Antitumor promoting effect of components of Chorei-to on rat urinary bladder carcinogenesis in a short-term test with concanavalin A

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Abstract

The effects of 5 components of Zhu-Ling-Tang (Chorei-to), *i.e*, Polyporus, Talcum, Asini Gelatinum, Alismatis Rhizoma and Hoelen, on the bladder tumor promotion by 5 % sodium saccharin (SS), 3 % DL-tryptophan (Trp), 2 % butylated hydroxyanisole (BHA) or 0.01 % N-butyl-N-(4-hydroxybutyl) nitrosamine (BHBN) were examined using a short-term test with concanavalin A in Wistar rats. Rats were given 0.01 % BHBN in drinking water for one week, and then each promoter alone or test samples (orally) and the promoters were administered for 3 weeks. Treatment with Polyporus (3.5 and 7 mg/kg/day), Talcum (1 and 2 mg/kg/day) and Asini Gelatinum (250 and 500 mg/kg/day) showed almost the same inhibitory effect as that of Chorei-to (270 and 540 mg/kg/day) against the tumor promotion by either SS or Trp. Meanwhile, both BHA and BHBN-induced tumor promotion was inhibited strongly by the treatment with Polyporus. Moreover, treatment with formulations omitting Polyporus, Talcum or Asini Gelatinum or Polyporus and Talcum significantly decreased the inhibitory effect against the SS-induced tumor promotion by 38 %, 31 %, 23 % or 54 % compared with that of the Chorei-to treated group. These findings indicate that Polyporus, Talcum and Asini Gelatinum are important components in the antitumor promoting effect of Chorei-to. Especially, Polyporus seems to be a key component in the inhibitory actions of Chorei-to against all bladder tumor promoters tested.

Key words antitumor promoter, concanavalin A, Chorei-to, urinary bladder carcinogenesis, sodium saccharin, tryptophan, Polyporus.

Abbreviations BHA, butylated hydroxyanisole ; BHBN, N-butyl-N-(4-hydroxybutyl) nitrosamine ; Chorei-to (Zhu-Ling-Tang), 猪苓湯 ; Con A, concanavalin A ; ED_{50}, 50 % effective dose ; EDTA, ethylenediaminetetraacetic acid : α-MM, α-methyl mannoside ; PBS, phosphate buffered saline ; SS, sodium saccharin ; Trp, DL-tryptophan.

Introduction

One of the main problems in the treatment of human superficial bladder cancer is the high frequency of recurrence after transurethral resection of the tumors.[1] The process of this recurrence may consist of multiple steps, and bladder tumor promoters are thought to play a key role in the recurrence.[2,3] Kakizoe *et al.*[4-7] established a new short-term bioassay to detect the early phase of bladder carcinogenesis by measuring the agglutination of isolated bladder epithelial cells by concanavalin A (Con A) : when rats were given bladder carcinogens, their bladder epithelial cells showed increased agglutinability with Con A. N-butyl-N-(4-hydroxybutyl) nitrosamine (BHBN),[4] sodium saccharin (SS),[5] DL-tryptophan (Trp)[6] and butylated hydroxyanisole (BHA)[7] were found to have a tumor promoting effect by this method, and were later proved to be bladder tumor promoters in a long-term carcinogenicity test.[8-10] Moreover, aspirin, α-difluoromethylornithine

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and all-trans aromatic retinoid were shown to have an antitumor promoting activity by this method.[11] Thus, this assay is very useful for screening not only bladder tumor promoters but also antipromoters on bladder carcinogenesis.

Previously, we screened 15 Kampo formulations[12] for antitumor promoting activity in bladder tumorigenesis in rats by using the short-term assay, and demonstrated that oral treatment with Chorei-to possessed a strong inhibitory effect on the tumor promotion by 5 % SS, 3 % Trp, 2 % BHA and 0.01 % BHBN. In this report, we examined the effect of the components of Chorei-to, Polyporus, Talcum, Asini Gelatinum, Alismatis Rhizoma and Hoelen, on the bladder tumor promotion by SS, Trp, BHA and BHBN, to clarify the role of these components in the antitumor promoting effect of Chorei-to.

Materials and Methods

Animals : Five-week-old, male, Wistar rats were obtained from Japan SLC, Inc., Hamamatsu, Japan, and kept in a room with controlled temperature (23± 1°C), humidity (50±5 %) and 12-h light/12-h dark cycles. They were fed commercial rat chow (MF : Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*, and were used after one week of acclimation (average weight, 140 g).

Chemicals : The following chemicals were used : BHBN (Tokyo Kasei Organic Chemicals, Co., Ltd., Tokyo) ; SS and BHA (Wako Pure Chemical Industries, Ltd., Tokyo) ; Trp (Takara Kohsan, Co., Ltd., Tokyo) ; Con A and α-methyl mannoside (α-MM, Sigma Chemical, Co., St. Louis, MO).

Herbal medicines and administration doses : Raw herbal medicines were purchased from Yamamoto Yakuhin Kogyo, Co., Ltd., Tokyo. Chorei-to, formulations omitting one or two components from Chorei-to and each raw herb were extracted with 400 ml of boiling water for 60 min, and then lyophilized. Each lyophilized water extract was dissolved in water just before use.

The usual daily dose of Chorei-to (15 g)[13] consists of 5 raw herbs : Polyporus (3 g), Talcum (3 g), Asini Gelatinum (3 g), Alismatis Rhizoma (3 g) and Hoelen (3 g). The yield of a water extract of Chorei-to was 2.7 g (18.0 %) from the mixture of 5 raw herbs (15 g). In this experiment, the water extract of Chorei-to was administered orally (p.o.) to rats at doses of 270 and 540 mg/kg/day. These doses correspond to 5- and 10-fold the usual daily dose of Chorei-to, respectively.

Meanwhile, the yield of water extracts of each raw herb, which were extracted separately with water, was as follows : Polyporus (0.035 g, 1.2 %), Talcum (0.01 g, 0.3 %) , Asini Gelatinum (2.5 g, 83.3 %), Alismatis Rhizoma (0.275 g, 9.2 %) and Hoelen (0.07 g, 2.3 %) from 3 g of each raw herb. Rats were treated p.o. with the water extracts of 3.5 and 7 mg/kg/day Polyporus, 1 and 2 mg/kg/day Talcum, 250 and 500 mg/kg/day Asini Gelatinum, 27.5 and 55 mg/kg/day Alismatis Rhizoma, and 7 and 14 mg/kg/day Hoelen. These doses were calculated on the basis of their yield of the water extracts and their compounding ratios in the usual daily dose of Chorei-to. The lower dose and the higher dose correspond to 5- and 10-fold the usual daily dose of Chorei-to, respectively.

The yield of water extracts of the formulations omitting one or two components from Chorei-to was as follows: Chorei-to minus Polyporus (2.60 g, 21.7 %), Chorei-to minus Talcum (2.34 g, 19.5 %), Chorei-to minus Asini Gelatinum (0.41 g, 3.4 %), Chorei-to minus Alismatis Rhizoma (1.94 g, 16.2 %), Chorei-to minus Hoelen (2.22 g, 18.5 %), Chorei - to minus Polyporus and Talcum (1.84 g, 20.4 %). Rats were administered p.o. 260 mg/kg/day Chorei-to minus Polyporus, 234 mg/kg/day Chorei-to minus Talcum, 41 mg/kg/day Chorei-to minus Asini Gelatinum, 194 mg/kg/day Chorei-to minus Alismatis Rhizoma, 222 mg/kg/day Chorei-to minus Hoelen, 184 mg/kg/day Chorei-to minus Polyporus and Talcum : these doses correspond to 5- fold the usual daily dose.

Treatment of animals : The rats were randomly divided into 3 groups consisting of 6 rats each. All rats were given 0.01 % BHBN in their drinking water for one week. For the next three weeks, rats in control groups were given the basal diet alone, those in promoter - treated groups were given the basal diet containing the promoters (SS ; 5 %, Trp ; 3 %, BHA ; 2 %) or drinking water containing 0.01 % BHBN, and those in test sample-treated groups were given the promoters and test samples. The BHBN solution was freshly prepared every three days in water at a con-

centration of 0.01 % and given to rats using a shaded bottle. All test samples were administered p.o. once a day at 11 in the morning during the exposure period.

Agglutination assay : All rats were killed in week four and the urinary bladder was excised. The Con A agglutination assay was performed as reported previously.[14] Briefly the urinary bladder was washed with 0.9 % saline, everted and incubated in 0.15 M NaCl containing 5 mM EDTA. Epithelial cells were separated by sonicating and squashing the bladder mucosa. In each group, the epithelial cells from 2 animals were combined and collected by centrifugation. Ten micro liters of the cell suspension ($4\text{-}6\times10^6$ cells/ml) was mixed with 10 μl of Con A (3.2 mg/ml), with or without 10 μl of α-MM (6.4 mg/ml), in a total volume of 40 μl of PBS (pH 7.4), and gently shaken for 30 min at 37°C on a microplate. Then the number of aggregates of more than 4 cells per 200 free or aggregated cells was counted in a hemocytometer. The number of Con A - dependent aggregates was calculated from the difference between the mean numbers of aggregates without and with α-MM. Three assays for each composite from 2 rats, were performed in each group of 6 animals. All data are expressed as mean±S.D.. The statistical significance of differences of agglutination values between test sample - treated and untreated groups was examined by using Student's t test.

The inhibitory percent was calculated by the following formula : Inhibition (%)= $\{1-(Y-Z)/(X-Z)\}\times100$, where X,Y and Z are the mean number of Con A - dependent aggregates of the promoter - treated groups, the test sample-treated groups and the control groups.

Results

Effects of Chorei-to and its components on bladder tumor promotion by SS, Trp, BHA and BHBN

Fig. 1 summarizes the inhibitory effects of Chorei-to and its components on bladder tumor promotion by 5 % SS, 3 % Trp, 2 % BHA or 0.01 % BHBN.

No significant increase in the number of aggregates was observed after treatment with 0.01 % BHBN for one week (data not shown). However, on treatment with SS for 3 weeks after treatment with BHBN for one week, the number of Con A-dependent

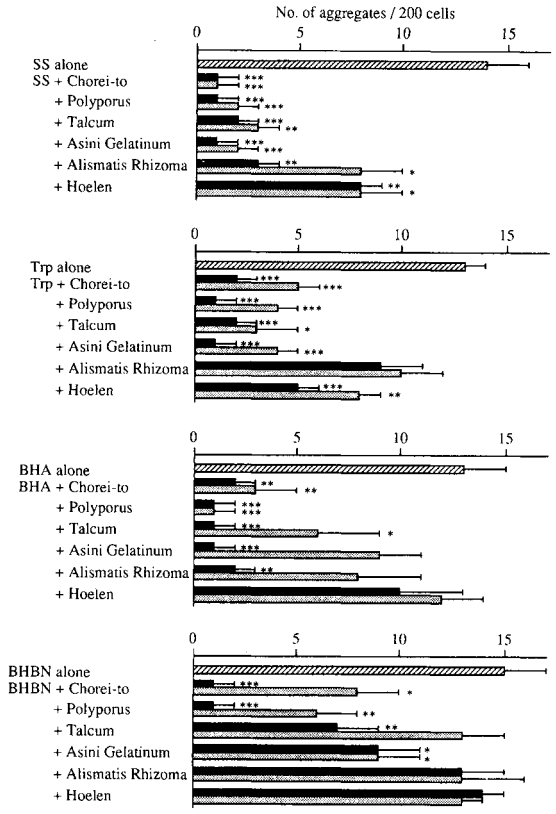

Fig. 1 Effects of Chorei-to and its components on bladder tumor promotion by SS, Trp, BHA or BHBN.
All the rats in each group were given 0.01 % BHBN in drinking water for one week, and then administered each promoter for 3 week, without or with a low-dose (▨) or a high-dose (■) of Chorei-to and its components. All values are mean±S.D. (n=3). The number of Con A-dependent aggregates of the control group was 1±1. Significant differences from the promoter alone groups,
***: $p<0.001$, **: $p<0.01$, *: $p<0.05$.

aggregates increased markedly (14±2). The increase in Con A - dependent aggregates was strongly and significantly suppressed in the treatment with Polyporus, Talcum and Asini Gelatinum, and these inhibitory effects were similar to those of Chorei-to. However Alistatis Rhizoma and Hoelen showed a weak inhibitory effect.

Similarly, the increase in Con A-dependent aggregates (13±1) of bladder epithelial cells of rats treated with Trp for 3 weeks was suppressed significantly by Polyporus, Talcum and Asini Gelatinum, and weakly by Alismatis Rhizoma and Hoelen.

Treatment with BHA alone increased the number of Con A-dependent aggregates to 13±2 per 200 cells. The increase in the aggregates was inhibited com-

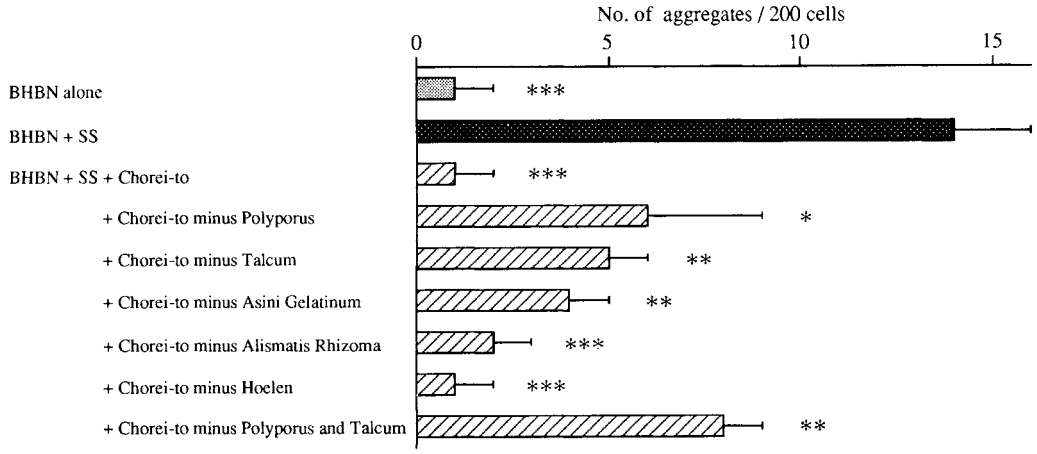

Fig. 2 Effects of Chorei-to and formulations omitting one or two components from Chorei-to on SS-induced bladder tumor promotion.
All the rats in each group were given 5 % SS without or with a 5-fold the usual daily dose of formulations for 3 weeks, after administration of 0.01 % BHBN for one week. All values are mean± S.D.(n=3). Significant differences from the BHBN and SS-treated group, ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$.

pletely by 3.5 and 7 mg/kg Polyporus. Although treatment with the higher dose of Talcum, Asini Gelatinum and Alismatis Rhizoma inhibited completely the increase in the aggregates, treatment with the lower dose of them showed a weak inhibitory effect. Hoelen slightly inhibited the increase.

Treatment with 0.01 % BHBN for 4 weeks increased the number of Con A-dependent aggregates to 15 ± 2 per 200 cells. Among the components, only Polyporus showed a strong inhibitory effect against the BHBN-induced increase in the aggregates.

Treatment with each component alone for 3 weeks did not show any significant effect on the body weights and food intake of rats compared with the rats treated with only the basal diet for 3 weeks (data not shown).

Effects of Chorei-to and formulations omitting component (s) from Chorei-to

Fig. 2 shows the inhibitory effects of Chorei-to and its modified formulations, which were prepared by omitting one or two components from Chorei-to, on SS-induced bladder tumor promotion.

In a preliminary experiment, we found that the SS-induced increase in Con A-dependent aggregates was suppressed by 69 %, 85 %, 92 %, 100 % and 100 % in the treatment with 1-, 2-, 4-, 5- and 10-fold the usual daily dose of Chorei-to, respectively (data not shown). Therefore, we examined the effect of the formulations omitting one or two components from Chorei-to at a dose of 5-fold the usual daily dose.

Administration of the formulations omitting Polyporus, Talcum or Asini Gelatinum alone from Chorei-to decreased the inhibitory effect against the SS-induced increase in the aggregates by 38 %, 31 % or 23 % compared with that of the Chorei-to treated group, respectively. However, the inhibitory effects of the formulations omitting Alismatis Rhizoma or Hoelen alone against the SS-induced increase were almost the same as that of Chorei-to. Moreover, the formulation omitting Polyporus and Talcum from Chorei-to showed the lowest inhibitory effect with a 46 % inhibition.

Discussion

We examined the effect of 5 components of Chorei-to on the bladder tumor promotion by SS, Trp, BHA and BHBN, to prove the role of the components in the antitumor promoting action of Chorei-to. Treatment with Polyporus, Talcum or Asini Gelatinum alone strongly and significantly inhibited the SS-induced increase in the aggregates, and these

inhibitory effects were almost the same as that of Chorei-to (Fig. 1). Therefore, we considered at first that each of the formulations omitting Polyporus, Talcum, Asini Gelatinum alone or both Polyporus and Talcum from Chorei-to probably possesses similar inhibitory activity to that of Chorei-to. However, treatment with these formulation showed much weaker inhibitory effects than that of Chorei-to. This indicates that mixture of the components produces a decrease in the inhibitory effect against the SS-induced bladder tumor promotion due to an interaction of each component in Chorei-to.

In Kampo medicine, a combination of raw herbs is usually used as a formulation.[13] The various constituents in the formulations are generally considered to have a synergistic effect and/or an additive effect and may mitigate the side effects of certain constituent (s) in the formulations. However, there is still little scientific evidence supporting this view. The present findings provide direct evidence of the interactions of components in a Kampo formulation Chorei-to.

Though Polyporus[15] and Hoelen[16] have been traditionally used as diuretics, only Polyporus showed strong inhibitory effects. We demonstrated previously that a diuretic furosemide had only a slight inhibitory effect.[12] These findings indicate that the inhibitory effects of Polyporus against the tumor promotion may not be due to its diuretic property.

Polysaccharides in Polyporus[17] and Hoelen[18] are known to possess the antitumor activity against sarcoma 180. We demonstrated previously that an antitumor polysaccharide in Coriolus versicolor, Krestin,[19,20] exhibited a moderate inhibitory effect against SS-induced tumor promotion in this model.[12] These findings suggest that the antiumor promoting effect of Polyporus and Hoelen against the SS-induced tumor promotion are partly due to the antitumor polysaccharides.

In conclusion, one of the components of Chorei-to, Polyporus, showed inhibitory effects similar to those of Chorei-to against the bladder tumor promotion by SS, Trp, BHA and BHBN. Moreover, treatment with a formulation omitting Polyporus alone produced a decrease in the inhibitory effect against the tumor promotion compared with that of the Chorei-to treated group. These findings indicate that Polyporus is an important component in the antitumor promoting actions of Chorei-to. Further studies to identify the active constituent (s) are being conducted.

和文抄録

5種の猪苓湯配合生薬(猪苓，滑石，阿膠，沢瀉，茯苓)の5％ sodium saccharin (SS)，3％ DL-tryptophan (Trp)，2％ butylated hydroxyanisole (BHA) あるいは0.01％ N-butyl-N-(4-hydroxybutyl) nitrosamine (BHBN) 誘発膀胱発癌プロモーション作用に対する抑制効果を，concanavalin A 依存性凝集活性を指標とする短期試験法を用いて検討した。本研究ではWistarラットに0.01％ BHBNを飲料水として1週間自由摂取させた後，各種プロモーター単独，または被験試料と共に3週間与え，被験試料の膀胱発癌プロモーション抑制作用を検討した。その結果，SS及びTrp誘発プロモーションが猪苓(3.5及び7 mg/kg/day)，滑石(1及び2 mg/kg/day)，あるいは阿膠(250及び500 mg/kg/day)単独投与により，猪苓湯投与(270及び540 mg/kg/day)と同程度に抑制されること，さらにはBHA及びBHBN誘発プロモーション作用を，猪苓のみが強く抑制することが明らかとなった。また，猪苓湯より配合生薬を1味あるいは2味除去した方剤について抗プロモーション作用を検討した結果，猪苓湯去猪苓，猪苓湯去滑石，猪苓湯去阿膠，猪苓湯去猪苓・滑石の各方剤投与により，猪苓湯に比し，抗プロモーション作用がそれぞれ38％，31％，23％及び54％減弱することがわかった。

これらの結果より，猪苓湯の抗プロモーション作用において，猪苓，滑石，阿膠が重要な役割をはたしていることが示された。特に猪苓は猪苓湯の有するすべてのプロモーターに対する抑制作用において，鍵となる生薬であることが示唆された。

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