analysis of free TS (14). One ml of the homogenate (duplicate samples) was centrifuged at 4°C and 4 000 × g for 20 min. To 50 µl of the cytosol, 25 µl of homogenisation buffer, 50 µl [^{3}H]FdUMP (about 200.000 dpm) in H_2O and 25 µl of 46,2 µmole CH_2FH_4 was added. These procedures were carried out at 2–4°C. The tubes were incubated at 37°C for 10 min, and then 3 ml of 3% acid charcoal (rapidly stirred at 4°C) was added followed by vortex mixing and centrifugation at 4°C and 4 000 × g for 15 min to precipitate unbound radioactivity. Thereafter 0.8 ml of the supernatant was subjected to beta scintillation counting. TS concentrations were expressed as pmole bound FdUMP per gram tissue (wet weight).

Tumor tissue levels of CH_2FH_4 and FH_4 were assayed with some modifications according to the Priest methodology (15). To 50 µl aliquots of cytosol or homogenisation buffer alone (blank) were added 50 µl of the [^{3}H]FdUMP (about 800 000 dpm), 4 pmole of L. Casei TS in 25 µl of homogenisation buffer (with 0,2% BSA). Buffer with or without formaldehyde was used for the estimation of $FH_4 + CH_2FH_4$ and CH_2FH_4 respectively. These procedures were carried out at 2–4°C and the tubes were then incubated at 37°C for 10 min, whereafter 1 ml ice-cold 3% acid charcoal was added for separation of protein-bound radioactivity. The samples were then centrifuged at 4°C and 4 000 × g for 15 min, and scintillation counting performed. Folate concentrations were expressed as nmoles per gram tissue (wet weight).

Statistics

Differences between groups of patients were analyzed with a multifactorial ANOVA. Multiple regression analysis was performed for the analysis of variable correlation. All data are presented as mean ± standard error of the mean (SEM).

Results

Baseline TS levels were 2.4 ± 0.31 pmol/g in liver metastases of colorectal cancer (n = 87), 4.2 ± 1.0 pmol/g in primary colorectal cancer (n = 13), 2.7 ± 0.93 pmol/g in gastric cancer (n = 13), 3.1 ± 1.7 pmol/g in pancreatic cancer (n = 10), 3.4 ± 1.4 pmol/g in breast cancer (n = 13) and 0.58 ± 0.08 pmol/g in normal liver parenchyma (n = 24). The distributions of TS levels were skewed in both gastrointestinal and breast cancers (Fig. 1), but close to normally distributed in healthy liver parenchyma. Baseline TS levels were significantly higher in malignant tissues than in normal livers, but the differences between the various types of malignances were not statistically different. The TS level in primary colorectal cancers was higher than in hepatic metastases of colorectal tumors.

The degree of differentiation was correlated to tumor tissue TS levels (Fig. 2). The small number of primary

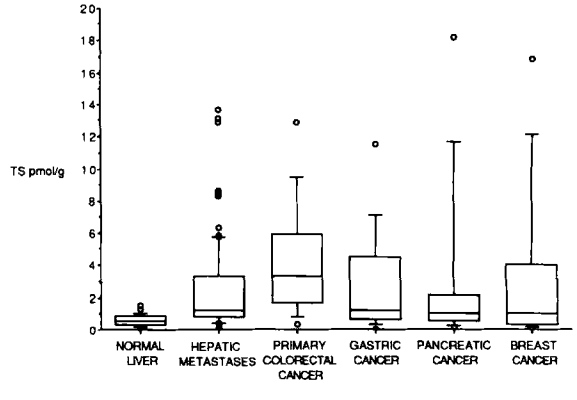

Fig. 1. TS levels in biopsies from normal liver parenchyma, (n = 24), liver metastases of colorectal cancer, (n = 87), primary colorectal cancer, (n = 13), gastric cancer, (n = 13), pancreatic cancer, (n = 10), and breast cancer, (n = 13). Lines and boxes represent the 10th, 25th, 50th, 75th and 90th percentile, circles represent individual extreme values below the 10th and above the 90th percentile. Baseline TS values in normal liver parenchyma are significantly lower than in the malignant tissues.

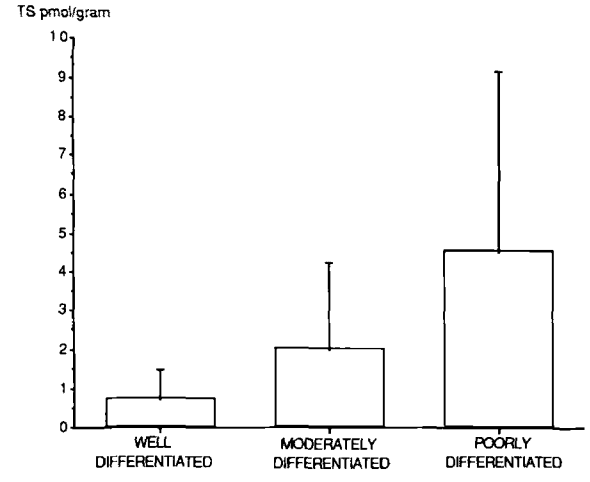

Fig. 2. Tumor tissue TS levels (mean ± SEM) in 136 cancer patients in relation to differentiation grade, well differentiated (n = 21), moderately differentiated (n = 66) and poorly differentiated (n = 49). The difference between poorly and well differentiated tumors was statistically significant ($p < 0.05$)

colorectal cancers did not allow reliable correlation analysis between Dukes' classification and intratumor TS levels.

In a limited number of samples, the levels of methylene tetrahydrofolates in tumor tissue were also measured but there was no significant correlation between folate and TS levels (Fig. 3). Mean CH_2FH_4 levels were in normal livers 2.2 ± 0.52 nmol/g (n = 11), in liver metastases of colorectal cancers 0.24 ± 0.08 nmol/g (n = 23), in gastric cancers 0.25 ± 0.9 nmol/g (n = 4), in pancreatic cancers 0.12 ± 0.09 nmol/g (n = 7), and in breast cancers 0.70 ± 0.28 nmol/g (n = 4). CH_2FH_4 was significantly lower in tumor tissue compared with normal liver parenchyma ($p < 0.05$) but the differences between different tumor types were not statistically significant. From primary colorectal cancers we did not obtain enough biopsy material for the analysis of CH_2FH_4.

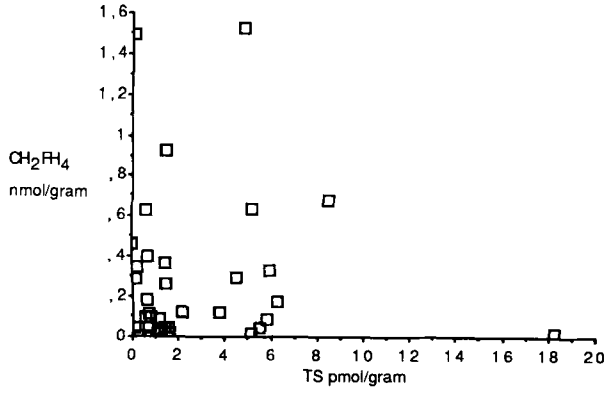

Fig. 3. Tumor tissue CH2FH4 levels in 49 cancer patients in relation to tumor tissue TS levels. There was no statistically significant correlation between the two parameters.

Discussion

The results in the present study reveal that TS levels are higher in malignant tissues than in normal liver parenchyma. Although no significant differences between tumor types were found, TS levels in malignant tumors were correlated to histological differentiation (16). Earlier reports, utilising different techniques, have also linked high TS levels to poor prognosis and metastatic spread in ovarian and colorectal cancer (12, 13, 17–19). In our previous studies of total and free TS levels after 5-FU therapy total TS in malignant tumors was significantly higher than in normal tissues (11, 20). However, in the earlier studies, free TS (TS_{free}) was found to be higher in liver metastases than in primary colorectal tumors. On the other hand it has recently been reported that TS protein levels are equal in primary tumors and lymph node metastases of lung cancers (21).

Baseline TS levels were found to be higher in primary colorectal cancers than in liver metastases. However, the time course of tissue TS after i.v. 5-FU injections makes it difficult to estimate the true baseline, pre 5-FU TS, from TS after i.v. 5-FU injections. Earlier studies regarding TS levels after 5-FU i.v. push injections have shown a decline of inhibition starting 60 min after the injection (11) in accordance with a rebound increase of TS_{free} (20). In a recent study we have also found a divergence of TS_{tot} levels measured in tumor tissues and normal livers from one to five hours after i.v. 5-FU injections (Spears et al. unpublished study). Increasing TS_{tot} has also been observed in experimental tumors resistant to 5-FU, whereas decreasing TS_{tot} levels were found after i.v. 5-FU injections in the experimental tumors sensitive to 5-FU (22). This divergence may be related to if apoptosis is induced and further sustains the theory that 5-FU as other cell cycle specific agents do not kill cells by their biochemical actions per se but by dissociation of normally integrated cell cycle events, with induction of programmed cell death (7). 5,10-CH_2FH_4-polyglutamate in combination with dUMP/FdUMP protects TS against proteolytical degradation which may be the key mechanism whereby the normal proteolytic mechanism of TS turnover is potentially slowed by TS protection within the TS-FdUMP-folatepolyglutamate ternary complex (23).

Measurements of TS in human tissues are associated with considerable problems due to low tissue levels of this enzyme. The samples in the present study had not been exposed to 5-FU and consequently FdUMP inhibition of enzyme posed no analytical problems. The advantages of the ligand binding assay of TS include high reproducibility and precision with a sensitivity requiring only 10 mg of tissue for duplicate assay (14, 24). The whole tissue is assayed and so the contribution of normal stromal elements is also included. If normal adjacent stromal cells produce a lot of thymidine, this could produce a bystander effect for contributing to DNA salvage pathway resistance

in the tumor cells, thus making whole tissue measurements important. The ligand binding assay is also conveniently performed in parallel with the folate assays. This helps to correct for tissue cellularity, in addition to describing the kinetic situation for optimal TS inhibition, predicting the response to 5-FU treatment and assessing the requirements for folate supplementation. Other available methods for TS analysis, such as the ^{3}H-displacement assay (25, 26), immunohistochemical labeling methods with monoclonal antibodies against TS protein (12), and reverse transcription polymerase chain reaction for the analysis of TS gene expression (27) may also be used for TS baseline studies. However, the optimal method for screening of large materials has not yet been determined.

Further prospective trials analysing tumor tissue TS and folate levels in relation to therapy response will be necessary in order to improve our tools for optimizing 5-FU + folate treatment in advanced cancer.

ACKNOWLEDGEMENTS

This work was supported by grants from The Swedish Medical Research Foundation (B94-04X-07155-10C and B94-17X-07184-10C), The Swedish Cancer Society (0512-B93-06XCC and 1081-B93-08XAC), Assar Gabrielsson Foundation for Cancer Research, Gustav V Jubilee Clinic Foundation for Cancer Research and NCI CA 39629.

REFERENCES

1. The Nordic Gastrointestinal Tumor Adjuvant Therapy Group. Superiority of sequential methotrexate, fluorouracil and leucovorin to fluorouracil alone in advanced symptomatic colorectal carcinoma: a randomized trial. J Clin Oncol 1989; 7: 1437–46.
2. The Nordic Gastrointestinal Tumor Adjuvant Therapy Group. Biochemical modulation of 5-fluorouracil: A randomized comparison of sequential methotrexate, 5-fluorouracil and leucovorin versus sequential 5-fluorouracil and leucovorin in patients with advanced symptomatic colorectal cancer. Ann Oncol 1993; 4: 235–40.
3. Peters GJ, van der Wilt CL, van Groeningen CJ, Smid K, Meijer S, Pinedo HM. Thymidylate synthase inhibition after administration of fluorouracil with or without leucovorin in colon cancer patients: Implications for treatment with fluorouracil. J Clin Oncol 1994; 12: 2035–42.
4. Weber G. Biochemical strategy of cancer cells and the design of chemotherapy: G.H.A. Clowes memorial lecture. Cancer Res 1983; 43: 3466–92.
5. Heidelberger C, Danneberg PB. Fluorinated pyrimidines: A new class of tumor inhibitory compounds. Nature 1957; 179: 663–6.
6. Danenberg P. Thymidylate synthase- A target enzyme in cancer chemotherapy. Biochem Biophys Acta 1977; 473: 73–92.
7. Spears C. Clinical resistance to antimetabolites. Heamatol Oncol Clin North Am 1995; 9: 397–413.
8. Chu E, Koeller D, Johnston P, Zinn S,Allegra C. Regulation of thymidylate synthase in human colon cancer cells treated 5-fluorouracil and interferon-g. Mol Pharmacol 1993; 43: 527–33.
9. Swain SM, Lippmann ME, Egan EF, Steinberg SM, Allegra CJ. Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. J Clin Oncol 1989; 7: 890–9.
10. van der Wilt CL, Pinedo HM, Smid K, Peters GJ. Elevation of thymidylate synthase following 5-FU treatment is prevented by the addition of leucovorin in murine colon tumors. Cancer Res 1992; 52: 4922–8.
11. Spears CP, Gustavsson BG, Berne M, Frösing R, Bernstein L, Hayes AA. Mechanisms of innate resistance to thymidylate synthase inhibition after 5-fluorouracil. Cancer Res 1988; 48: 5894–900.
12. Johnston P, Fisher E, Rockette H, et al. The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. J Clin Oncol 1994; 12: 2640–7.
13. Suzuki M, Ohwada M, Tamada T, Tsuru S. Thymidylate synthase activity as a prognostic factor in ovarian cancer. Oncology 1994; 51: 334–8.
14. Spears CP, Gustavsson BG. Methods for thymidylate synthase pharmacodynamics: serial biopsy, free and total TS, FdUMP and dUMP, and H_4PteGlu and CH_2FH_4PteGlu assays. Adv Exp Med Biol 1988; 244: 97–106.
15. Priest DG, Bunni M, Sirotnak FM. Relationship of reduced folate changes to inhibition of DNA synthesis induced by methotrexate in L1210 cells in vivo. Cancer Res 1989; 49: 4204–9.
16. Carriaga MT, Henson DE. The histologic grading of cancer. Cancer 1995; 75: 406–21.
17. Leichman L, Danenberg P, Leichman C, et al. Intratumoral thymidylate synthase expression and response to 5-fluorouracil (5FU)-leucovorin (LV) in patients with colorectal cancer. Proc Am Assoc Cancer Res, 1993.
18. Gustavsson BG, Carlsson G, Frösing R, Spears CP. Pharmacodynamics of 5-FU and leucovorin. Adv Exp Biol Med 1988; 244: 219–24.
19. Spears CP, Carlsson G, Muggia FM, Waugh W, Jaresko G, Gustavsson BG. Aspects of one-carbon folate cycling related to fluoropyrimidine and antifolate therapy. In Muggia FM, ed. Chemotherapy: concepts, mechanisms, and new targets for chemotherapy. Boston, MA: Kluwer Academic Publ., 1994.
20. Spears CP, Gustavsson BG, Mitchell MS, et al. Thymidylate synthetase inhibition in malignant tumors and normal liver of patients given intravenous 5-fluorouracil. Cancer Res 1984; 44: 4144–50.
21. Mattern J, Volm M. Detection of resistance proteins in matched primary lung tumors and lymph node metastases. Anticancer Res 1994; 14: 417–20.
22. Spears CP, Shahinian AH, Moran RG, Heidelberger C, Corbett TH. In vivo kinetics of thymidylate synthetase inhibition in FUra-sensitive and resistant murine colon adenocarcinomas. Cancer Res 1982; 42: 450–6.
23. Galivan J, Maley F, Baugh C. Protective effect of pteroylpolyglutamates and phosphate on the proteolytic inactivation of thymidylate synthetase. Arch Biochem Biophys 1977; 184: 346–54.
24. Spears CP, Gustavsson BG, Frösing R. Folinic acid modulation of fluorouracil: tissue kinetics of bolus administration. Invest New Drugs 1989; 7: 27–36.
25. Roberts D. An isotopic assay for thymidylate synthetase. Biochemistry 1966; 5: 3546–8.
26. Houghton JA, Radparvar S, Torrance PM, Williams LG, Houghton PJ. Determination of thymidylate synthase activity in colon tumor tissues after treatment with 5-fluorouracil. Biochem Pharmacol 1987; 36: 1285–9.
27. Horikoshi T, Danenberg KD, Stadlbauer THW, et al. Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the polymerase chain reaction. Cancer Res 1992; 52: 108–16.