Effects of Chinese herbal medicines, Keishi-bukuryo-gan and/or Shakuyaku-kanzo-to, on normal, preneoplastic and neoplastic mammary glands in mice

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Abstract

Mastopathy has conservatively been treated by a pharmacotherapeutic administration of progesterones or other drugs. Recently, we have prescribed Chinese herbal medicines, Keishi-bukuryo-gan (KBG) and/or Shakuyaku-kanzo-to (SKT) to patients with symptomatic mastopathy. In the present study, we investigated the effects of KBG and/or SKT on normal, preneoplastic and neoplastic mammary glands in mice. The combined administration of KBG and SKT (1 g/kg/day, each) for 2 months slightly regulated the intermittent and irregular appearance of estrus in estrous cycle, and reduced the formation of normal mammary end-buds and the number of preneoplastic mammary hyperplastic alveolar nodule (HAN) by decreasing the activity of thymidylate synthetase (TS), a key enzyme in *de novo* pathway for pyrimidine nucleotide synthesis. The 20-day combined administration lowered the neoplastic mammary gland growth by suppressing the TS gene expression. These results suggest that the combined administration of KBG and SKT is promising for the therapy of symptomatic mastopathy with little harmful side effects.

Key words Chinese herbal medicines, mastopathy, SHN mouse.

Abbreviations Gn-RH,gonadotropin-releasing-hormone ; KBG, Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan) 桂枝茯苓丸 ; SKT, Shakuyaku-kanzo-to (Shao-Yao-Gan-Cao-Tang) 芍薬甘草湯 ; RT-PCR, reverse transcription-polymerase chain reaction ; TK, thymidine kinase ; TS, thymidylate synthetase ; HAN, hyperplastic alveolar nodule.

Introduction

Mastopathy is a chronic cystic mastitis without infection or inflammation and occurs with mastodynia (pain and tenderness) in late twenties or thirties, and must be considered as the first stage of a mammary dysplasia. The patients are often nervous and underweight, and nullipara with an irregular menstrual cycle and a low fertility resulted in a luteal dysfunction with relative hypersecretion of estrogens.[1] The incidence of mammary cancer in the patients with mastopathy is higher (2 to 6-fold) than that of the over-all population in the same age groups.[1] The patients with mastopathy, if the presence of mammary cancer can be excluded with certainty, have conservatively been treated by progesterone therapy, or pharmacotherapeutic administration of gonadotropin-releasing-hormone (Gn-RH) analogs[2] or danazol.[3] As previously reported, Chinese herbal medicines, Keishi-bukuryo-gan (KBG) or Shakuyaku-kanzo-to (SKT) suppressed the development of preneoplastic mammary hyperplastic alveolar nodules (HAN) with a decrease of the activity of thymidylate synthetase

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(TS : EC 2.1.1.45), a key enzyme in *de novo* pathway for pyrimidine nucleotide synthesis in mice.[4] Recently, we have prescribed KBG and / or SKT to the patients with symptomatic mastopathy and have got a satisfactory result.[5]

In the present study, we investigated the effects of KBG and/or SKT on normal, preneoplastic and neoplastic mammary glands in mice.

Materials and Methods

Materials : The components of the medical herbs (KBG and SKT : Tsumura Co. Ltd., Tokyo) are listed in Table I ; *i.e.,* KBG and SKT are composed of five and two herbal drugs, respectively. Both mixtures consisting of each chopped ingredient were prepared and extracted with hot water, filtered, lyophilized, and stored at 4°C as KBG and SKT extracts.

Animals and treatments : SHN/Mei virgin mice, which have been maintained in the Experimental Animal Research Laboratory, Meiji University and established as a strain with high potential for the incidence of mammary tumors and uterine adenomyosis associated with hyperprolactinemia,[6,7] were used in the present study. At one month of age, female litter mates were divided into 8 groups of 10 mice each. All mice were kept in plastic cages with wood shavings, 4 to 6 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.*

Table I Components of Keishi-bukuryo-gan (KBG) and Shakuyaku-kanzo-to (SKT).

KGB	
1 bark of *Cinnamomum cassia* Bl. (Lauraceae)	3.0 g
2 root of *Paeonia lactiflora* Pall. (Paeoniaceae)	3.0 g
3 seed of *Prunus persica* Batsch or *P. persica* Batsch var. *davidiana* Maxim. (Rosaceae)	3.0 g
4 carpophores of *Poria cocos* Wolf (Polyporaceae)	3.0 g
5 root bark of *Paeonia suffruticosa* Andr. (Paeoniaceae)	3.0 g
SKT	
1 root of *Glycyrrhiza glabra* L. var. *glandullifera* Reg. et Herd. or *G. uralensis* Fisch. (Leguminosae)	6.0 g
2 root of *Paeonia lactiflora* Pall. (Paeoniaceae)	6.0 g

Experiment I : Beginning at 3 months of age in 4 of 8 groups, the estrous cycle of each mouse was checked every morning (8:00-9:00 h), and mice were weighed every 10 days. Experimental groups were fed laboratory diet (CE-2, CLEA Japan Co., Ltd., Tokyo) containing 1 % of KBG and/or SKT and the control group was given the diet only for 2 months.

Experiment II : Beginning at 6 months of age, the estrous cycle of the remaining 4 groups were checked and weights were recorded as in Experiment I. After the appearance of palpable mammary tumor (approximately 5 mm in diameter), the experimental groups were fed laboratory diet containing 1 % of KBG and/or SKT and the control was given diet only. Tumor sizes expressed in terms of the geometric mean of the two major diameters were recorded every 7 days for 20 days.

After each experiment (I or II) was finished, each animal was sacrificed by decapitation under light ether anesthesia and blood was collected from the trunk. Sera, inguinal mammary glands and mammary tumors were stored at -80°C for measurement of serum prolactin level and assay of activities of TS and thymidine kinase (TK : EC 2.7.1.21), a key enzyme in salvage pathway for pyrimidine nucleotide synthesis.

Organ weights : At autopsy, anterior pituitary, adrenals, ovaries and spleen were removed and weighed.

Normal and preneoplastic mammary gland growth : At autopsy, the bilateral third thoracic mammary glands were prepared for the wholemount evaluation and examined under 10-fold magnification. The degree of formation of normal end-buds was rated from 1 to 7 in increments of one, and the mean value for the bilateral glands represents the rating of the individual. The area of each mammary gland was automatically measured by a computerized digitizer (PIAS, Model LA-525, Tokyo, Japan). The number and area of HAN were also measured and recorded.

Serum prolactin levels : Serum prolactin levels were determined by homologous radioimmunoassay (RIA) using a mouse prolactin RIA kit (a gift from Dr. A.F.Parlow) and expressed in terms of ng per ml.

Enzyme preparation and assay : 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, containing 0.1 mM EDTA, 1 mM mercaptoethanol and 0.25 M sucrose, at final concentration, at 0°C. The homogenate was centrifuged for one hour at 4°C at 105,000×g, and the supernatant was used as the crude enzyme preparation. As previously reported,[8] activities of TS and TK were determined by the methods of Dunlap *et al.*[9] and Taylor *et al.*,[10] respectively. Enzyme activities were normalized to tissue contents of protein and expressed as fmol/mg protein/minute. Values were 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.[11] TS and TKmRNA expressed in the mammary tumor was determined by reverse transcription - polymerase chain reaction (RT - PCR) method using oligo (dT). The RT-PCR was performed with SUPERSCRIPTTM Preamplification System (GIBCO BRL) and recombinant Taq DNA polymerase (Nippon Gene, LtD., 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 (TScDNA and TKcDNA) increased linearly from 26 th to 32 th cycle. With 34 or more cycles the specific cDNA signal increased only slightly. The production of cDNA was demonstrated to be proportional to the amount of input RNA. With 30 cycles of amplification, the signals for TScDNA and TKcDNA increased linearly between 0.75 and 3.0 μg and between 0.025 and 0.2 μg, respectively, of mouse mammary tumor RNA added to the RT-PCR reaction. Based on these results, 3.0 μg and 0.1 μg of RNAs for the RT-PCR reactions using the primers for TS and TKcDNA, respectively, were used with 30 cycles (each cycle consisting of a denaturing step of 94°C for 40 sec, annealing at 55°C for 40 sec, and extending at 72°C for 40 sec) in a programmable temperature control system (ATTO, Tokyo, Japan). Two sets of primers for each TS, TK and β-actin were used for PCR, respectively (Table II).

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

Gene	Primer sequences	Amplified mRNA sequence length (base pairs)
TS	5'TAGCACAGGCGGCACACGGAGT3' 5'TGCTCCGCGATGTGACCCAGGA3'	311
TK	5'TGAATGGGGAGCTATCTTGCCA3' 5'TCGTTGGATGTGGATTATACCC3'	327
β-actin	5'AGGCCCAGAGCAAGAGAGGCAT3' 5'CATGGCTGGGGTGTTGAAGGTC3'	227

Statistical analyses : The statistical significance of difference between groups was evaluated by Student's t-test, and $p<0.05$ was considered significant.

Results

Effects of Chinese herbal medicines on body growth, organ weights, normal and preneoplastic mammary gland growth, serum prolactin levels and estrous cycle

Administration of SKT (1 g / kg / day) for 2 months enhanced the body growth to 1.8-fold that of the control ($p<0.01$) (Table III). Although there were little differeces among groups in organ weights, the formation of end-buds in normal mammary glands of mice treated with KGB+SKT (1 g/kg/day,each) was reduced to two thirds of that of the control ($p<0.05$). The combined administration of KBG and SKT markedly reduced the number of HAN to less than 35 % of that of the control ($p<0.05$). The area of normal and preneoplastic mammary glands and serum prolactin levels differed little among groups (data not shown). The relatively irregular estrous cycle of SHN mice was slightly improved by the combined administration of KBG and SKT ($p<0.05$), because the intermittent appearance of estrus in the control SHN mice was altered to relatively regular appearance by the conbined administration ; *i.e.*, percent of days of proestrus plus estrus in the estrous cycle was increased from 20.1±2.6 % to 27.9±1.6 % (Table III).

Effects of Chinese herbal medicines on TS and TK activities in mammary glands

Although there were no differences among groups in TK activity, TS activities in mammary tissues were

Table III Body growth, organ weights, normal and preneoplastic mammary gland growth and estrous cycle in each group (mean±S.E.M).

	Control	KBG	SKT	KBG+SKT
Body weight				
Initial	25.8±0.7	25.4±0.5	25.0±0.6	25.9±0.5
Final	27.2±0.7	26.3±0.4	27.3±0.6	26.8±0.6
%Change	5.2±0.5	3.5±1.2	9.6±0.3**	3.3±1.1
Organ weight				
AP	2.30±0.20	2.46±0.33	1.80±0.15	2.10±0.23
AD	12.8±0.7	11.4±0.6	12.2±0.6	12.7±0.4
OV	21.0±1.0	18.3±0.4	19.7±0.5	19.7±0.8
SP	75.1±3.9	79.2±3.4	69.4±2.0	73.0±2.3
Mammary glands				
Rating of normal mammary gland (1-5)	2.7±0.2	3.2±0.1	2.6±0.2	1.8±0.2*
Number of HAN	34.6±10.4	39.0±17.9	14.2±3.1	12.1±2.6*
Estrous cycle				
%(P+E)	20.1±2.6	26.1±5.1	23.6±1.7	27.9±11.6*

% (P+E) : Percent of proestrous and estrous days in estrous cycle.
AP : anterior pituitary, AD : adrenals, OV : ovaries, SP : spleen.
* and **Significantly different from the control at $p<0.05$ and 0.01, respectively.

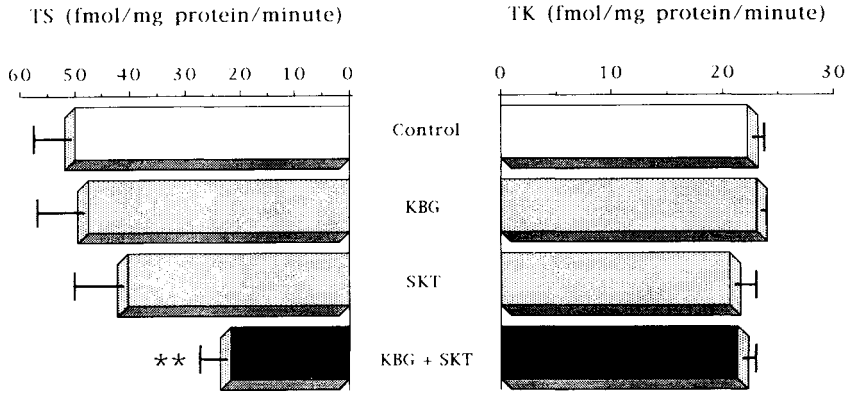

Fig. 1 Activities of thymidylate synthetase (TS) and thymidine kinase (TK) (fmol/mg protein/min) in mammary glands in each group ; Control, Keishi-bukuryo-gan (KBG), Shakuyaku-kanzo-to (SKT) and KBG+SKT (Experiment I).
**Significantly different from the control at $p<0.01$.

markedly suppressed to less than a half of the control by the combined administration of KBG and SKT ($p<0.01$) (Fig. 1).

Effects of Chinese herbal medicines on neoplastic mammary glands

Little differences were shown in body growth, organ weights and serum prolactin level of mammary tumor-bearing mice among groups (data not shown).

Tumor growth was significantly reduced by the combined administration of KBG and SKT, but not KBG or SKT alone, compared with that of the control ($p<0.05$) (Fig. 2).

The expression of TSmRNA in the experimental groups was lowered compared with that of the control

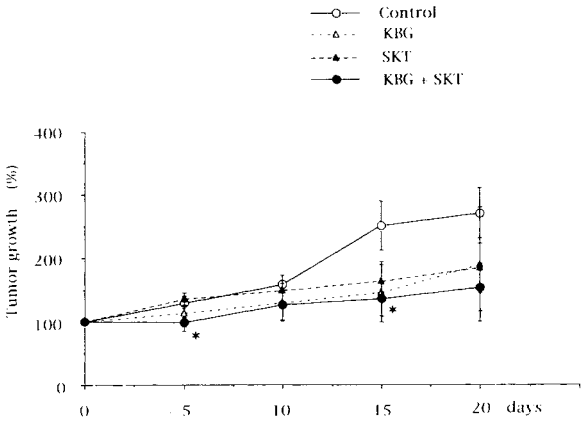

Fig. 2 Mammary tumor growth for 20 days in each group ; Control, Keishi-bukuryo-gan (KBG), Shakuyaku-kanzo-to (SKT) and KBG+SKT (Experiment II).
*Significantly different from the control at $p<0.05$.

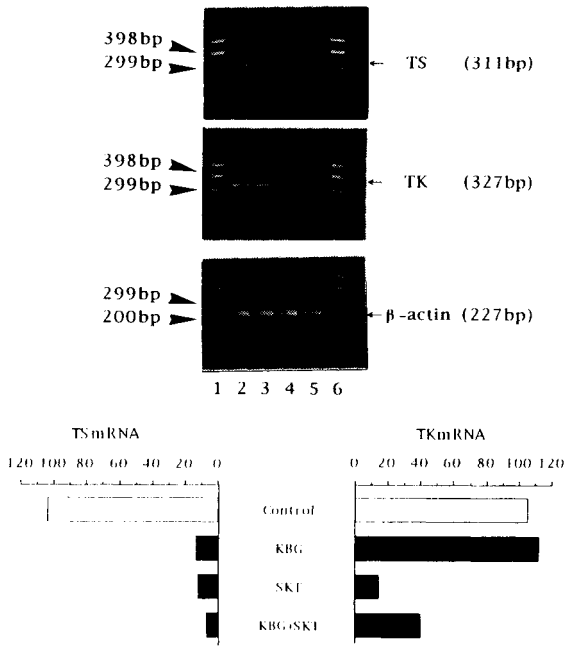

Fig. 3 The expression of TSmRNA in the experimental groups was lowered compared with that of the control (Control>KBG, SKT and KBG+SKT). The expression of TKmRNA in SKT and KBG+SKT groups was lowered compared with those of the control and KBG groups (Control and KBG>SKT and KBG+SKT). The expression of β-actin mRNA differed little among groups.
Three μg and 0.1 μg of RNA were used with 30 cycles for the RT-PCR reactions using TS and TK primers, respectively. The signal strength of each RNA was determined by densitometry in photographs using an image analyzer (AE-6920-MF Densitograph, ATTO, Tokyo, Japan).
1 and 6; Marker, 2; Control, 3; Keishi-bukuryo-gan (KBG), 4; Shikuyaku-kanzo-to (SKT), 5; KBG+SKT.

(Control>KBG, SKT and KBG+SKT). The expression of TKmRNA in SKT and KBG+SKT groups was lowered compared with those of the control and KBG groups (Control and KBG > KBG+SKT and SKT). The expression of β-actin mRNA differed little among groups (Fig. 3).

Discussion

The patients with uterine adenomyosis and/or endometriosis, if malignancy can be excluded with certainty, are generally treated with a surgical operation or a pharmaceutical administration including Gn-RH analogs, danazol and some Chinese herbal medicines.[2, 12-14] As previously reported, KBG suppressed the development of uterine adenomyosis in patients[14] and mice.[15] Administration of KBG or SKT reduces the degree of dysmenorrhea in patients.[16, 17] Recently we have prescribed KBG[5] and/or SKT to patients with symptomatic mastopathy.

TS and TK catalyze the formation of deoxythymidine monophosphate (dTMP) by the methylation of deoxyuridine monophosphate (dUMP), with the concomitant conversion of N^5,N^{10}-methyleneterahydrofolic acid to 7,8-dihydrofolic acid *via* the *de novo* pathway and by thymidine phosphorylation *via* the salvage pathway, respectively. The activities of TS and TK are high in rapidly proliferating normal, fetal and neoplastic tissues.[4, 8-10] In regenerating rat bone marrow, TS activity transiently increases and peaks, followed by the increase in TK activity and nucleated cell number, with the TS and TK peaks being observed in the $G_{0/1}$ and S phases, respectively, of the cell cycle.[18] In human gastric cancer, TS activity is relatively higher compared with that of the TK in poorly differentiated type but not well differentiated type.[19] Thus, the *de novo* pathway, but not the salvage pathway, in DNA replication would be related to the cellular malignancy.

In the present study, we investigated the effects of KBG and/or SKT on normal, preneoplastic and neoplastic mammary glands in mice, in an attempt to define the effects of the Chinese herbal medicines on patients with mastopathy.

The combined administration of KBG and SKT slightly improved the irregular estrous cycle, and

reduced the number of preneoplastic mammary HAN resulting in the decrease of TS activity in mammary glands without relation to the serum levels of prolactin of which elevation was known to accelerate the incidence and development of mammary tumors and uterine adenomyosis in SHN mice.[4,7,15] Furthermore, the combined administration lowered the tumor grouth by suppressing TS gene expression.

These results suggest that the combined administration of Chinese herbal medicines, KBG and SKT, is promising for the therapy of symptomatic mastopathy with little harmful side effects.

和文抄録

乳腺症は各種薬剤により，保存的な対症療法が施されているが，近年，私共は桂枝茯苓丸および芍薬甘草湯の投与を行っている。そこで，乳癌好発系マウスの正常乳腺，乳腺癌化過程，乳癌に与える桂枝茯苓丸および芍薬甘草湯の影響について検討した。桂枝茯苓丸と芍薬甘草湯の同時投与により，本マウス特有の不整性周期がやや改善し，乳腺胞および乳癌前癌状態の一つである乳腺過形成葉状結節の形成が抑制され，DNA デ・ノボ合成経路のチミジル酸合成酵素（TS）活性が乳腺組織で減少していた。さらに，乳癌組織でもTS遺伝子の発現および腫瘍増大が抑制される傾向が認められた。以上の結果，桂枝茯苓丸および芍薬甘草湯の併用療法は，乳腺症の治療に有効である可能性が示唆された。

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