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Research paper

Additive effects of medroxyprogesterone acetate and 5-fluorouracil derivative on 7,12-dimethylbenz-[a]anthracene-induced rat mammary tumors

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Chronic oral administration of 1-(2-tetrahydrofuryl)-5-fluorouracil in combination with uracil suppressed thymidylate syntetase (TS) gene expression followed by reduction of TS activity in rat mammary tumors induced with 7,12-dimethylbenz[a]anthracene. Addition of medroxyprogesterone acetate (MPA) to the anticancer drug caused an additional decrease in TS and thymidine kinase activities in the tumor growth and restoration of bone loss. These results suggest that the simultaneous adminstration of MPA and anticancer drugs causes increased inhibition of mammary tumor growth and also diminishes the bone loss. [© 1998 Lippincott-Raven Publishers.]

Key words: 5-Fluorouracil, gene expression, medroxyprogesterone acetate, rat mammary tumor, thymidylate synthetase.

Introduction

Medroxyprogesterone acetate (MPA) is a progesterone derivative which shows antiestrogenic, antiandrogenic, antigonadotropic[1] and antitumor activities against hormone-target organs and hormone-depended tumors.[1-3] Pannuti *et al.* demonstrated the high-dose administration of MPA in advanced breast cancer patients in 1974.[2] It has also been reported that MPA has beneficial effects such as the improvement of nausea, vomiting, anorexia, weight loss and hematological toxicity induced by chemotherapy.[2-5] However, efficacy of adjuvant chemoendocrine therapy using MPA against mammary tumors is less certain in pre- or postmenopausal studies.

In the present study, we investigated the effects of MPA and/or 1-(2-tetrahydrofuryl)-5-fluorouracil in combination with uracil (UFT) on the growth of 7,12-dimethylbenz[*a*]anthracene (DMBA) induced rat mammary tumors, although the results were so preliminary that the sample size was small.

Materials and methods

Animals and treatments

Young female Sprague-Dawley rats (Sankyo Laboratory Service, Tokyo, Japan) were used. Throughout the experiment, all rats were housed under controlled lighting and temperature, given a commercial diet (CE-2; CLEA Japan, Tokyo, Japan) with tap water *ad libitum*, and weighed every 7 days.

At 48 days of age, the animals were given a single i.v. injection of 5 mg of DMBA (special 15% fat emulsion with DMBA, 5 mg/ml, Upjohn, Kalamazoo, MI; a gift from Professor Dr CB Huggins). The appearance of palpable mammary tumor and tumor size expressed in terms of the geometric mean of the two major diameters were recorded every 7 days.

At 201 days of age, tumor-bearing rats were divided into four groups of seven each. (1) Rats were given a commercial diet and s.c. injections of sesami oil twice a week for 90 days (DMBA-Control). (2) Rats were given s.c. injections of MPA (10 mg/100 g of body

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weight; Aldrich, Milwaukee, WI) dissolved in sesami oil twice a week (DMBA-MPA). (3) Rats were given diet containing UFT (Taiho Pharmaceutical, Tokyo, Japan) [500 mg of 1-(2-tetrahydro-furyl)-5-fluorouracil and 1.12 g of uracil in 1 kg of diet] throughout the experiment (DMBA-UFT). (4) The remaining seven rats were given the same UFT diet and MPA injections (DMBA-MPA+UFT).

At 290 days of age, two animals in each group were given a single i.v. injection of bromodeoxyuridine (BrdU; 10 ml/kg body weight, Cell proliferation kit, RPN 20, batch 12, Amersham, Amersham, UK) into the tail vein 6 h before autopsy. Mammary tumors removed from rats given BrdU were immediately fixed in 10% formaldehyde buffer solution (pH 7.2). The remaining five animals without BrdU injection in each group were bled by cardiac puncture under deep anaesthesia with urethane (1.5 g/kg body weight; Merck, Darmstadt, Germany), and the mammary tumors, anterior pituitary, adrenals, spleen and bilateral femurs were removed and weighed. Each femur was fixed in 99.5% ethanol and stored. All experimental procedures conformed to the regulations described in the NIH Guide to the Care and Use of Laboratory Animals.

Plasma levels of hormones

Plasma levels of prolactin were determined by a radioimmunoassay kit donated by Dr A F Parlow. Plasma levels of estradiol and progesterone were also determined by a radioimmunoassay kit (Diagnostic Products, Los Angeles, CA). Prolactin and progesterone levels are expressed as ng/ml and estradiol as pg/ml.

Plasma levels of 5-fluorouracil (5-FU), 1-(2-tetrahydrofuryl)-5-FU and uracil were measured by HPLC in the Laboratory of Taiho Pharmaceutical (Tokushima, Japan).[6]

Immunohistochemistry with BrdU in mammary tumors

BrdU incorporated into cellular DNA was detected by a monoclonal anti-BrdU antibody by the procedure described in the protocol. Six sections of each tumor were randomly chosen and BrdU-immunoreactive cells were counted in 400 cells per each section. The results were expressed in terms of BrdU-immunoreactive (S phase) cells as a percentage of total cells.

Preparation and assay of tissue thymidylate synthetase (TS) and thymidine kinase (TK)

TS (EC 2.1.1.45) and 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}-methylenetetrahydrofolic 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.[7-9] Each mammary tumor from animals without BrdU injection was used for enzyme assay. As previously reported,[10] the activities of TS and TK were determined by the methods of Dunlap *et al.*[11] and Taylor *et al.*,[12] respectively. Enzyme activities were normalized to tissue content of protein and expressed as fmol/mg protein/min. Values are means of duplicate assays.

RNA isolation and the detection of TS and TK mRNA

Total RNA was isolated from 100 mg of frozen mammary tumor tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method. TS and TK mRNA expressed in the mammary tumor was determined by the reverse transcription-polymerase chain reaction (RT-PCR) method. Reverse transcription was performed using a combination of oligo(dT) primers [0.5 μl oligo$(dT)_{12-18}$ primers (0.5 μg/ml) (Gibco/BRL, Gaithersberg, MD)] and random primers [0.5 μl random hexamers (0.05 μg/ml) (Gibco/BRL)] with Superscript™ Preamplification System (Gibco/BRL) according to the procedure of each supplier's recommendation.[13] Once the cDNA copy has been created using the mRNA template, the PCR was conducted immediately, as outlined below. Alternatively, the cDNA was stored at $-20°C$, until required for analysis. The PCR was performed with recombinant Taq DNA polymerase (Nippon Gene, Tokyo, Japan) according to the procedure of each supplier's recommendation.

In order to optimize the RT-PCR assay, the relationship of signal strength to cycle number and to the amount of RNA added was determined by densitometry in photographs using an image analyzer (AE-6920-MF Densitograph; ATTO, Tokyo, Japan). Each signal for both products (TS and TK cDNA) increased linearly from the 26th to 36th cycle. With 38 or more cycles the specific cDNA signal increased only slightly. The production of cDNA was demonstrated to be propor-

tional to the amount of input RNA. With 34 cycles of amplification, the signals for TS and TK cDNA increased linearly between 0.75 and 3.0 mg and between 0.025 and 0.2 mg, respectively, of rat mammary tumor RNA added to the RT-PCR reaction. Based on these results, 2.0 and 0.1 mg of RNAs for the RT-PCR reactions using the primers for TS and TK cDNA, respectively, were used for 34 cycles (each cycle consisting of a denaturing step of 94°C for 40 s, annealing at 56°C for 40 s and extending at 72°C for 40 s) in a Gene Amp PCR System 2400 (Perkin Elmer, Foster City, CA). Two sets of primers for each TS, TK and β-actin were used for PCR, respectively (Table 1). TS and TK mRNA levels were determined by densitometry in photographs using the image analyzer and expressed as a ratio of β-actin mRNA.

Bone mineral density in femur

A microradiograph of each fixed femur was taken with soft X-ray apparatus (Type-Softex; Softex, Tokyo, Japan). A step-wedge made of synthetic hydroxyapatite plates (Mitsubishi Kasei, Tokyo, Japan) of different thicknesses was placed on the same radiographic films (Soft X-ray film, Type FR; Fuji Photofilm, Tokyo, Japan) to serve as a standard for measuring the bone mineral density (BMD) of the femur, i.e. the BMD was determined by analyzing the gray levels of the target area in the microradiographs with an image analyzer (Aspect; Mitani, Fukui, Japan). A standardized relationship was established between the gray levels (0–255) and hydroxyapatite content expressed as mg Ca/mm^2 by analyzing the image of the standard step-wedge.[14] Since the gray levels and the logarithm of the hydroxyapatite content were well correlated positively ($y = 245.7x - 540.8$), with a correlation coefficient of 0.99 under the conditions used for taking micrographs (85 V, 1 mA for 60–120 s), the BMD (mg Ca/mm^2) was calculated and determined using the measured gray levels of the target area.

Table 1. Primers to amplify cDNA formed by reverse transcriptase on mRNA templates of TS, TK and β-actin

Gene	Primer sequences	Amplified mRNA sequence length (bp)
TS	5′-TAGCACAGGCGGCACACGGAGT-3′ 5′-TGCTCCGCGATGTGACCCAGGA-3′	311
TK	5′-TGAATGGGGAGCTATCTTGCCA-3′ 5′-TCGTTGGATGTGGATTATACCC-3′	327
β-actin	5′-AGGCCCAGAGCAAGAGAGGCAT-3′ 5′-CATGGCTGGGGTGTTGAAGGTC-3′	227

Statistical analysis

The significance of differences between groups was evaluated by Student's *t*-test, Wilcoxson's rank test, one-way ANOVA followed by Scheffe's multicomparison test or χ^2 test with Yates' correlation and $p < 0.05$ was considered significant.

Results

Body growth, organ weights and bone mineral density

The body growth of rats given consecutive injections of MPA in the DMBA-MPA group was markedly increased compared with that in the other groups ($p < 0.05$) (Table 2).

The anterior pituitary weights in the DMBA-MPA group were significantly reduced compared with that in the DMBA-Control group ($p < 0.05$). The weights of ovaries and adrenals in rats given MPA injections with or without the UFT diet were markedly reduced compared with those of the DMBA-Control group ($p < 0.01$). There were no differences among groups in spleen weight.

The bone mineral density of femur in rats treated with MPA was significantly increased to 106% of that in the DMBA-Control ($p < 0.01$).

Plasma levels of hormones and 5-FU derivatives

In rats given MPA injections with the UFT diet, the plasma levels of estradiol and progesterone were markedly reduced compared with those in the DMBA-Control group ($p < 0.01$) (Table 3). The plasma estradiol levels in the DMBA-MPA+UFT group were also not detected. There were significant differences between groups in the plasma prolactin levels.

The plasma levels of 1-(2-tetrahydrofuryl)-5-FU and 5-FU were clearly detected in rats given the UFT diet with or without MPA injections.

Mammary tumor development and immunohistochemistry with BrdU in tumors in each group

Although the number of palpable mammary tumors in the DMBA-Control group markedly increased to 2-fold for 90 days ($p < 0.01$), the tumor development in the other three groups was not continued but altered, e.g.

the number of tumors was reduced to approximately 35% of that before treatment by MPA injections with UFT diet ($p<0.01$) (Table 4).

The percentages of BrdU-immunoreactive (S phase) cells in rats given MPA injections with or without the UFT diet were markedly reduced compared with that in the DMBA-Control ($p<0.01$).

Expression of mRNA of TS and TK, and those activities in mammary tumors in each group

Although the expression ratio of TK mRNA (TK mRNA/β-actin mRNA) differed little among groups, the expression ratio of TS mRNA (TS mRNA/β-actin

Table 2. Effects of MPA and/or UFT on body growth, organ weights and bone mineral density in DMBA-treated rats (mean ± SEM)

	DMBA-Control (n=7)	DMBA-MPA (n=7)	DMBA-UFT (n=7)	DMBA-MPA+UFT (n=7)
Body growth				
initial (g)	278.1 ± 6.4	287.5 ± 17.5	280.0 ± 9.8	301.7 ± 10.5
final (g)	300.1 ± 6.4	342.5 ± 25.6[a]	297.7 ± 11.0	306.7 ± 17.1
change (%)	8.0 ± 0.2	18.8 ± 2.0[a]	6.4 ± 1.7	1.5 ± 3.0
Organ weight (mg)				
anterior pituitary	18.9 ± 1.0	14.3 ± 1.2[a]	20.2 ± 1.0	15.7 ± 1.9
ovaries	81.3 ± 7.0	27.0 ± 2.5[b]	71.0 ± 6.4	21.0 ± 2.6[b]
adrenals	81.9 ± 2.5	18.0 ± 0.7[b]	84.2 ± 6.9	17.7 ± 2.4[b]
spleen	557 ± 44	544 ± 100	513 ± 62	571 ± 73
Bone mineral density (mg Ca/mm^2)	790.6 ± 8.1	834.8 ± 7.6[b]	786.1 ± 18.7	812.0 ± 49.2

Significantly different from that of the DMBA-Control at [a]$p<0.05$ and [b]$p<0.01$, respectively

Table 3. Effects of MPA and/or UFT on the plasma levels of prolactin, estradiol, progesterone, 5-FU, 1-(2-tetrahydrofuryl)-5-FU and uracil in DMBA-treated rats (mean ± SEM)

	DMBA-Control (n=7)	DMBA-MPA (n=7)	DMBA-UFT (n=7)	DMBA-MPA+UFT (n=7)
Prolactin (ng/ml)	54.7 ± 16.8	22.7 ± 9.3	51.2 ± 29.2	57.9 ± 25.8
Estradiol (pg/ml)	5.79 ± 1.82	ND[a]	5.30 ± 2.36	ND[a]
Progesterone (ng/ml)	16.7 ± 2.3	0.9 ± 0.2[a]	26.1 ± 8.3	8.4 ± 6.9
1-(2-tetrahydrofuryl)-5-FU (mg/ml)	ND	ND	3.91 ± 0.53	3.05 ± 0.60
5-FU (mg/ml)	ND	ND	0.03 ± 0.00	0.04 ± 0.03
Uracil (mg/ml)	0.72 ± 0.05	0.81 ± 0.15	0.62 ± 0.06	1.21 ± 0.34

Significantly different from that of the DMBA-Control at [a]$p<0.01$.

Table 4. Effects of MPA and/or UFT on mammary tumor growth and percentages of BrdU-immunoreactive (S phase) cells in the mammary tumors of DMBA-treated rats (mean ± SEM)

	DMBA-Control (n=7)	DMBA-MPA (n=7)	DMBA-UFT (n=7)	DMBA-MPA+UFT (n=7)
Number of tumors/rat				
initial	2.14 ± 0.40	2.14 ± 0.34	2.29 ± 0.42	2.43 ± 0.37
final	4.29 ± 0.36[b]	1.57 ± 0.48[a]	1.14 ± 0.40[a]	0.86 ± 0.34[a,b]
BrdU-immunoreactive (S phase) cells	7.0 ± 0.5	0.1 ± 0[a]	6.0 ± 0.6	1.9 ± 0.3[a]

Significantly different from that of the [a]DMBA-Control group and [b]initial number of tumors at $p<0.01$, respectively.

mRNA) in the DMBA-UFT and DMBA-MPA+UFT groups were markedly lowered to 66.0 and 44.7% of that of the DMBA-Control, respectively ($p<0.01$) (Figure 1a).

The mammary TS activities in the DMBA-MPA, DMBA-UFT and DMBA-MPA+UFT groups were markedly reduced to 35, 47 and 18% of that of the DMBA-Control, respectively ($p<0.01$) (Figure 1b). The mammary TK activities in the DMBA-MPA, DMBA-UFT and DMBA-MPA+UFT groups were markedly reduced to 20, 46 and 21% of that of the DMBA-Control, respectively ($p<0.01$).

Discussion

Although endocrine therapy in combination with chemotherapy for advanced or recurrent breast cancer is expected to be more beneficial than each therapy alone, the efficacy of adjuvant chemo-endocrine therapy using MPA against mammary tumors is less certain in pre- or postmenopausal studies.[4,15] Aldaz *et al.*[16] and we[17] have reported that MPA increased the incidence of mouse mammary tumors, but it has been known that MPA suppressed the proliferation of human mammary cancer cell lines, MCF-7[18] and MFM-223.[19] Ishikawa *et al.* reported that chemo-endocrine therapy using MPA was more effective in DMBA-induced mammary tumor in rats.[20] In the present study, we investigated the additive effects of MPA and 5-FU derivative UFT on body growth, organ weights, bone mineral density, plasma hormone levels and DMBA-induced mammary tumors indicating tumor multiplicity, immunohistochemistry using BrdU, activities of DNA-synthesizing enzymes, TS and TK, and expression of those enzymes' mRNA in rats.

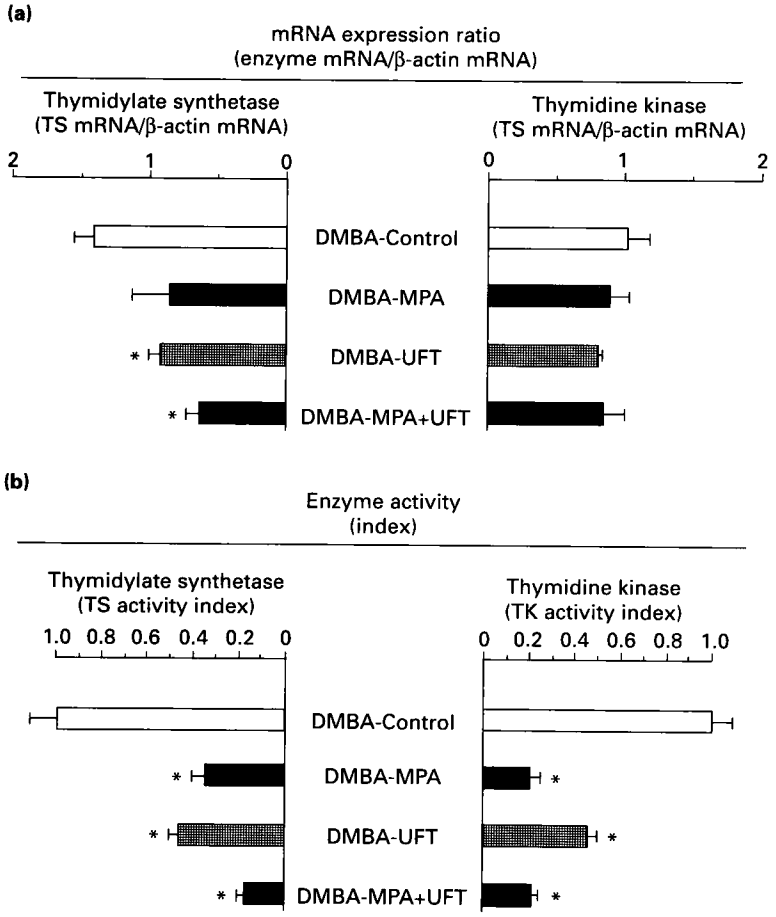

Figure 1. Effects of MPA and/or UFT on mRNA expression and the activities of TS and TK in DMBA-induced mammary tumors in rats. The signal strength of TS and TK mRNA was determined by densitometry in photographs using an image analyzer and expressed as a ratio of β-actin mRNA (a). The enzyme activity was normalized to tissue content of protein and expressed as the standardized index (percentage), i.e. the activity in a tissue divided by the average value in the DMBA-Control group (b). Mean ± SEM. Significantly different from that of the DMBA-Control at $*p<0.01$.

Although MPA administration alone increased body weight as reported by other investigators ($p<0.05$),[3,4,15] MPA with UFT diet did not enhance body growth, i.e. the UFT diet might induce appetite loss throughout the experiment. MPA administration with or without the UFT diet reduced weights of anterior pituitary, ovaries and adrenals ($p<0.01$ or 0.05), and resulted in the reduction of plasma levels of estradiol ($p<0.01$) and progesterone (NS and $p<0.01$). Sala *et al.* reported that administration of 2000 mg a day of MPA for 30 days markedly reduced the plasma levels of follicle stimulating hormone and luteinizing hormone.[21] As Muccioli *et al.* reported,[22] MPA administration slightly reduced the plasma prolactin level.

Although MPA administration led to a dysfunction in hypothalamo-pituitary-ovarian axis and completely suppressed the plasma levels of ovarian hormones, BMD was markedly raised compared with that of the DMBA-Control ($p<0.01$). As other investigators reported,[2-5] MPA is suggested to increase the body weight, resulting in the elevation of bone volume. Isserow *et al.*[23] showed that MPA alone had no significant effect on cancellous bone volume, though Barengolts *et al.*[24] reported that MPA prevented postoophorectomy-induced bone loss in the ovariectomized aged rat. In the present study also, MPA with the UFT diet slightly elevated the BMD although the UFT diet slightly reduced appetite and body growth.

Pannuti *et al.*,[15] Hupperets *et al.*[4] and Tominaga *et al.*[3] demonstrated that chemoendocrine therapy using MPA appeared to be more beneficial than each therapy alone in the treatment of advanced or recurrent breast cancer. In the present study, development of mammary tumors was markedly suppressed by the UFT diet and/or MPA injections ($p<0.01$ or 0.05). MPA with the UFT diet was the most effective of all groups ($p<0.01$).

Conclusions

Chronic oral administration of UFT as a diet content suppressed TS gene expression, but not TK gene expression, followed by reduction of TS activity in tumor cells. Although MPA administration little affected gene expression of TS and TK, MPA lowered activities of TS and TK, followed by alteration of BrdU-immunoreactive (S phase) cells in tumor cells. Higher dosage and/or longer duration of therapy using UFT or MPA appears to suppress not only *de novo* but also salvage pathways for pyrimidine nucleotide synthesis. The present study suggests the beneficial effects of MPA combined with chemotherapy using oral administration of UFT on the development of DMBA-induced mammary tumors and bone loss although the results are so preliminary that the sample size is small.

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