Antitumor-promoting activity of diallyl pentasulfide ; a constituent of garlic

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Abstract

Diallyl pentasulfide (DPS), a constituent of aged garlic, dose-dependently inhibited Epstein-Barr virus early antigen (EBV-EA) expression induced by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), a potent tumor-promoter. DPS could not affect TPA-enhanced ^{32}Pi incorporation into phospholipids of cultured cells, although a low dose of DPS increased the ^{32}Pi incorporation. *In vivo*, DPS inhibited TPA-induced epidermal ornithine decarboxylase activity in mouse, and also suppressed the promoting effect of TPA on skin tumor formation in mice initiated with 7, 12-dimethylbenz-[a]-anthracene. These results suggested that DPS seems to be one of the anti-carcinogenic constituents included in garlic extract.

Key words Garlic, Diallyl pentasulfide, Antitumor-promotion, two-stage skin carcinogenesis, ODC activity.

Abbreviations DMBA, 7,12-dimethylbenz-[a]-anthracene ; DPS, diallyl pentasulfide ; EBV-EA, Epstein-Barr virus early antigen ; ODC, ornithine decarboxylase ; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Introduction

Garlic has not only been used as a cooking herb, but also as a traditional medicine. A number of studies on the constituents of garlic revealed pharmacological activities, such as antiplatelet-aggregation effect[1,2] and greatest antibacterial activity.[3] Recently, the possible association between consumption of garlic and lower risk of cancer is becoming a suject of growing interest. Thus, the antitumor-effect of garlic was investigated using various experimental models. Topical application of garlic oil, garlic extract, or allixin, the constituent of garlic, inhibited the skin-tumor formation induced by TPA.[4-6] Allyl sulfuric compounds were isolated from garlic and other *Allium* plants. It was also reported that diallyl sulfide inhibited the effect of colon carcinogen,[7] and diallyl disulfide induced apoptosis of human colon cancer cells.[8]

However, there are some negative reports ; organosulfur compounds from garlic and onions enhanced glutathione S-transferase placental form positive focus formation in the liver,[9] and also enhanced hepatocarcinogenesis in rats.[10]

In epidemiological studies, some negative or positive relation between the consumption of garlic or other *Allium* vegetables and the lower risk of cancer were reported in case-control or cohort studies.[11,12] Thus, further studies are necessary to elucidate the usefulness and harmfulness of *Allium* genus vegetables for cancer.

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In the present study, we evaluated the effect of diallyl pentasulfide, a naturally occurring allyl sulfuric compound abundant in vegetables of *Allium* genus, against TPA-induced phenomena *in vitro* and *in vivo*.

Materials and Methods

Chemicals : Diallyl pentasulfide (DPS) was supplied by Wakunaga Pharmaceutical Co., Hiroshima, Japan. 7,12-Dimethylbenz-[a]-anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co., St. Louis, Mo., USA. Radioactive inorganic phosphate (^{32}Pi, carrier-free) and L-[2,3-^{3}H]-ornithine were purchased from Japan Radioisotope Associations, Tokyo, Japan. All other chemicals were biochemical reagent grade.

Assay for Epstein-Barr virus activation in Raji cells : Epstein-Barr virus (EBV) activation was assayed by the method of Ito *et al.*[13] Human lymphoblastoid cells (Raji cell), carrying genomes of the EBV, were incubated for 48 hr in 1 mL of medium containing *n*-butyric acid (4 μmol), TPA (32 pmol) and/or various concentrations of DPS. The induction rate of early antigen (EA) of EBV was monitored by staining individual smears with EA^{+}VCA^{+} serum [EA titer 1 : 320, EBV capsid antigen (VCA) titer 1 : 1280] or with EA^{-}VCA^{+} serum (VCA titer 1 : 640). Data are the mean value of duplicate experiments, and expressed as the percentage of EA induction.

Cell culture and assay for ^{32}Pi incorporation into phospholipids of HeLa 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 method of Nishino *et al.*[14]

For ^{32}Pi-labeled phospholipids analysis, phospholipid fractions extracted from each ^{32}Pi-labeled cells were subjected to thin layer chromathography (TLC, silica gel 60, Merck, in the solvent system of $CHCl_3$-acetone-MeOH-AcOH-H_2O=10 : 4 : 2 : 2 : 1, v/v), and the TLC plates were autoradiographed.

Assay for inhibition of ornithine decarboxylase : Induction of ornithine decarboxylase (ODC) was carried out by a single application of 10 μg (16.5 nmol) TPA on the back of 7-week-old male ICR mice (purchased from Japan CLEA Ind.). DPS (165 nmol ; molar ratio to TPA was 10 : 1) was applied with or without TPA. The blank group was treated with 200 μL of acetone, which was used as the solvent for TPA and DPS. After 4 hr, epidermis was collected in the buffer A (50 mM phosphate buffer, 5 mM DTT, 0.1 mM EDTA, 40 mM pyridoxal phosphate). The samples were sonificated and ultacentrifuged at 32,000 rpm for 20 min. Cell extracts were held on ice until the ODC assay was performed.

ODC assay was based on the method Djurhuus[15] by measuring the level of [^{3}H] putrescine derived from [^{3}H] ornithine. One hundred μL of reaction mixture containing 48 mM phosphate buffer (pH 7.2), 1 mM EDTA, 0.01 mM pyridoxal phosphate, 0.45 mM ornithine plus [2,3-^{3}H]-ornithine (46.5 Ci/mol), and 50 μL of cell extracts were incubated for 1 hr at 37°C under 5 % CO_2. The reaction was stopped by placing the samples on ice for 10 min. One hundred μL of the samples were transferred to Whatman P81 paper, a strong cation-exchanger, for selective binding of putrescine in excess ammonia (0.1 M) bath. After drying, the samples were counted in a liquid scintillation counter.

Two-stage mouse-skin carcinogenesis experiments : Initiation of skin carcinogenesis was carried out by a single application of 100 μg DMBA (390 nmol) on the back of 7-week-old female ICR mice (purchased from Japan CLEA Ind.). For promotion, TPA (2.0 μg, 3.25 nmol) was applied on the same place twice a week from 1 week after the initiation. DPS (3.25 μmol ; molar ratio to TPA was 1000 : 1) was applied simultaneously with TPA. TPA or/and DPS were dissolved in 100 μL of acetone. The number of tumors was determined once a week. Each experimental group consisted of 15 mice. The experiment was continued for 16 weeks.

Results and Discussion

TPA is one of skin specific potent tumor-promoters, and its various biological activities have been reported, e.g. enhancing effect on protease activity, eicosanoid metabolism, phospholipid synthesis, nucleic acid replication and transcription, ornithine

Table I Inhibitory effect of diallyl pentasulfide on EBV activation by tumorpromoter.

	Concentration (μg/ml) of diallyl pentasulfide			
	20	15	10	5
Relative ratio of	0	13.2	37.4	69.0
EBV activation	(10)	(70)	(100)	(100)

Data are expressed as relative ratio of activation with respect to positive control Data in parenthesis : viability of cells (%)
Raji cells, Epstein-Barr-virus (EBV) genome-carrying human lymphoblastoid cells, were incubated for 48 hr in 1 mL of medium containing *n*-butyric acid (4 μmol), TPA (32 pmol) and various concentration of test compound. After 48 hr, the induction rate of early antigen (EA) of EBV was measured.

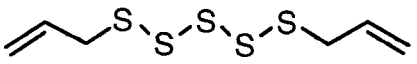


Fig. 1 Structure of diallyl pentasulfide.

decarboxylase activity,[16] *etc.* These effects may be closely related to edema, and finally tumor-promotion. Thus, inhibiting the TPA-induced various phenomena may lead to anti-tumor promotion.

TPA-induced activation of Epstein-Barr virus (EBV) in Raji cells has been used to evaluate the potency of antitumor-promoting activity.[17] DPS inhibited EBV-EA expression in a dose-dependent manner (see Table I). Ten μg/mL of DPS (41.2 μM ; molar ratio to TPA was 1 : 1289) was inhibited EBV activation about 60 %, and no cytotoxicity was observed in this dose. ID_{50} was between 5 μg/mL and 10 μg/mL. Cytotoxicity of DPS was observed at more than 15 μg/mL (61.9 nmol/mL).

To evaluate the potency of antitumor-promoting effect, we have also used the *in vitro* assay system, the inhibitory effect on tumor-promoter enhanced ^{32}Pi-incorporation into phospholipids of HeLa cells.[14] DPS at the dose of 15 μg/mL and more could not affect the phospholipids synthesis, although some potentiation was observed at a lower concentration, 10 μg/mL and 5 μg/mL (Fig. 2). The phospholipid fractions containing ^{32}Pi were analyzed by TLC. (Data not shown) Some potentiation of incorporation into phosphatydyl-choline was observed at the dose of 10 μg/mL. In TPA-untreated fractions, 10 μg/mL DPS did not induce significant difference in comparison with control. Taken the case of DPS, this compound could not affect the ^{32}Pi-incorporation without TPA at the same dose. From these results, it was uncertain whether this compound was an antitumor-promoting agent or not. So, we tried to evaluate *in vivo* carcinogenesis experiment.

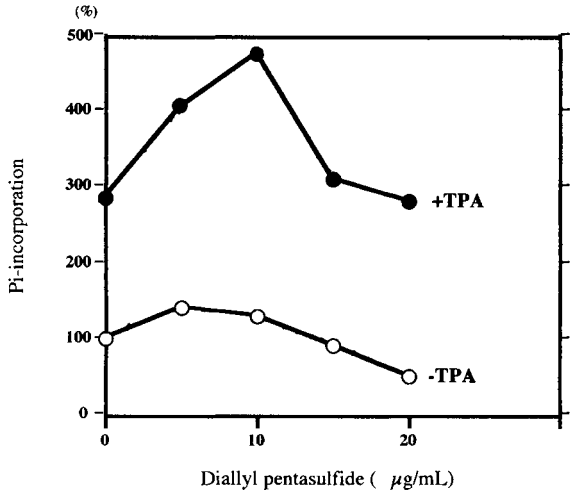
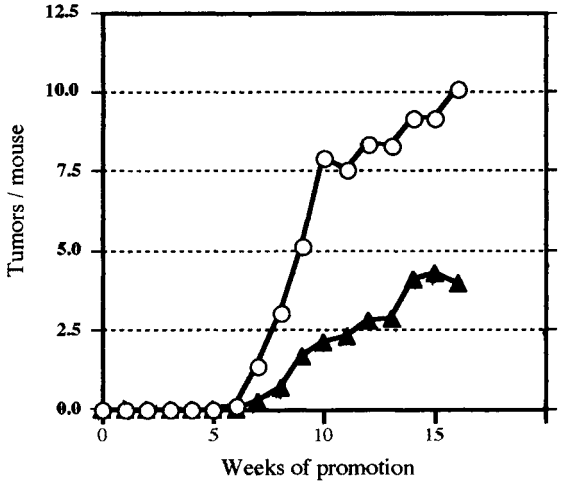


Fig. 2 Effect of diallyl pentasulfide on TPA-enhanced phospholipid synthesis.
HeLa cells were treated with various concentrations of DPS. After 1 hr, ^{32}Pi (74 kBq/culture) was added with or without TPA (50 nM). Incubation was continued for 4 hr, and then radioactivity incorporated into phospholipid fraction was measured. Data, expressed as percentage on TPA-enhanced ^{32}Pi incorporation, are mean values of duplicate experiments.

TPA-induced two-stage skin carcinogenesis model was useful to evaluate the potency of inhibitory activity on tumor-promotion, simply.[18,19] The dose of DPS was decided as 3.25 μmol by *in vitro* results, 10 μg/mL of DPS (molar ratio to TPA was 1 : 1000) suppressed TPA-induced EBV-EA activation, however, enhanced TPA-induced phospholipids synthesis. Fig. 3a and b show the time course of skin-tumor

a) Average number of tumors per mouse.



b) Percentage of tumor-bearing mice.

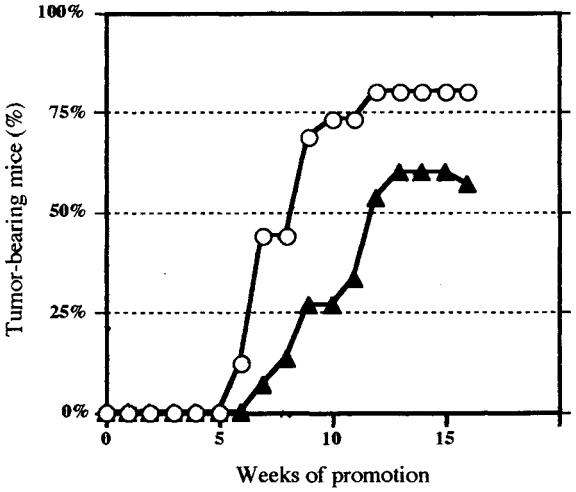


Fig. 3 Effect of diallyl pentasulfide on the promotion of skin tumor formation by TPA in DMBA-initiated mice.
—○— Group treated with DMBA plus TPA (3.25 nmol/painting)
—▲— Group treated with DMBA plus TPA (3.25 nmol/painting) and DPS (3.25 μmol/painting)

formation in the groups treated with DMBA plus TPA, with or without DPS. The number of tumors per mouse decreased significantly in the DPS-treated group. The gross appearance of the tumors was not significantly different between the group treated with DPS and the group without DPS. DPS reduced the percentage of tumor-bearing mice. The body weight gain of experimental animals was not different between the control and DPS-treated group (data not shown).

In two-stage epithelial carcinogenesis model, one of the earliest events following a single application with TPA is a rapid and transient induction in ODC activity.[16] ODC was considered as a rate-limiting enzyme of polyamin-biosynthesis cascade and related to tumor-promotion.[20] ODC catalyzes the decarboxylation of L-ornithine to putrescine. Polyamines (putrescine, spermine, and spermidine) are essential for normal cell proliferation and differentiation, and ODC are highly expressed in neoplastic cells and tissues.[21] Thus, activity of this enzyme is regarded as a marker of neoplastic region in human and experimental animals.

As shown in Table II, DPS suppressed ODC activation induced by TPA (53%, inhibition %), and DPS alone treatment did not affect ODC activity. The anti-ODC-activating effect of DPS may play an important role of the antitumor-promoting activity on mouse skin carcinogenesis.

Table II Effect of diallyl pentasulfide on ODC activity induced by TPA in mouse skin.

Condition	ODC activity (nmole putrescine/mg protein/hr)	% to Control
Acetone	0.5	0.0
+TPA	25.4	100.0
+TPA+DPS	12.1	46.6
+DPS	0.3	−0.8

Mice were treated with 165 nmol of DPS and/or 16.5 nmol of TPA in 200 μL acetone. Control was treated with TPA and vehicle in acetone. Mice were killed 4 hr after TPA treatment to determine ODC activity. Data are mean value of three mice.

In these experiments, DPS inhibited the TPA-induced EBV-EA in a dose dependent manner *in vitro*, and also suppressed the tumor promotion on two-stage skin carcinogenesis *in vivo*. However, this compound enhanced the tumor-promoter induced phospholipids metabolism *in vitro*, DPS without TPA did not affect this phenomenon. The mechanisms of TPA-induced phospholipids metabolisms are not clear,

though these results suggested us that DPS may inhibit some metabolic enzymes of chemical mediator which is induced by TPA and induce phospholipids synthesis.

Diallyl pentasulfide is a component of aged garlic, not fresh garlic. The yield of the compound was less than 1%, at most. The yield may depend on the condition of store.

There is a report that garlic extract suppressed ^{32}Pi-incorporation into phospholipids of HeLa cells.[5] It is reasonable to suppose that the combined use of DPS with other antitumor-promoting agents in garlic, which have the activity to suppress TPA-enhanced phospholipid metabolism, may result in the improvement of the anti-carcinogenic effect.

Conclusions

In present study, it was found that DPS exhibits the antitumor-promoting activity *in vitro* and *in vivo*. DPS seems to be not such a potent antitumor-promoting agent, but to be one of the anti-carcinogenic constituents included in garlic extract. It is necessary to further study the effects of these constituents on anti-carcinogenic activity.

和文抄録

大蒜の成分である Diallyl pentasulfide (DPS) の発がんプロモーション抑制効果を検討した。DPS は強力な発がんプロモーターである TPA により誘発される Epstein-Barr ウイルス早期抗原誘示に対し濃度依存的に阻害効果を示した。また，TPA により誘導される放射性無機リンの培養細胞リン脂質への取り込みにほとんど影響を与えなかったが，低濃度においては逆に TPA の効果を増強する傾向が認められた。*in vivo* において DPS は同じく TPA により誘導されるオルニチン脱炭酸酵素活性を阻害し，また，マウス皮膚二段階発がんにおいて抗プロモーション効果を示した。その効果は，大蒜の発がん予防作用の一端を担っているものと思われる。

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