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THYMIDYLATE SYNTHETASE OF IN VITRO CHEMICALLY AND VIRALLY TRANSFORMED RAT CELLS

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Summary

Thymidylate synthetase (EC 2 1.1.-) from in vitro 3-methylcholanthrene- or Rauscher leukemia virus-transformed rat embryo cells was studied The enzyme from control or transformed rat cells exhibited a K_m for 2′-deoxyuridylic acid of 4 5 ± 0.2 μM, but the transformants had a higher level of enzyme activity than did control cells. Titration of the enzyme with tritiated 5-fluoro-2′-deoxyuridylic acid indicated that the increased enzyme activity in the transformants was due to a greater level of cellular enzyme. While the level of enzyme activity in control cells was dependent on both the availability of nutrients in the medium and cell density, the level of enzyme activity in the transformants appeared to depend only on the availability of nutrients.

Introduction

Thymidylate synthetase (methylenetetrahydrofolate dUMP-C-methyltransferase, EC 2 1.1.-) catalyzes the synthesis of deoxythymidylic acid (dTMP) from 2′-deoxyuridylic acid (dUMP) and 5,10-methylenetetrahydrofolate This reaction is the only known de novo pathway for the synthesis of dTMP and may be one of the rate-limiting steps for DNA biosynthesis [1] Furthermore, thymidylate synthetase is the target enzyme for 5-fluoro-2′-deoxyuridylic acid (FdUMP), which is the activated form of the potent tumor inhibitory drug 5-fluorouracil [2,3]

Elevated activities of thymidylate synthetase have been observed in rapid-

Abbreviations The abbreviations used are dUMP, 2′-deoxyuridylic acid, dTMP, deoxythymidylic acid, FdUMP, 5-fluoro-2′-deoxyuridylic acid The nomenclature for the cell lines is Rauscher leukemia virus transformant, rat embryo cells transformed with Rauscher leukemia virus, 3-methylcholanthrene transformants I and II, two sublines of rat embryo cells transformed with 3-methylcholanthrene

ly dividing cell populations in vivo [1,4] and in vitro [1,5,6] and in neoplastic cell populations [1,3,7,8] These observations suggest that an understanding of the cellular control mechanism which governs the expression of thymidylate synthetase may aid in the elucidation of differences between normal and neoplastic cells Comparative biochemical studies of tumors arising in vivo and normal tissue are complicated by the difficulty of assessing the progenitor cells of the tumor However, in vitro chemical and viral malignant transformation has been achieved [9,10] and it is now possible to compare biochemical properties of transformed cells to the normal cells from which they were derived

In the present study, thymidylate synthetase was investigated using in vitro 3-methylcholanthrene-transformed and Rauscher leukemia virus-transformed rat embryo cells [11] The K_m values and changes in the specific activity of the enzyme during the culture cycle were compared in control and transformed cells The ability of FdUMP to form an isolatable covalent complex with thymidylate synthetase [12—15] was used to determine the amount of enzyme in the control and transformed cells

Materials and Methods

Cells and cell culture Two control rat embryo cell lines, one Rauscher leukemia virus-transformed line and two 3-methylcholanthrene-transformed lines, were obtained from Drs A. Freeman and P. Price of Microbiological Associates, Bethesda, Md The derivation of the transformed lines and some of their characteristics have been reported by Freeman et al. [11] and by Ryan and Curtis [16] Cells used in the experiments were grown in Petri dishes (100 mm, Falcon Industries, Oxnard, Calif.) in Eagle's basal medium and 10% heat-inactivated fetal calf serum (both from Grand Island Biological Co., Grand Island, N Y) at 37° C in an atmosphere of 5% CO_2 in air without antibiotics or fungizone Tests for mycoplasma were negative. The procedures for determining plating efficiency, doubling time, saturation density, and for passing the cells have been described [17]

Enzyme assay Assay of thymidylate synthetase was accomplished by modification of the isotopic procedure of Roberts [18] After isolating the cells by trypsinization, subsequent steps were carried out at 4° C The cells were suspended in phosphate-buffered saline for counting and aliquots, which contained a known number of cells, were removed After centrifugation, the buffer was replaced with a known volume of the cofactor solution used for spectrophotometric assay of the enzyme [7] The cells were broken in this solution by two 10-s sonifications with a Sonifier Cell Disruptor (Heat Systems-Ultrasonic, Inc , Model W185) with a micro tip at an intensity of 30 W Microscopic examination indicated that more than 99% of the cells were disrupted. Aliquots (0 225 ml) were immediately preincubated for 5 min at 37° C prior to the addition of 25 μl of the [5-^{3}H]dUMP solution (4 0 · 10^7—3.2 10^8 cpm/μmol) The same procedure was used for all cell lines and the enzyme assays were initiated within 25 min after the removal of the cultures from the incubator The final concentrations of the components in 0 25 ml of the standard reaction mixture were 3 32 mM formaldehyde, 50 mM β-mercaptoethanol, 0 22 mM 5,10-methylenetetrahydrofolate, 0 1 mM dUMP and 100 mM potas-

sium phosphate, pH 6.8. An increase in the concentration of either substrate did not increase the reaction velocity. The reaction was terminated by adding 0.2 ml of 33% trichloroacetic acid, followed by 1 0 ml of charcoal solution (2 g/10 ml) The solution was mixed with a Vortex and allowed to stand for 10 min before centrifugation In most experiments it was necessary to re-centrifuge the supernatant to remove the charcoal completely. 0 2 ml of the clear supernatant was counted in a scintillation counter Blanks (trichloroacetic acid added prior to [5-^{3}H]dUMP) gave counts of 300—800 cpm The efficiency of the scintillation counter for both assay and titration samples (below) was determined by internal standards to be 17% Samples were counted until a minimum of 10 000 cpm above background were recorded

Enzyme titration The binding of 6-^{3}H-labeled FdUMP to thymidylate synthetase was measured by a previously reported procedure [13] A cell suspension ($25 \cdot 10^6$—$35\ 10^6$ cells) in phosphate-buffered saline was divided into three aliquots One aliquot (approx 10% of the cells) was used to determine soluble cellular protein, approx 20% of the cells for enzyme assay, and the remainder for binding studies. For binding, the cofactor solution and 6-^{3}H-labeled FdUMP were added simultaneously. The cells for protein determination and enzyme titration were then broken by sonification and incubated at 37°C for 2 h Both samples were centrifuged at 105 000 × *g* for 1 h to remove cell debris [7] The supernatants from the binding experiments were applied to a Sephadex G-25 column (0 7 × 30 cm) to separate the enzyme-bound inhibitor from the unbound inhibitor and other components of the reaction mixture [13] The enzyme eluted from the column was 100% inhibited Protein was determined in the cell supernatant and column effluent by the procedure of Lowry et al [19], with bovine serum albumin as a standard The enzyme-bound inhibitor was determined by liquid scintillation counting (efficiency of 17%) of aliquots of the effluent containing the protein. The lowest levels of bound 6-^{3}H-labeled FdUMP (3—4 times a background of 17 cpm) were obtained with confluent control I cells From the amount of protein per 10^6 cells and the dpm of FdUMP bound per μg protein, the data were calculated as pmol of FdUMP bound per 10^6 cells

Chemicals Reagents were obtained from the following sources β-mercaptoethanol from Eastman Chemicals, Rochester, N Y , (±), L-tetrahydrofolic acid, Sigma Chemical Co , St Louis, Mo , and [5-^{3}H]dUMP (55 mCi/mol), New England Nuclear, Boston, Mass Drs Danenberg and Heidelberger of McArdle Laboratory for Cancer Research (University of Wisconsin, Madison) generously supplied the 6-^{3}H-labeled FdUMP (1 2 Ci/mmol)

Results

Properties of the cell lines Selected characteristics of the rat embryo cells are presented in Table I Some properties of the rat cell lines and their electron microscopic appearances were previously reported [17]. All cell lines were passed in culture between 110 and 125 passages, therefore, passage number should not be a factor for comparisons The plating efficiencies of the transformants were between 90 and 95% and they had a more rapid doubling time than the control cells The transformed cells had a greater number of surface

TABLE I

PROPERTIES OF CONTROL AND TRANSFORMED RAT EMBRYO CELLS

Cell line	Plating efficiency * (%)	Generation time (h)	Saturation density (cells/100 mm dish)** ($\times 10^6$)
Control line I	54	24	6—7
Control line II	49	24	6—7
Rauscher leukemia virus transformant	94	17	13—17
3-Methylcholanthrene transformant I	91	12	12—20
3-Methylcholanthrene transformant II	90	n d	12—20

* Mean of 10 dishes

** Range from three separate studies, each conducted in duplicate

n d , not determined

microvilli and blebs [17] and showed little contact inhibition of growth, as they grew to saturation densities between 2 and 4 times that of the control cells (Table I)

Apparent K_m and V values Preliminary studies with the cell lines indicated the rate of enzyme reaction was linear for 55 min and linear with cell numbers between $1 \cdot 10^6$ and 8 10^6 cells per assay Therefore all enzyme reactions were conducted for 50 min with cell numbers between 3 10^6 and 8 10^6 per assay. The apparent K_m and V values for thymidylate synthetase from normal and transformed rat embryo cells, with dUMP as the variable substrate (5—36 μM) and 5,10-methylenetetrahydrofolate as the fixed substrate (0 22 mM), were determined from linear double reciprocal plots Apparent V values of 15, 55, and 1520 pmol TMP/h per 10^6 cells were observed for the enzyme from confluent cultures of the control, the Rauscher leukemia virus transformant, and the 3-methylcholanthrene transformant I cells, respectively The apparent K_m for dUMP of the enzyme from all cell lines was 4 5 ± 0 2 μM This value is in agreement with reported values for the enzyme from other mammalian cell systems [1,7] The data suggest that the increased enzyme activity in the transformants is not due to an alteration which affects the interaction of enzyme and dUMP Furthermore, the possibility of an endogenous enzyme activator in the transformed cells or an endogenous inhibitor in the control cells was eliminated by assay of mixtures of the two cell lines Mixture of normal and transformed cells prior to assay yielded the same total activity as the sum of their individual activities

Variation in thymidylate synthetase activity during the culture cycle Following subculture of the rat embryo cell lines, thymidylate synthetase was assayed at 24-h intervals for 6 days and the results are shown in Fig 1 The number of cells per dish and the enzyme activity per 10^6 cells are presented for the control and the two transformed cell lines For the contact-sensitive control rat embryo cells (Fig. 1A), the enzyme activity was elevated during the first 2 days of growth, but declined rapidly as the cultures approached confluency By microscopic examination, all cell lines appeared to have cell-to-cell contact

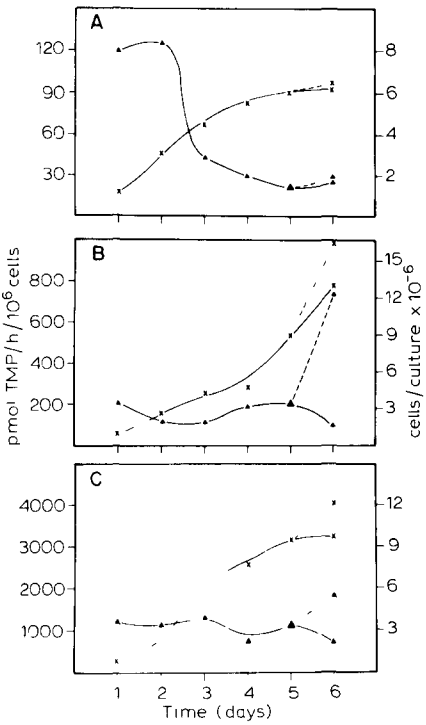

Fig 1 Culture cycle of normal and transformed rat embryo cells A, Control II cells, B, Rauscher leukemia virus transformant, C, 3-Methylcholanthrene transformant I 10^6 cells were seeded per dish on Day 0 for each cell line, and the cells harvested at 24-h intervals for 6 days For determination of Day 1 enzyme activity, cells from 5 to 10 individual cultures were mixed and assayed As the number of cells per culture increased, fewer cultures were required for assay and by Day 6, cells from 2 to 3 individual cultures were mixed for each assay The data are the mean of two experiments each conducted in duplicate The concentrations of dUMP and 5,10-methylenetetrahydrofolate were 0 1 and 0 22 mM, respectively X, number of cells per culture, ▲, thymidylate synthetase activity per 10^6 cells, - - - - -, effect of medium change on Day 5 on cell number and enzyme activity

at approx $5 \cdot 10^6$ cells per dish. The enzyme activity in the control cells then remained low, as the cells ceased to divide Even a medium change on Day 5 failed to stimulate a substantial increase in enzyme-specific activity or cell number. However, the Rauscher leukemia virus transformant (Fig 1B) and the 3-methylcholanthrene transformant (Fig 1C) showed different growth and enzyme activity patterns Both of these lines demonstrated a relatively constant thymidylate synthetase-specific activity and a continual increase in cell number throughout the 6-day culture cycle Furthermore, the addition of fresh medium on Day 5 to already "piled-up" cultures caused large increases in both the cell number and specific activity of the enzyme

Quantitative analyses of thymidylate synthetase in normal and transformed cells The fluorinated pyrimidine, FdUMP, is a potent covalent inhibitor of thymidylate synthetase and the enzyme-FdUMP complex can be isolated by gel filtration [13] This procedure can therefore be used to determine the number of enzyme molecules per cell The data in Table II show the amount of soluble protein per 10^6 cells, the enzyme activity per 10^6 cells, and the calculated pmol of FdUMP bound per 10^6 cells The data indicate that the trans-

TABLE II

QUANTITATIVE ANALYSIS OF THYMIDYLATE SYNTHETASE ACTIVITY AND FdUMP BINDING SITES

Cell line	μg protein/ 10^6 cells*	Enzyme activity (pmol/h per 10^6 cells)**	FdUMP bound (pmol/10^6 cells)***
Control line I	102 ± 10	50 ± 7	0 0710 ± 0 015
Control line I (at confluency)	78 ± 2	12 ± 4	0 025 ± 0 008
Control line II	n d	125 ± 14	n d
Rauscher leukemia virus transformant	81 ± 5	220 ± 20	0 148 ± 0 004
3-Methylcholanthrene transformant I	101 ± 3	1480 ± 85	1 13 ± 0 040
3-Methylcholanthrene transformant II	n d	260 ± 22	n d

* Mean ± S E , values from four determinations are rounded off to the nearest whole number

** Mean ± S E , determined on Day 2 or at confluency (Day 6) The concentrations of dUMP and 5,10-methylenetetrahydrofolate were 0 1 and 0 22 mM, respectively Each enzyme assay was carried out twice in duplicate

*** Mean ± S E , the concentration of 6-^{3}H-labeled FdUMP was 5 μM After gel filtration, the contents of two test tubes containing the cellular protein were each analyzed in duplicate for both radioactivity and protein Each binding experiment was carried out twice

n d , not determined

formants had a higher enzyme activity and greater FdUMP binding capacity than the control cells, but there was considerable variation of enzyme activity between the two control cell lines and between the two 3-methylcholanthrene transformants. Clonal variation of thymidylate synthetase activity has been previously reported for other cells in culture [5,20] Furthermore, the data in Table II, in agreement with data in Fig. 1A, show that both the enzyme activity and the FdUMP binding capacity decreased by factors of 3 to 4 as the control cells became confluent (Day 6)

Discussion

In the control rat embryo cells, at least two factors are involved in determining the level of enzyme activity during the culture cycle the availability of nutrients or serum factors and the degree of cell-to-cell contact (Fig 1A). The rapid decline in enzyme activity as the cells approach confluency indicates that the enzyme is closely regulated as cell-to-cell contact occurs Furthermore, contact-sensitive regulation of the enzyme cannot be overcome by the addition of fresh medium The decrease in enzyme activity as the cells approach confluency is probably due to both the dilution of enzyme concentration by cell division and the decreased enzyme synthesis, as a greater number of cells accumulate in the G_1 interval of the cell cycle [5,21] By contrast, the availability of nutrients or serum components appears to be the major factor affecting thymidylate synthetase levels in transformants, with cell-to-cell contact having little or no effect (Figs 1B and 1C)

The gel filtration procedure which utilizes 6-^{3}H-labeled FdUMP as an

active site titrant is a specific method for quantitatively analyzing thymidylate synthetase levels [13] Santi et al. [15] have described a nitrocellulose filter assay using radioactive FdUMP for the quantitative analysis of the enzyme From the titration data (Table II) and the assumption that each enzyme molecule has two binding sites [13,15], one can calculate that there are approx 3 4 $\cdot 10^5$ molecules of thymidylate synthetase per cell in the exponentially growing 3-methylcholanthrene transformant I cells and approx 2.1 $\cdot 10^4$ molecules of enzyme per cell in exponentially growing control I rat embryo cells

Because regenerating and rapidly proliferating tissues as well as neoplastic tissue have elevated thymidylate synthetase activity [1], increased levels of this enzyme may not be related to malignancy per se. However, the elevated and cell contact-insensitive thymidylate synthetase activity in the transformants was caused by oncogenic chemical or viral treatment of the control cells. Thus, treatment with these agents either directly or indirectly altered the level and cellular regulation of thymidylate synthetase Further studies utilizing in vitro transformed cells may aid in elucidating differences in the regulatory mechanisms for this enzyme as well as others in normal and neoplastic cells

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