Studies on cancer chemoprevention by traditional folk medicines. XV.[1] Antitumor-promoting effect of White pepper, *Piper nigrum*, and piperine *in vitro* and *in vivo* carcinogenesis

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Abstract

Three kinds of extracts (*n*-hexane, ethyl acetate and methanol) derived from White pepper, the fruits of *Piper nigrum* L. (Piperaceae), were examined for antitumor-promoting activity on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-enhanced ^{3}H-choline incorporation into phospholipids of C3H10T1/2 cells. Piperine isolated from this material exhibited the remarkable inhibitory effect on TPA-enhanced ^{3}H-choline and ^{32}Pi-incorporation into phospholipids of cultured cells *in vitro* screening tests.

In the *in vivo* system, each extract and piperine were also seen to have an inhibitory activity on TPA-induced mouse ear edema. In addition, the acetone soluble part derived from the finely crushed powder of White pepper and piperine were proved to have antitumor-promoting activity in two-stage mouse skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene (DMBA) as initiator plus TPA as promoter, and the topical treatment of the acetone soluble part affected the effect of decrease and delaying emergence of skin tumor formation, although that of piperine was not of such a high potency. Moreover, the acetone soluble part derived from White pepper strongly exhibited an inhibitory effect in the 4-nitroquinoline N-oxide (4 NQO) as initiator plus glycerol as promoter.

Key words White pepper, *Piper nigrum*, piperine, ^{3}H-choline incorporation, ^{32}Pi-incorporation into phospholipid, two-stage mouse skin carcinogenesis, two-stage mouse lung carcinogenesis.

Abbreviations TPA, 12-*O*-tetradecanoylphorbol-13-acetate ; 4NQO, 4-nitroquinoline N-oxide ; DMBA, 7,12-dimethylbenz[a]anthracene ; ^{32}Pi, radioactive inorganic phosphate.

Introduction

White pepper has been widely used in the world as a foodstuff and spice, and also has been used as a traditional folk medicine. A number of interesting pharmacological and physiological substances have been isolated from *Piper nigrum* L. (Piperaceae), and their pharmacokinetics have been reported by many authors,[2-5] although the antitumor activity of piperine has been rarely studied.

It has recently become apparent that environmental factors play an important role in lowering the incidence of several adult diseases, especially human cancer. In a continuing search for potential anticancer agents under "Studies on Cancer Chemoprevention by Traditional Medicines", we are surveying effective methods to employ the antitumor-promoting activity on TPA-stimulated ^{3}H-choline and ^{32}Pi-incorporation into phospholipids of cultured cells *in vitro* and on the inhibition of the promotion of skin, lung and colon tumor formations *in vivo* carcinogenesis experiments. We have reported the inhibitory effect of many Umbelliferous plants,[6] *Allium* spp.,[7] spices,[8] traditional

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Chinese medicines,[9] and Kampo medicines.[10]

In the previous report,[8] we clarified the antitumor-promoting effect by 111 kinds of extracts derived from 37 spices on TPA-enhanced ^{3}H-choline incorporation into phospholipids of C3H10T1/2 cells and on TPA-induced mouse ear edema. Furthermore, by using antitumor-promoting activity as an indicator, we isolated ursolic acid from Sage, luteolin from Celery seed, laxogenin from Xiebai and piperine from White pepper, respectively.

The present study examined the antitumor effects *in vitro* system in cultured cells, and *in vivo* experiments mouse ear edema on the extracts and piperine obtained from White pepper, the fruits of *Piper nigrum*, moreover, two-stage mouse skin and lung carcinogenesis using the acetone soluble part and piperine.

Materials and Methods

Materials : White pepper, very fine crushed powder (c.p. White pepper), and the fruit of *Piper nigrum* L. (Piperaceae), were purchased from Tokyo Spice Co. (Tokyo in Japan) and was deposited in the Herbarium, Meiji College of Pharmacy. White pepper (500 g) was successively extracted with *n*-hexane, ethyl acetate, methanol and water (each 600 ml 2hr × 2, per extraction) under reflux. The *n*-hexane, ethyl acetate and methanol solutions were evaporated *in vacuo* to yield the corresponding *n*-hexane (He), ethyl acetate (Ac) and methanol (Me) extracts, respectively. 100 g of White pepper was extracted with hot acetone two times at 6 hr each to yield an acetone soluble part.

Chemicals : TPA, DMBA and 4 NQO were purchased from Sigma Chemical Co., USA. ^{3}H-choline and ^{32}Pi were purchased from Japan Radioisotope Associations, Japan.

Assay for ^{3}H-choline incorporation into phospholipids of cultured cells : Incorporation of ^{3}H-choline into phospholipids of C3H10T1/2 cells was assayed as described previously.[7,8] Each extract was dissolved in DMSO to give a corresponding test solution, which was applied to C3H10T1/2 cells. Mouse fibroblast C3H10T1/2 cells were incubated with 50 μg/ml of each test solution. After 1 hr, ^{3}H-choline (370 kBq/culture) was added with or without TPA (50 nM). After 4 hr incubation, the radioactivity incorporated into phospholipids of cells were measured. Data are means for duplicate assays.

Assay for ^{32}Pi incorporation into phospholipids of cultured cells : HeLa cells were cultured in Eagle's minimum essential medium supplemented with 10 % calf serum under a humidified atmosphere of 5 % CO_2 in air. Incorporation of ^{32}Pi into phospholipids of HeLa cells was assayed by the same method described previously.[6,7,9,11,12]

Animals : Male ddY and female ICR mice (6 weeks of age) were obtained from SLC, which were assigned to each group, housed together, and acclimatized under standard conditions with free access to food and water for a week.

TPA-induced mouse ear edema : Induction of ear edema was based on the method of Okuyama *et al.*[8] and Inoue *et al.*[13,14] The *n*-hexane and ethyl acetate extracts (each 10 mg) were dissolved in acetone (200 μl) and methanol extract (10 mg) was dissolved in ethanol (200 μl) to give the test solution, respectively. Each 20 μl of test solution was applied for topical application to both inner and outer surfaces of the right ear only by using a micropipette. After 30 min. treatment of test solutions, TPA was dissolved in acetone at a concentration of 100 μg/ml, 2 μg per ear which was treated on both inner and outer surfaces of the ear. Ear thickness was measured 5 hr after TPA treatment with a pocket thickness gauge (Ozaki MFG. Co. Ltd).

Two-stage carcinogenesis experiments :

(1) Two stage mouse skin carcinogenesis : Six-week old ICR female mice were used. The back of each ICR female mouse was shaved with surgical clippers. The mice were carcinogenecally initiated with DMBA 150 μg (585 nmol) in acetone (0.1 ml) topically. One week after initiation, TPA (2 μg, 3.25 nmol) in acetone was applied on the same part of the back of mice twice a week. Each sample was administrated both topically and orally. The piperine topical treated group (5) was given a dose of 1 mg per mouse, 30 min. early for each TPA-treatment. In the oral administration group (4), piperine was dissolved in drinking water (1.25 mg/100 ml), and given *ad libitum* for the promotion period. Topical application of

White pepper (group 3) was carried out with 5 mg of acetone soluble part obtained from the fine powder. And group 2 was given the crushed powder of White pepper in drinking water (12.5 mg/100 ml) for the promotion period. TPA and test sample were continuously applied for 18 weeks. The number of tumors was measured once a week. Each experimental group consisted of 15 mice.

(2) Two-stage mouse lung carcinogenesis : The fine crushed powder of White pepper with HCO-60 (0.04 g/100 ml) piperine in drinking water were prepared for oral administration.

This experiment was performed by the same method as the mouse lung tumor formation by our previous method.[8] Six week old ddY male mice were initiated with 4 NQO (0.3 mg/mouse) as an initiator which was dissolved in a mixture of olive oil and cholesterol (20 : 1) by single subcutaneous (s.c.) application on the first experimental day. For promotion, 5 % glycerol with (control 2) or without 0.04 % HCO-60 (control 1 ; Nikko chemicals, Japan) in drinking water were given *ad libitum* from 4 weeks after initiation continuously for 25 weeks. The test sample was dissolved in drinking water with 0.04 % HCO-60 containing 5 % glycerol. After mice were sacrificed, the lungs were removed and fixed in a formaldehyde solution. Each experimental group consisted of 15 mice.

Results

The *n*-hexane (71.5 %), ethyl acetate (64.2 %) and methanol (73.3 %) extracts derived from the White pepper exhibited an inhibitory effect on TPA-enhanced ^{3}H-choline incorporation into phospholipids of C3H10T1/2 cells *in vitro*. Piperine was also carried out the screening tests on TPA-enhanced ^{3}H-choline incorporation and on TPA-stimulated ^{32}Pi-incorporation into phospholipids of cultured cells as shown in Table I.

Next, the same extracts were supplied for the screening test on TPA-induced mouse ear edema *in vivo* to show the strong inhibitory effect such as *n*-hexane (69.6 %), ethyl acetate (51.9 %) and methanol (69.1 %) extracts, respectively. The methanol extract and piperine had a dose-dependent inhibitory effect on

Table I Inhibitory effect of extracts and piperine obtained from White pepper on TPA-enhanced ^{3}H-choline incorporation into phospholipids of C3H10T1/2 cells and ^{32}Pi-incorporation into phospholipids of HeLa cells.

Sample	^{3}H-Choline Inhibition[1] (%)	^{32}Pi Inhibition[2] (%)
piperine (50 μg/ml)	60.8	95.0
piperine (25 μg/ml)	19.8	55.5
piperine (10 μg/ml)	0	43.3

[1]C3H10T1/2 cells were treated with 3 concentrations of piperine. After 1 hr, ^{3}H-choline (370 kBq/culture) was added with or without TPA (50 nM).
[2]HeLa cells cultured in Petri dishes were incubated with each concentration of piperine. After 1 hr, ^{32}Pi (74 kBq/culture) was added with or without TPA (50 nM).
Each incubation was continued for 4 hr, and then radioactivity incorporated into phospholipid fraction was measured. Data are mean values of duplicate experiments and are expressed as % inhibition.

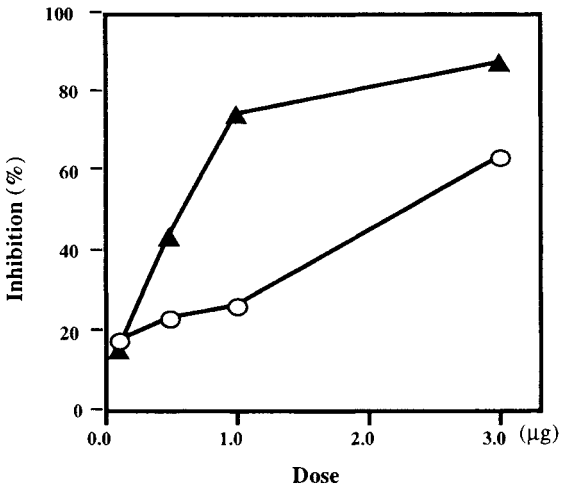

Fig. 1 The dose-dependent inhibition of piperine and methanol extr. obtained from White pepper on TPA-induced mouse ear edema.
—○— Piperine
—▲— MeOH extr.

TPA-induced mouse ear edema, which the IC_{50} of the former was 0.7 mg/ear and the latter was 2.2 mg/ear as shown in Fig. 1. Thus, piperine may be a less active constituent in the extract of White pepper.

In addition, we tried also to evaluate the next *in vivo* antitumor-promoting activity on two-stage mouse skin and lung carcinogenesis. Now, the inhibit-

ing potency is relatively parallel without some cases to the antitumor promoting effect. The evaluation of the inhibitory potency for the tumor-promoter enhanced-phospholipids metabolism has been shown to be practically useful for the screening of new antitumor-promoting agents *in vitro* system. In some cases, it shows a correlation between the inhibitory effect against TPA-induced inflammation on mouse ear edema and inhibition on the skin tumor promotion.[8, 13, 15] In general, White pepper is used in a variety of ways as a spice or condiment as powder of the fruit. Thus, the acetone soluble part of the very fine crushed powder (c.p. White pepper) obtained from the fruits of White pepper and piperine were used to carry out the two-stage mouse skin and lung carcinogenesis experiments under the oral administration and topical treatment.

In the first set up, treatment one week later with DMBA, the mice were treated topically with TPA 2 μg (3.24 nmol) and treatment continued twice a week for 18 weeks. From the Fig. 2a, the first tumor formation of the control mouse appeared at week 5 and was completely formed at week 7. Of the test samples, the percentage of tumor-bearing mice of White pepper (2) and piperine (4) as an oral treatment and piperine as topical treatment (5) had almost the same result (87-93 %), whereas that of the c.p. White pepper as topical treatment (3) was delayed 3 weeks behind the control and the average number of tumors per mouse decreased the tumor formation (47 %). Fig. 2b shows that the average number of tumors per mouse of each test material was less than that of control. The control group (1) produced 19.6 tumors per mouse at week 18, on the other hand, samples 3, 5 and 6 had less tumor formation 12.6, 9.1 and 11.4 items, respectively, whereas that of (4) suppressed excellently the induction of tumors per mouse by 0.8 item.

In the next two-stage experiment, the effect of the same material on lung carcinogenesis test treated with 4 NQO as an initiator and 5 % glycerol alone (control-1 ; group I) or plus 0.04 % HCO-60 (control-2 ; group II) was studied for 25 weeks. As shown in Table II, each control of the number of tumors-bearing mice was 100 % on the lung carcinogenesis test, respectively. And the intake on the number of tumor-bearing mice of piperine (93 %) and the c.p. White

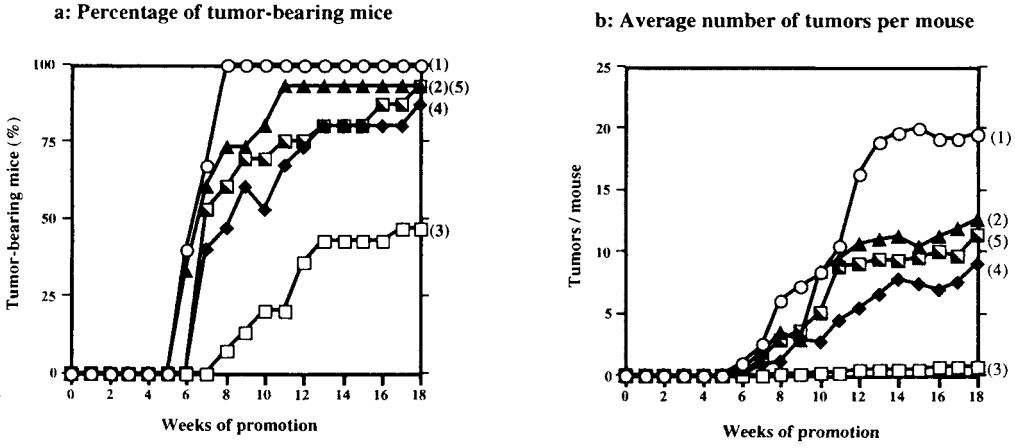

Fig. 2 Inhibitory effect of White pepper and piperine on skin tumor formation induced by TPA in DMBA-initiated mice.

—○— Control (1)
—▲— White Pepper Oral (2) (125 mg/l in drinking water)
—□— White Pepper Topical (3) (5 mg/mouse painting)
—◆— Piperine Oral (4) (12.5 mg/l in drinking water)
—◪— Piperine Topical (5) (1 mg/mouse painting)

From 1 week after initiation by DMBA (150 μg), TPA (2 μg) was applied twice a week for 18 weeks. The incidence of papillomas was observed weekly. Each sample was prepared and treated as described in Materials and Methods.

Table II Inhibitory effect of White pepper and piperine on the promotion of lung carcinogenesis by glycerol in 4 NQO-initiated mice.

Group		Number of Tumor-bearing mice (%)	Tumors per mouse	Inhibition (%)*
I	5 % glycerol	100	8.33	—
II	+0.04 % HCO-60	100	7.82	—
III	+0.04 % HCO-60 +White pepper	75	2.58	67
IV	+0.04 % HCO-60 +piperine	93	4.00	49

For initiation, 4 NQO dissolved in a mixture of olive oil and cholesterol (20 : 1), was given by single s.c. injection on first experimental day (0.3 mg/mouse). Glycerol, as tumor promoter, was dissolved in drinking water of a concentration of 5 %, and given *ad libitum* from experimental week 5 to 30, Piperine and White pepper powder were administrated as shown in Materials and Methods.
*The inhibition rate compared to group II (5 % glycerol+0.04 % HCO-60).

pepper with HCO-60 as surface active agent by oral administration (75 %), of which tumor per mouse of latter was 2.58 items, moreover, remarkably reduced the formation of lung tumor (inhibition rate : 67 %) compared with control 2.

The HCO-60 as surface active agent did not affect tumor formation in this experiment. The increase of body weight rate by each test compound was the same grade as that of both controls. Thus, each test compound did not seem to have general toxicity.

Tumors of each lung were analyzed as to histopathological findings and the alveolar-bronchiolar adenoma was determined. All of adenoma existed in contact with the pleura and not the center of lung.

Discussion

The decrease of a high incidence of cancer and cancer prevention is now the most urgent problem in public health. We have been investigating the use of the antitumor-promoting activity *in vitro* as a primary screening for discovery of active agents from natural resources. We are now especially interested in daily foodstuffs, spices and traditional Chinese medicines. In this paper, we would like to report the inhibitory activity of White pepper against tumor-formation, which is known to be one of our daily foodstuffs and has been widely used for a long time as a traditional folk medicine.

As reported in the previous paper,[8] some spices, Basil, Ginger, Marjoram, Rosemary, White pepper and Xiebai showed an excellent inhibitory effect on TPA-enhanced ^{3}H-choline incorporation into phospholipids of C3H10T1/2 cells, and then Allspice, Basil, Bay (Laurel), Cardamom seed, Cinnamon, Cumin, Dill seed, Dry ginger, Ginger, Horseradish, Japanese parsley, Marjoram, Oregano, Parsley, Pink pepper, Red pepper, Rosemary, Sage, Tarragon, Thyme, Turmeric and White pepper exhibited potent activity on TPA-induced mouse ear edema. Of these spices, White pepper had a high inhibitory effect on antitumor-promotion in both experiments.

Recently, Yasukawa *et al.* tested the inhibitory effects of spices, vegetables and fruits on TPA-induced inflammation in mice.[15] It was demonstrated that Black pepper, *Piper nigrum*, had an inhibitory effect on TPA-induced ear edema, but the active component was not reported in the paper. We can find some reports on the carcinogenesis induced by black pepper. Members of the *Piper* genus, such as White pepper and Black pepper, contain similar compounds and may prove a fruitful source for studying the role of dietary factors in carcinogenesis.[16-18]

In the present study, piperine demonstrates a lesser effect, but the c.p. White pepper as topical treatment strongly exhibits an inhibitory effect on tumor promotion by DMBA-TPA in the two-stage mouse skin and 4 NQO-glycerol in the two-stage mouse lung initiation promotion model. This suggests a need for additional studies with other constituents of White pepper.

和文抄録

白胡椒 *Piper nigrum*（コショウ科）から得た3種のエキス（*n*-ヘキサン，酢酸エチルエステル，メタノール）でTPAによるC3H10T1/2細胞のリン脂質への^{3}H-コリンの取り込みを調べた。単離したpiperineでは，TPAによるC3H10T1/2細胞のリン脂質への^{3}H-コリンの取り込みと，TPAによって誘発されるHeLa細胞への^{32}Piの取り込みに対する抑制効果を検討した。piperineは両試験で抑制効果を示した。*In vivo* の実験系として，同3種の各エキス及びpiperineのTPAによるマウス耳浮腫実験で，いずれも阻害効果が認められた。更に，白胡椒微細粉末のアセトン可溶部とpiperineを経口投与と背中の皮膚に塗布する方法でマウスの皮膚二段階実験を行ったところ，アセトン可溶部を皮膚に塗布する投与方法では非常に強い抑制効果を示した。しかし，piperineにはいずれの投与方法においてもさほど強い抑制効果は認められなかった。次に，イニシエーターとして4NQO，プロモーターとしてglycerolを用いる経口投与法による肺二段階発がん試験で，白胡椒微細粉末のアセトン可溶部とpiperineはいずれも腫瘍発生を抑制する結果が得られた。

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