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Suppression of mammary tumors by oral administration of 1-(2-tetrahydrofuryl)-5-fluorouracil in combination with uracil in SHN virgin mice

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Chronic oral administration of 1-(2-tetrahydrofuryl)-5-fluorouracil in combination with uracil suppressed not only *de novo* but also salvage pathways for pyrimidine nucleotide synthesis, and reduced mammary tumorigenesis and tumor growth in SHN virgin mice, which have a high potential for the incidence of mammary tumors.

Key words: DNA synthesizing enzyme, 5-fluorouracil, mouse mammary tumor, SHN virgin mouse.

Introduction

1-(2-Tetrahydrofuryl)-5-fluorouracil, a 5-fluorouracil (5-FU) analog, in combination with uracil (UFT) in a 1:4 molar ratio,[1] has been used as an oral anticancer drug in Japan.[2,3] The drug is believed to be metabolized to 5-FU mainly in the hepatic enzyme pathways.[4] Thymidylate synthetase (TS; EC 2.1.1.45) and thymidine kinase (TK; EC 2.7.1.21) catalyze the formation of deoxythymidine monophosphate (dTMP) by the methylation of deoxyuridine monophosphate (dUMP) with the concomitant conversion of N^5,N^{10}-methylene-tetrahydrofolic acid to 7,8-dihydrofolic acid via the *de novo* pathway and by the phosphorylation of thymidine via the salvage pathway, respectively. High TS and TK activities have been found in rapidly proliferating tissues of normal, fetal and neoplastic tissues.[5,6]

Previously we reported that 5 month administration of low-dose UFT, at a dosage of 100 mg kg diet of 1-(2-tetrahydrofuryl)-5-FU, suppressed preneoplastic hyperplastic alveolar nodule (HAN) formation in SHN mice associated with a reduction in the activities of DNA synthesizing enzymes.[5]

In the present study, we investigated the effect of long-term administration of UFT on spontaneous mammary tumorigenesis, and on TS and TK activities in mammary tumors of SHN virgin mice.

Materials and methods

Animals and treatments

SHN/Mei virgin mice with high potential for the incidence of mammary tumors and uterine adenomyosis[8,9] were used. Beginning at 1 month of age, female litter mates were divided into two groups of 20 mice each; 18 and 17 mice were finally used for the control and the experimental groups in the present study, respectively. Synthetic standard diet (AIN-76TM; Nihon Nosan Kogyo KK, Yokohama, Japan) with UFT [100 mg of 1-(2-tetrahydrofuryl)-5-FU and 224 mg of uracil in 1 kg of diet; Taiho Pharmaceutical, Tokyo, Japan] was given to the experimental group from 1 month of age for 8 months and, from 10 months of age for 4 months the dose of UFT in the diet was increased to 5-fold (i.e. 500 mg/kg diet of 1-(2-tetrahydrofuryl)-5-FU). All mice were kept in plastic cages with wood shavings, two to four mice in each cage, in an animal room that was air-conditioned (21–22°C and 50–70% relative humidity) and lighted (14 h of light from 5:00 to 19:00 h). Diet and tap water were given *ad libitum*. Beginning at 6 months of age, the estrous cycle was checked every morning (8:00–9:00 h) and each mouse was weighed every 10 days. After the appearance of palpable mammary tumor, tumor sizes expressed in terms of the geometric mean of the two major diameters were recorded every 7 days

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for 3 weeks, followed by decapitation under light ether anesthesia. Blood was collected from the trunk. Non-tumorous animals in both the experimental and the control groups were killed at 13 months of age. Separated serum samples and removed mammary tumors were stored at −80°C for the measurements of 5-FU concentration, and TS and TK activities.

Organ weight

At autopsy, the anterior pituitary, adrenals, ovaries and liver were removed and weighed.

Uterine adenomyosis

At autopsy, uteri were removed, fixed in Bouin's solution, sectioned at 6 μm and stained with hematoxylin–eosin. Each specimen was microscopically examined for uterine adenomyosis.

Serum levels of 5-FU and uracil

Serum levels of 5-FU, 1-(2-tetrahydrofuryl)-5-FU and uracil were measured by HPLC in the Laboratory of Taiho Pharmaceutical (Tokushima, Japan).[10]

Enzyme preparation and assay

Eight and nine tumors monitored for 3 weeks of tumor growth from 6 to 9 months of age in the control and the experimental groups, respectively, were used for enzyme assay as low-dose groups, and eight and five tumors monitored from 10 to 13 months of age in the control and the experimental groups, respectively, were used as high dose groups. As previously reported,[11,12] activities of TS and TK were determined by the methods of Dunlap *et al.*[13] and Taylor *et al.*,[14] respectively.

Each specimen was pulverized with an autopulverizer under liquid nitrogen and then homogenized with 10 volumes of 5 mM Tris–HCl buffer, pH 7.5, containing 0.1 mM EDTA, 1 mM mercaptoethanol and 0.25 M sucrose at final concentration 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.

The TS assay mixture (700 μl), consisting of a 0.1 M potassium phosphate buffer, pH 6.8, containing 5 mM NaF, 1 mM *dl*,L-5,10-methylene-tetrahydrofolate and 1 mM [5-^{3}H]dUMP (10.6 Ci/mmol; Amersham, UK), was incubated with the enzyme preparation at 37°C for 10 min, and the reaction was stopped by addition of 100 μl of 10% (v/v) $HClO_4$. Two hundred microliters of 8% (w/v) Norit A was added and the mixture was centrifuged for 10 min at 4°C at 1500 ***g***, and 200 μl of the supernatant was added to 5 ml of scintillant (16 g PPO, 0.2 g POPOP, 1.0 l Triton X-100 and 3.0 l toluene). The radioactivity in the supernatant was counted in a liquid scintillation counter.

The TK assay mixture (200 μl), consisting of 5 mM $MgCl_2$, 10 mM ATP, 2 μM [6-^{3}H]thymidine (21.0 Ci/mmol; Amersham) and 0.1 M Tris–HCl buffer, pH 7.5, was incubated with the enzyme preparations at 30°C for 15 min, and the reaction was stopped by boiling the assay mixture. Then 100 μl of supernatant of the mixture was spotted onto 1.8 × 1.8 cm DEAE–cellulose paper (Toyo Filter, Japan). The paper was washed successively with 1 mM ammonium formate and methanol, dried, and inserted into vials containing scintillant (25.0 g PPO, 1.5 g POPOP and 5.0 l toluene), and the radioactivity was counted in a liquid scintillation counter.

Enzyme activities were normalized to tissue contents of protein and DNA, the content of which was determined by the method of Schneider,[15] and were expressed as pmol/μg DNA/mg protein. Values were means of duplicate assays.

Statistical analyses

The statistical significance of differences between groups was evaluated by the multiple classification method of analysis of variance[16] in mammary tumorigenesis, i.e. this method allowed the difference to be checked while taking into account both the incidence and the onset time of tumors, and by Student's *t*-test or Wilcoxon's rank test in other parameters; $p < 0.05$ was considered significant.

Results

There was little difference in body growth and the weights of anterior pituitary, adrenals, ovaries and liver between groups, i.e. the control [UFT(−)] and the experimental [UFT(+)]. Little difference was observed in the incidence and the grade of uterine adenomyosis between groups. The pattern

Table 1. UFT concentration and serum levels of 5-FU, 1-(2-tetrahydrofuryl)-5-FU and uracil in each group

	Age (months)			
	1–9		10–13	
	control ($n = 8$)	experimental ($n = 9$)	control ($n = 10$)	experimental ($n = 8$)
UFT concentration in diet as 1-(2-tetrahydrofuryl)-5-FU (mg/kg diet)	0	100	0	500
Serum level (μg/ml)				
5-FU	0	0	0	0.04 ± 0.02[a]
1-(2-tetrahydrofuryl)-5FU	0	0	0	0.90 ± 0.44
uracil	1.48 ± 0.03	2.00 ± 0.24	2.30 ± 0.27	2.82 ± 0.28

[a] mean ± SEM.

of estrous cycle differed little between groups (data not shown).

The serum levels of 5-FU, 1-(2-tetrahydrofuryl)-5-FU and uracil in the experimental group with high dose UFT were elevated (Table 1).

The first incidence of a mammary tumor was at 6 months of age in each group. There was little difference in mammary tumor incidence between the control and the experimental groups with low dose UFT from 1 to 9 months of age for 8 months, but there was a trend towards a decrease in mammary tumor incidence in the experimental group with high dose UFT, from 10 to 13 months of age for 4 months ($p < 0.05$; by the multiple classification method of analysis of variance[16] (Table 2).

Although little difference was observed in the growth of palpable mammary tumors between groups from 6 to 9 months of age for 4 months, this slight difference proved of significance, since the average growth rate of mammary tumors in the experimental group with high dose UFT, from 10 to 13 months of age for 4 months, was reduced to 56% of that in the control ($p < 0.05$) (Table 2).

The average activities of TS and TK in mammary tumors obtained from 6 to 9 months of age in each group markedly increased to approximately 3 times those of the non-tumorous regions of mammary glands in the control group, respectively ($p < 0.05$), but both enzyme activities differed little between groups.

On the other hand, TS and TK activities in the experimental group with high dose UFT from 10 to 13 months of age were reduced to 26% ($p < 0.01$) and 66% (not significant) of those of the control, respectively (Table 3). Both enzyme activities of the non-tumorous regions in mammary glands in all groups were roughly similar to those of the specimens obtained from 6 to 9 months of age in the control group (data not shown).

Table 2. Cumulative incidence of mammary tumors and the percent changes of tumor growth for 3 weeks in each group

	Age (months)			
	1–9		10–13	
	control (−)	experimental (+) low-dose	control (−)	experimental (+) high-dose
Cumulative incidence of mammary tumors throughout whole experiment	8/18 (44.4%)	9/17 (52.9%)	16/18 (88.9%)	14/17 (82.4%)
Tumour incidence after 10 months of age			8/10 (80.0%)	5/8 62.5%)
Percent change of tumor growth for 3 weeks	336 ± 23	335 ± 37	409 ± 53	231 ± 21[a]

[a] Significantly different from the respective control at $p < 0.05$.

Table 3. TS and TK activities of mammary tumors in each group 3 weeks after the appearance (mean ± SEM)

	Enzyme activity (pmol/μg DNA/mg protein)	
	TS	TK
Normal mammary gland (non-tumorous region of mammary gland in control group)	0.104 ± 0.023	0.028 ± 0.003
1–9 months of age		
control	0.281 ± 0.058	0.081 ± 0.011
experimental (low-dose UFT)	0.270 ± 0.043	0.078 ± 0.013
10–13 months of age		
control	0.283 ± 0.042	0.080 ± 0.013
experimental (high-dose UFT)	0.074 ± 0.025[a]	0.053 ± 0.017

[a] Significantly different from the control at $p < 0.01$.

Discussion

SHN mice, which were established and maintained by Nagasawa *et al.*,[8] are known as a strain having a high potential for the incidence of mammary tumors and uterine adenomyosis. TS and TK are known as the key enzymes for pyrimidine nucleotide synthesis in *de novo* and salvage pathways, respectively. The anticancer effect of 5-FU and its derivatives has been ascribed to three major mechanisms:[17] (i) metabolism to 5-fluoro-2-deoxyuridine-5-monophosphate (FdUMP), which is a potent suicide inhibitor of TS; (ii) incorporation of the ribonucleoside triphosphate of 5-FU into some species of RNA; and (iii) incorporation into DNA.

Previously we reported that 5-FU derivative combined with uracil, UFT, suppressed preneoplastic mammary hyperplastic alveolar nodule formation in mice[5] as well as chemical tumorigenesis in the colon[18] and liver[19] in rats.

In the present study, we examined the anticancer effects of chronic oral administration of UFT, the doses being the same as those in the previous study[5] [100 mg/kg diet as 1-(2-tetrahydrofuryl)-5-FU in a low dose group] and the 5-fold dose (500 mg/kg diet in a high dose group).

Although the chronic oral administration of low dose UFT, at a dose which markedly suppressed the mammary hyperplastic alveolar nodule formation,[5] did not reduce the tumor incidence, high dose UFT, 5-fold that in the low dose group, was suggested to suppress not only the incidence but also the growth of mammary tumors, resulting in the reduction of both TS and TK activities in mammary tumors. As Nakajima *et al.*[20] reported, the serum level of 1-(2-tetrahydrofuryl)-5-FU peaked 2 h after the oral administration of UFT, the dose of which was 300 mg as 1-(2-tetrahydrofuryl)-5-FU, and showed 13.7 ± 1.1 μg/ml in human beings; thus, high dose UFT in the present study was even less than 10% of the human clinical dose.

Conclusions

Our data indicate that the chronic oral administration of low dose UFT (0.9 ± 0.4 μg/ml 1-(2-tetrahydrofuryl)-5-FU in the serum) could prevent mammary tumorigenesis without severe liver dysfunction in SHN virgin mice. Long-term administration of low dose UFT reduced not only *de novo* but also salvage pathways for pyrimidine nucleotide synthesis, resulting in the prevention of mammary tumorigenesis with slight suppression of mammary growth in SHN virgin mice.

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