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THYMIDYLATE SYNTHETASE AND THYMIDINE KINASE ACTIVITIES IN DMH-INDUCED COLON CARCINOMAS IN RATS AND EFFECTS OF UFT

BY

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ABSTRACT

Carcinoma of the colon was induced in rats by injection of a carcinogen 1,2-dimethylhydrazine (DMH), and thymidylate synthetase (TS) and thymidine kinase (TK) activities, which catalyze the biosynthesis of dTMP by the *de novo* pathway and the salvage pathway of pyrimidine synthesis, respectively, were measured in normal control colon, DMH-treated normal colon, and DMH-induced colon carcinoma with or without administration of two doses of an anti-cancer drug UFT (a combination of tegafur and uracil).

TS and TK activities were both increased after treatment with DMH, markedly in colon carcinoma tissue, and to a lesser degree in normal-appearing colon tissue. This phenomenon is well explained by the hypothesis that biochemical alterations of DNA-synthesizing enzyme activities occur as a preliminary step prior to the development of overt cancerous transformation.

A low dose of UFT inhibited TS activity but enhanced TK activity, therefore, the salvage pathway may compensate for the reduced level of the *de novo* synthesis. On the other hand, a large dose of UFT reduced both TS and TK activities, perhaps due to cytotoxic effects of UFT incorporation into RNA.

Key words: 1,2-dimethylhydrazine (DMH), thymidylate synthetase (TS), thymidine kinase (TK), UFT.

INTRODUCTION

It has been shown that carcinoma of the colon can be induced in rats by weekly subcutaneous injections of a carcinogen 1,2-dimethylhydrazine (DMH), and that such tumors are analogous to human colon carcinoma in their mode of growth and metastasis (Deschner [1]; Thurnherr *et al.* [2]). Such experimentally induced colon carcinoma has proved to be a useful model both for investigation of the mechanisms underlying carcinogenesis (Richards [3]; Maskens [4]), and for the evaluation of new anti-cancer therapies (Hamada *et al.* [5]).

Thymidylate synthetase (TS*; EC 2.1.1.45) is a key regulatory enzyme in the *de novo* pathway of pyrimidine synthesis; TS catalyzes the methylation of dUMP to form dTMP, with the concomitant conversion of N^5, N^{10}-methylenetetrahydrofolic acid to 7,8-dihydrofolic acid. TS activities have been studied in regenerating liver (Imura *et al.* [6]), in bone marrow cells (Sakamoto *et al.* [7]), and in neoplastic cells (Chello *et al.* [8]).

Thymidine kinase (TK*; EC 2.7.1.21)

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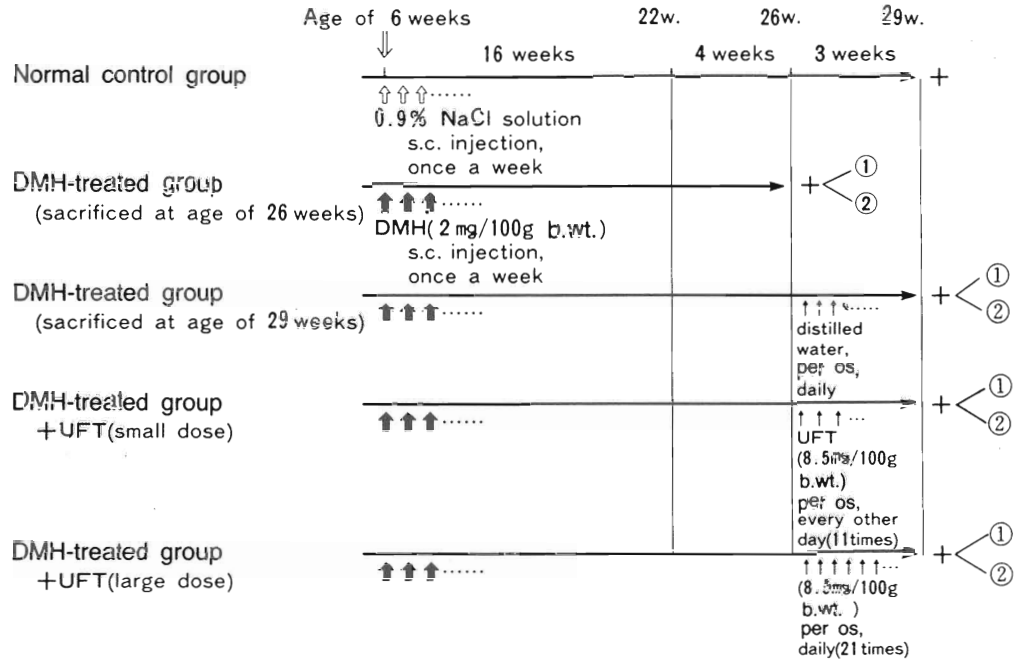
Table 1. Experimental Schedule



+: Sacrificed, specimens were divided into 2 groups (①DMH-treated normal colon, ②DMH-induced colon carcinoma)

catalyzes the formation of dTMP by the phosphorylation of thymidine in the salvage pathway (Hashimoto *et al.* [9]). High TK activities have been found in rapidly proliferating normal and neoplastic tissues (Taylor *et al.* [10]); Greengard *et al.* [11]; Sakamoto *et al.* [12]).

It has been reported that TK activity is increased in both human colon carcinoma (Herzfeld *et al.* [13]; Sakamoto *et al.* [14, 15]), and in DMH-induced murine colon carcinoma (Thurnherr *et al.* [2]; Weber *et al.* [16]). Furthermore, it has also been shown that increased TS activity in DMH-induced colon carcinoma is inhibited after administration of 5-fluorouracil (Spears *et al.* [17]).

In the present study, we investigated and compared changes in TS and TK activities in DMH-induced carcinogenesis, and used them as parameters to evaluate the effect of UFT on carcinoma of the colon.

Materials and Methods

I. Experimental protocol

As shown in Table 1, six-week-old male Donryu rats (Nihon Rat Co. Ltd., Japan) were divided into five groups of ten animals in each group. One group was designated as the normal control group, and received subcutanous injections of 0.9% NaCl solution once a week for 16 weeks, and was sacrificed 7 weeks later. The other four groups all received subcutaneous injections of the carcinogen, 1,2-dimethylhydrazine (DMH*; 2.0 mg/100 g of body weight; Nakarai Chemicals, Kyoto, Japan) once a week for 16 weeks. Induction of colon carcinomas was confirmed by testing the stool for occult blood. One DMH-treated group was sacrificed at the age of 26 weeks, and specimens of normal colon tissue ① and colon carcino-

ma tissue ② were removed and stored at −80°C. The other three groups received, respectively, daily oral administration of distilled water, oral administration of a small dose of 8.5 mg/100 g of body weight of UFT (a combination of tegafur and uracil at a molar ratio of 1:4, Taiho Pharmaceutical Co., Tokushima, Japan) every other day and oral administration of a large dose of 8.5 mg/100 g of body weight of UFT daily for 3 weeks. These latter three groups were all sacrificed at 29 weeks of age, and specimens of DMH-treated normal colon ① and DMH-induced colon carcinoma ② were removed and stored at −80°C.

II. Enxyme preparation

All specimens were pulverized with an autopulverizer under liquid nitrogen, and then homogenized with 10 volumes of 5 mM Tris-HCl buffer (pH 7.5)/0.1 mM EDTA/1.0 mM mercaptoethanol/0.25 M sucrose at 0°C. The homogenate was centrifuged for 1 h at 4°C at 105,000 g and the supernatant was used as the crude enzyme preparation.

III. Assay for TS activity

TS activity was determined by the method of Dunlap *et al.* [18]. The assay mixture (700 *μl*), consisting of 0.1 M potassium phosphate, 5 mM NaF buffer (pH 6.8), 1 mM d*l*,L-5,10-methylenetetrahydrofolate, and 1 mM [5-^{3}H] dUMP (10.6 Ci/mmol, amersham, U.K.), was incubated with the enzyme preparation at 37°C for 10 min, and then the reaction was stopped by the addition of 100 *μl* of 10% (v/v) $HClO_4$·200 *μl* of 8% (w/v) Norit A was added, and the mixture was centrifuged for 10 min at 0°C at 1,500 g, and then 200 *μl* of the supernatant was added to 5 m*l* of scintillant (16 g of PPO, 0.2 g of POPOP, 1.0 l of Triton X-100, and 3.0 l of toleune). The radioactivity of the aliquot was counted in a liquid scintillation counter.

IV. Assay of TK activity

TK activity was determined by the method of Taylor *et al.* [10]. The assay mixture (200 *μl*, consisting of 5 mM $MgCl_2$, 10 mM ATP, 2 *μl* [6-^{3}H] thymidine (21.0 Ci/mmol, Amersham, U.K.), and 0.1 M Tris-HCl buffer (pH 7.5), was incubated with the enzyme preparation at 30°C for 15 min, and then a 100 *μl* sample was spotted onto 1.8 cm×1.8 cm DEAE-cellulose paper squares (Toyo Filter, Tokyo, Japan). The paper was washed successively with 1 mM ammonium formate and methanol, dried, and inserted into vials containinag scintillant (25.0 g of PPO, 1.5 g of POPOP, and 5.0 l of toluene), and the radioactivity was counted in a liquid scintillation counter.

Enzyme activities are expressed as pmol/mg protein/min or as standard scores (activity quotients), i.e., the activity in a tissue divided by that in normal control colon. Values are means of duplicate assays. The protein content of the enzyme solution was measured by the method of Lowry *et al.* [19], using bovine serum albumin as a standard.

V. Statistical analysis

The significance of the results was assessed using Fischer's exact test or Student's t test, and a p value <5% was considered significant.

Results

I. TS and TK activities in normal control colon, DMH-treated normal colon, and DMH-induced colon carcinoma

Table 2 shows the differences in TS and TK activities between normal control colon, and normal-appearing colon tissue or colon carcinoma tissue 4 weeks or 7 weeks after completion of DMH administration. Activities are expressed as pmol/mg protein/min (mean±SEM), and as standard scores (in parentheses). It can be observed that TS and TK activities in DMH-treated

Table 2. TS and TK Activities in Normal Control Colon, DMH-treated Normal Colon, and DMH-induced Colon Carcinoma

		TS activity (pmol/mg protein/min)	(Ratio)	TK activity (pmol/mg protein/min)	(Ratio)
Normal control colon	②	0.221±0.019[a]	(1.00)	0.018±0.002[f]	(1.00)
DMH-treated normal colon	①	0.245±0.028[b]	(1.11)	0.022±0.003[g]	(1.22)
DMH-induced colon carcinoma	②	0.549±0.025[c]	(2.48)	0.032±0.004[h]	(1.78)
	①	0.766±0.090[d]	(3.47)	0.050±0.006[i]	(2.78)
	②	1.086±0.120[e]	(4.91)	0.107±0.012[j]	(5.94)

①Removed at age of 26 weeks, ②Removed at age of 29 weeks
a ; mean±SEM, a vs c and e($p<0.001$), b vs c($p<0.001$), d vs e(N.S.), b vs d, c vs e($p<0.01$), f vs h ($p<0.05$), f vs j($p<0.01$), g vs h(N.S.), i vs j($p<0.05$), g vs i, h vs j($p<0.01$)

normal colon were not significantly increased at 4 weeks, but were signifiantly increased to, respectively, 248% and 178% of control at 7 weeks after DMH treatment. Furthremore, TS and TK activities were significantly increased in colon carcinoma tissue at both 4 and 7 weeks after DMH treatment. In particular, TK activity of DMH-induced colon carcinom tissue more than doubled during this three-week period.

II. Correlation between the standard scores for TS and TK activities

Enzyme activities in normal control colon, DMH-treated normal colon, and DMH-induced colon carcinoma of rats sacrificed at 29 weeks of age, were expressed as standard scores and plotted two-dimensionally as TS versus TK (Fig. 1). The activities thus plotted were found to cluster into three distinct groups, with normal control colon represented by a closely packed group of low TS and TK scores, DMH-treated normal colon represented by another distinct group of higher TS and TK scores, and DMH-induced colon carcinoma represented by a rather widely spread out group with more variable, but consistently very high TS and TK scores.

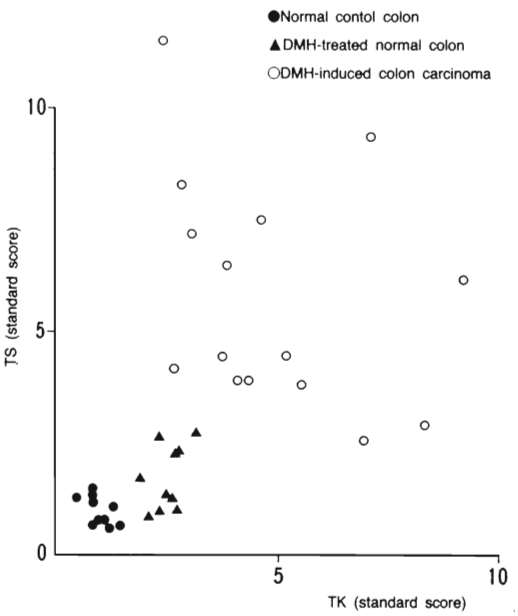


Fig. 1. Correlation between Standard Scores for TS and TK Activities. Means of the TS and TK Activities in 10 Normal Control Colons, 10 DMH-treated Normal Colons and 15 DMH-induced Colon Carcinomas were transformed into Standard Scores.

III. Effects of UFT on TS activity

Fig. 2 shows the effects of low (every-other-day administration) doses and high (daily administration) doses of UFT on TS activity in DMH-induced colon carcinoma tissue, as compared with TS activities in normal control colon, DMH-treated nor-

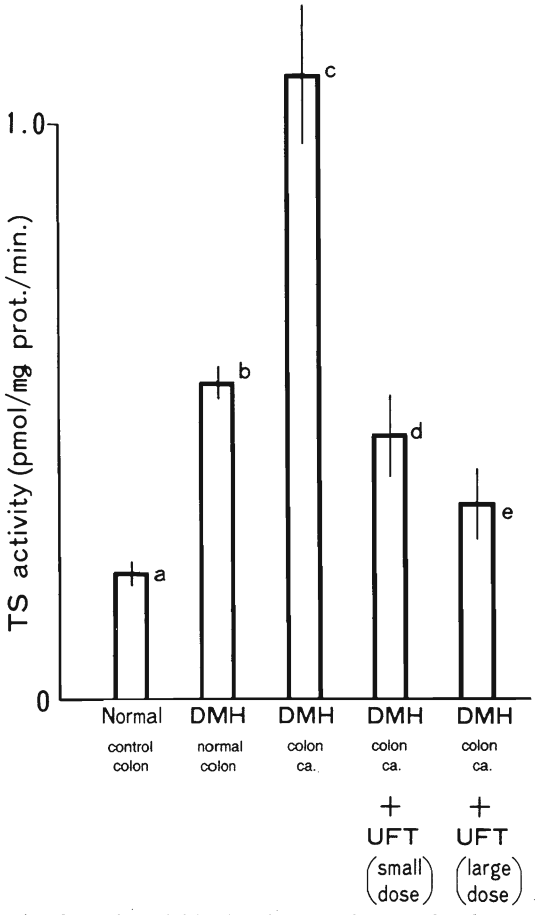


Fig. 2. TS Activities in 10 Normal Control Colons, 10 DMH-treated Normal Colons and DMH-induced Colon Carcinomas (15 without UFT Treatment, 10 with Small Dose of UFT and 10 with Large Dose of UFT) in Rats Sacrificed at 29 Weeks of Age.
Bars, mean±SEM. a vs b and c, b vs c, c vs d and e ($p<0.01$) d vs e (N.S.).

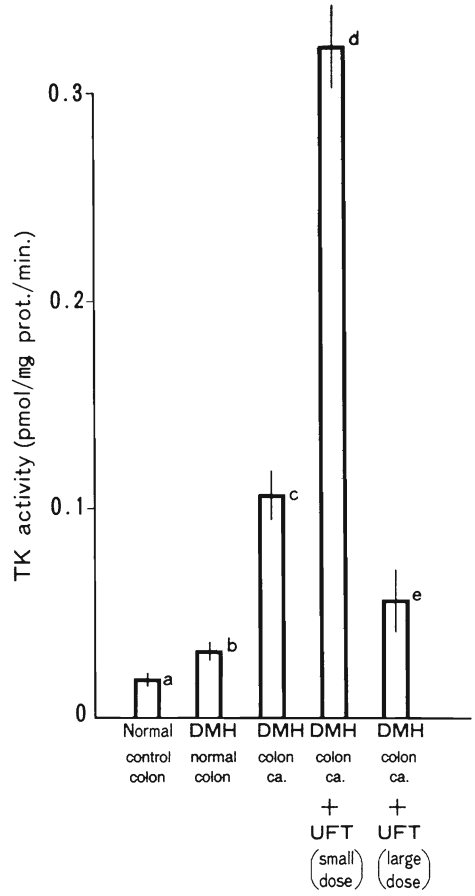


Fig. 3. TK Activities in 10 Normal Control Colons, 10 DMH-treated Normal Colons and DMH-induced Colon Carcinomas (15 without UFT Treatment, 10 with Small Dose of UFT and 10 with Large dose of UFT) in Rats Sacrificed at 29 Weeks of Age.
Bars, mean±SEM. a vs b ($p<0.05$), a and b vs c ($p<0.01$), c vs d ($p<0.01$), c vs e ($p<0.05$), d vs e ($p<0.01$).

mal colon, and DMH-induced colon carcinoma tissue without UFT treatment in rats sacrificed at 29 weeks of age. Both high and low doses of UFT caused a significant decrease in TS activity of DMH-induced colon carcinoma tissue, to less than one-half that of untreated colon carcinoma tissue. Thus, TS activities of UFT-treated colon carcinoma tissue approximated those of DMH-treated normal colon, though they were not as low as those of normal control colon. Daily administration of UFT seemed to result in a slightly greater decrease in TS activity than every-other-day administration; however, the difference was not significant.

IV. Effects on UFT on TK activity

Fig. 3 demonstrates the effects of low every-other-day administration) doses and high (daily administration) doses of UFT on TK activities in DMH-induced colon carcinomas, as compared with T K activi-

ties in normal control colon, DMH-treated normal colon, and DMH-induced colon carcinoma without UFT treatment in rats sacrificed at 29 weeks of age. Low doses of UFT were found to be associated with a significant increase in TK activity, to about three-fold that of untreated colon carcinoma tissue. Conversely, high doses of UFT were associated with significantly decreased TK activity, to about one-half that of untreated colon carcinoma tissue.

Discussion

These results demonstrate that (1) TS and TK activities are increased after DMH treatment, in both normal-appearing colon tissue and colon carcinoma tissue, (2) regardless of dosage, UFT treatment causes a decrease in TS activity, and (3) a low dose of UFT result in an increase, but a high dose result in a decrease of TK activity, in DMH-induced colon carcinoma in rats.

In the present study, TS and TK activities 7 weeks after completion of DMH treatment were 248% and 178%, respectively, that of control in DMH-treatmented "normal" colon, and, 491% and 594%, respectively, that of control in DMH-induced colon carcinoma. This finding is consistent with, and supplements, the work of Thurnherr *et al.* [2], who noted increased TK activity (140% of normal) in DMH-treated normal mucosa, and the work of Weber *et al.* [16], who reported that TK activity is increased (514% of normal) in DMH-induced colon carcinoma, as well as previous work which also demonstrated increased TK activities in humdan colon carcinoma (Herzfeld *et al.* [13]; Sakamoto *et al.* [14, 15]).

It is particularly interesting to note that normal-appearing colon tissue nevertheless exhibits increased activity of pyrimidine synthesizing enzymes after DMH treatment, and that such increased activity constitutes a distinct group intermediate between normal control colon and colon carcinoma when plotted on a two-dimensional grid. This suggests that DMH-induced changes in DNA synthesizing enxymes occur prior to the onset of overt malignancy, and offfers biochemical support for the hypothesis, hitherto based on previous studies of the morphology and dynamics of crypt cell populations in mouse colon after DMH treatment (Richards [3]; Maskens [4]), and that DMH-induced carcinogenesis may involve two steps: (1) initial increase of mitotically active cells, followed by (2) eventual transformation of some of these cells. This hypothesis is especially intriguing in light of the controversy concerning the so-called "polyp-cancer sequence", i.e., whether carcinoma of the colon in man originates from adenomatous polyps, whether the two entities are entirely unrelated, or whether both arise from a common precursor lesion (Fenoglio and Pascal [20]; Chu *et al.* [21]).

Furthermore, in view of the limitations of surgical treatment alone, anti-cancer drugs remain a mainstay of therapy for carcinoma of the colon. The present study offers insight into the mechanisms by which one such drug, UFT, exerts its therapeutic effects. Previous studies have shown that fluoropyrimidines inhibit the activity of thymidylate synthetase, but their effects on pyrimidine synthesis in the salvage pathway, and in particular, on the activity of thymidine kinase, are not yet clear (Ikenaka *et al.* [22]; Nakamura *et al.* [23]).

In the present study, small doses of UFT resultedl in significantly decreased TS activity, but markedly increased TK activity, in DMH-induced colon carcinoma tissue. However, large doses of UFT resulted in a decrease of both TS and TK activities.

UFT may have two possible mechanisms

for the expression of its cytotoxic effects: (1) by conversion to 5-fluoro-2-deoxyuridine monophosphate, which binds to thymidylate synthetase, thereby inhibiting the formation of thymidylic acid and arresting DNA synthesis (Cohen *et al.* [24]), and (2) by its incorporation into RNA, leading to miscoding, alterations in protein composition and physicochemical properties of enzymes, and the inhitition of enzyme induction (Spiegelman *et al.* [25]).

Thus, one likely explanation for the results obtained in this study, is that the larger dose (daily administration) of UFT was sufficient to produce both of the effects described above, leading to a decrease of both TS and TK activities. On the other hand, the smaller ever-other-day administration) dose of UFT may have been sufficient to inhibit thymidylate synthetase and *de novo* pyrimidine synthesis, but was not enough to cause significant inhibition of protein synthesis and enzyme induction by incorporation into RNA. Consequenly, it is conceivable that the activity of the salvage pathway, and in particular, thymidine kinase, did not decrease, but rather increased in order to compensate for the reduced level of TS activity and *de novo* pathway synthesis. In this way, the dosage of UFT is an important factor in determining the mechanism and extent of its cytotoxic effects, and inadequate consideration of this factor may be one reason for the hitherto conflicting reports concerning its effects on TK activity.

In summary, DMH-induced colon carcinoma may be a useful model with which to study the mechanisms of carcinogenesis, and the efficacy of chemotherapeutic regimens. The present results suggest that (1) biochemical alterations of DNA-synthesizding enzyme activity precede the development of overt cancerous tranformation, and (2) a low dose of UFT which inhibit TS activity may, conversely, enhance TK activity, whereas, a large dose of UFT inhibit both *de novo* and salvage pathway enzymes, perhaps due to the cytotoxic effects of its incorporation into RNA.

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