Special Issue

Anti-carcinogenic activity of 6-methylsulfinylhexyl isothiocyanate-, an active anti-proliferative principal of wasabi (*Eutrema wasabi* Maxim.)

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Abstract

Synthetic 4-methylsulfinylhexyl isothiocyanate (MITC)(a potent inducer of phase 2 detoxification enzymes from broccoli) and 6-MITC(a potent anti-proliferative principal from wasabi) slightly inhibited the induction of mouse skin tumor in a two-stage process of carcinogenesis (initiator, 9,10-dimethyl-1,2-benzanthracene; promotor, 12-o-tetradecanoylphorbol-13-acetate), but the effect was not significant. Both compounds, however, significantly inhibited the mutation of skin resulting from topical applications of the carcinogens. When a murine hepatoma cell line, Hepa 1c1c7, was treated with 2-, 4-, 6- and 8-MITCs, they augmented the induction of its quinone reductase, one of the phase 2 detoxification enzymes in a concentration dependent manner, and the 4- and 6-MITCs were much more potent on the reduction of the enzyme than the 2- and 8-MITCs. All 2-, 4-, 6- and 8-MITCs suppressed the growth of murine tumor cells, their suppressive activities being proportional to the length of their methyl residue. They were also cytotoxic to mouse peritoneal exudate macrophages which were not proliferating *in vitro*, indicating that the cellular targets of isothiocyanate may not be dependent upon the cell cycle. In addition, all the 2-, 4-, 6- and 8-MITCs inhibited the production of nitric oxide (a potent radical carcinogen) by peritoneal macrophages.

Abbreviations: DMBA – 9,10-dimethyl-1,2-benzanthracene; FBS – fetal bovine serum; IFN- γ – interferon- γ ; ITC – isothiocyanate; LPS – lipopolysaccharide; MITC – methylsulfinylhexyl-isothiocyanate; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; TPA – 12-o-tetradecanoylphorbol-13-acetate

Introduction

The biological activities and functions of plant dietary compounds have been extensively studied with much attention focused on compounds especially in the context of chemoprevention against carcinogenesis (Osawa *et al.*, 1995).

In fact, there has been a growing body of evidence that certain plant and vegetable constituents possess substantial biological activities, including the stimulation and/or induction of immune reactions, differentiation of tumor cells (Constantinou *et al.*, 1995), the prevention of chemical carcinogenesis (Zhang *et al.*, 1992, 1994; Prochaska *et al.*, 1992), proliferation

and so on. For example, 4-methylsulfinylhexyl isothiocyanate (MITC) has been isolated from broccoli and identified as a potent inducer of phase 2 detoxification enzymes. Meanwhile, wasabi (*Eutrema wasabi* Maxim.) a very popular spice in Japan, exhibits physiological activities such as appetitive effect (Kojima, 1988), anti-microbial activities (Issiki *et al.*, 1993), and inhibition of platelet aggregation (Kumagai *et al.*, 1994). The isolation and identification of its fresh greenish flavor have been extensively examined (Etoh *et al.*, 1990), and synthetic compounds that replicate its flavor have been manufactured.

We previously reported that the water-soluble fractions of wasabi inhibited the growth of a human stom-

Table 1. Suppressive effect of 6-MITC on tumor promotion

Group	Tumor-bearer/ total (%)	Papiloma/ Tumor-bearer	Not mutated mouse/total (%)
At 15 weeks:			
6-MITC (162 nmol/mouse)	17/31 (54.8) ^a	2.2 ^a	6/31 (19.4) ^a
6-MITC (32.4 nmol/mouse)	18/30 (60.0)	2.0	2/30 (7.0)
4-MITC (162 nmol/mouse)	15/30 (50.0)	2.9	4/30 (13.3)
4-MITC (32.4 nmol/mouse)	19/30 (63.3)	2.8	3/30 (10.0)
Control	24/35 (68.6)	2.5	2/35 (5.7)
At 20 weeks:			
6-MITC (162 nmol/mouse)	22/30 (73.3)	4.2	5/30 (16.7)
6-MITC (32.4 nmol/mouse)	23/29 (79.3)	3.7	3/29 (10.3)
4-MITC (162 nmol/mouse)	23/29 (79.3)	3.7	4/29 (13.8)
4-MITC (32.4 nmol/mouse)	28/30 (93.3)	5.9	1/30 (3.3)
Control	29/33 (87.9) ^b	6.1	1/33 (3.0)

^a P < 0.05.^b Two mice died with cancer.

ach tumor cell line, MKN-28 and the most potent principal of the fractions was 6-MITC (Fuke *et al.*, 1994; Ono *et al.*, 1996). The present study deals with the anti-carcinogenic activity of 6-MITC, an active anti-proliferative principal of wasabi, and clarification of possible mechanisms of its anti-carcinogenic activity. The anti-carcinogenic activity of 6-MITC was evaluated by a two-stage model of mouse skin carcinogenesis. In order to clarify possible mechanisms of its anti-carcinogenic activity, effects of 6-MITC on several cells culturing *in vitro* were examined.

Materials and methods

Reagents and cells

Fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Cosmo Bio. Co., Ltd., and Wako Pure Chemical Industries Ltd., Japan, respectively. Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). A murine hepatoma Hepa 1c1c7 cell line was obtained from ATCC (American Type Cell Culture).

A two-stage model of mouse skin carcinogenesis

Seven-week-old female CD-1 mice were obtained from the Japan SLC Co. (Hamamatsu Japan) and

their back was shaved 2 days before a topical treatment of 9,10-dimethyl-1,2-benzanthracene (DMBA) (100 µg/0.1 ml acetone/mouse). Seven days later, all mice were received 2 µg (3.4 n moles) of 2-o-tetradecanoylphorbol-13-acetate (TPA) in 0.1 ml acetone at the same portion. This treatment was carried out two times weekly for twenty weeks. Tested samples were topically applied one hour before TPA treatment. Mice were shaved periodically as necessary, and the skin tumors were counted. Each experimental group was comprised of 30 mice.

Measurement of quinone reductase (QR)

Induction of QR in Hepa 1c1c7 murine hepatoma cells was carried out according to the method described by Prochaska *et al.* (1992). Briefly, Hepa 1c1c7 cells were suspended in α -minimum essential medium (α -MEM) containing 10% FBS (treated with activated charcoal) and incubated at 37 °C under a humidified atmosphere of 5% CO₂ for 24 hr, with 1×10^4 cells/0.1 ml/well in duplicated 96-well microtiter plates. Serially diluted isothiocyanate (ITC) solution was then added to each well and the cells were incubated for 48 hr. QR activity (based on reduced MTT products) was optically determined in cell lysates in one microtiter plate, and cell densities were determined using another microtiter plate by staining with crystal violet.

Figure 1. Effect of 6-MITC on quinone reductase induction in Hepa 1c1c7 cells. The point represent the mean $\pm$ SD of four determinants. Hepa 1c1c7 cells were suspended in 10% FBS- α MEM and incubated 24 hr. ITC solution was added to each well and the cells were incubated for 48 hr.

Measurement of anti-proliferative activity and cytotoxicity

Hepa 1c1c7 and r/m HM-1 cells were respectively suspended in α MEM and a 1:1 mixture of Hams F12 and Dulbecco's modified Eagle medium (F/D, 1:1) (GIBCO BRL) supplement with 10% FBS. The cell suspension ($100 \mu\text{l}$; 5×10^4 cells/ml) was incubated at 37°C under a humidified atmosphere of 5% CO_2 for 24 hr in 96-well microtiter plates. Serially diluted ITC solutions were then added to each well and the plates were incubated for 48 hr. Cell growth was determined by a standard MTT assay (Camichael *et al.*, 1987).

Mouse peritoneal exudate cells were harvested 4 days after an intraperitoneal injection of thioglycolate broth, and adherent cells (macrophages) were obtained by 2 hr-incubation at 37°C under a humidified atmosphere of 5% CO_2 . The cells were incubated

with serially diluted ITC's solution (FD medium) and their viability was determined after 48 hr by the MTT assay.

Measurement of nitric oxide

This was determined as previously described (Ryoyama *et al.*, 1993). Briefly, mouse peritoneal macrophages (3×10^5 cells/well) were treated with ITCs for 24 hr at 37°C under a humidified atmosphere of 5% CO_2 and stimulated with lipopolysaccharide (LPS) (10 ng/ml) plus interferon- γ (IFN- γ) (2 U/ml) for another 24 hr. Some culture fluid ($100 \mu\text{l}$) from each well was withdrawn and the nitrites (a stable product of nitric oxide) in each fluid were determined with the Griess reagent (1% sulfanilamide/0.1% naphthylene diamine dihydrochloride/2.5% H_3PO_4).

Figure 2. Effect of 6-MITC on growth of Hepa 1c1c7 and r/m HM-1 cells. Hepa 1c1c7 and r/m HM-1 cells were suspended in α MEM and 10% FBS-F/D medium, respectively. The cell suspension was incubated for 24 hr; ITCs solution were added to each well and the plates were incubated for 48 hr. The points represent the mean $\pm$ SD of six wells.

Figure 3. Effect of 6-MITC on viability of mouse peritoneal macrophages. Mouse peritoneal macrophages were suspended in 10% FBS-F/D medium. The cell suspension was incubated with diluted ITC's solution and their viability was determined 48 hr by MTT assay. The points represent the mean $\pm$ SD of six wells.

Results and discussion

Suppressive effect on tumor promotion

Synthetic 6- and 4-MITC were evaluated by a two-stage model of mouse skin carcinogenesis (initiator, DMBA; promoter, TPA) (Table 1). Synthetic 4-MITC, an active anti-carcinogenic principal of broccoli (Zhang *et al.*, 1992) was selected as a positive control. Papillomas appeared 6 weeks after promotion, and both 6- and 4-MITCs slightly delayed their induction. Both ITCs slightly inhibited skin tumor induction in a dose dependent manner (papilloma bearers and papillomas per bearer) compared with the mice treated with DMBA plus TPA, but the inhibition was not significant. Both ITCs, however, significantly inhibited the mutation of skin induced with DMBA plus TPA. This anti-mutagenic activity was also in a dose dependent manner. Neither 6- nor 4-MITC produced any apparent toxic effects after the topical applications.

There were no effects on body weight and no macroscopically observable effects on the skin.

Quinone reductase induction in Hepa 1c1c7 cells

Since it has been suggested that the anti-carcinogenic activity of 4-MITC is produced through the induction of detoxification phase 2 enzymes (Zhang *et al.*, 1992; 1994), synthetic 2-, 4-, 6- and 8-MITCs were assessed on the basis of the induction of quinone reductase in Hepa 1c1c7 cells. Figure 1 shows that all ITCs stimulated induction of the enzyme in a dose-dependent manner. 4-MITC was the most potent, followed by 6-MITC. However the augmentation of the enzyme induction obtained here was not as high as the reported one by 4-MITC (Zhang *et al.*, 1992, 1994).

Anti-proliferative activity

Phase 2 enzymes detoxify toxic, mutagenic and carcinogenic agents, resulting in reduced susceptibility

Figure 4. Effect of 6-MITC on nitric oxide production by mouse peritoneal macrophages. Mouse peritoneal macrophages were treated with ITCs for 24 hr and stimulated with LPS plus IFN- γ for 24 hr. The points represent the mean $\pm$ SD of six wells. The SD is within the markers.

of animals and cells to them. 4-MITC augments the induction of quinone reductase in the liver, glandular stomach and small intestine when it is administered to animals by gavage (Zhang *et al.*, 1992). The anti-carcinogenic activity of 4-MITC has been examined following the oral administration of both a carcinogen and 4-MITC (Zhang *et al.*, 1994).

Meanwhile, in the present experiment the carcinogens were topically applied and the ITCs were applied to mouse skin. Therefore, it seems unlikely that the anti-carcinogenic activity of 6- and 4-MITCs results from induction of phase 2 enzyme in the liver, although there is a possibility that cells in the skin may induce the enzyme in response to the ITCs. 6-MITC is isolated from wasabi with respect to anti-proliferative activity against human stomach tumor cell line, MKN-28. Thus, 6-MITC is also likely to inhibit the growth of murine tumor cells. This was observed against two murine tumor cells, Hepa 1c1c7 and r/m HM-1 (Figure 2). Neither of the tumor cells was susceptible to below 1 μ M of 2-, 4-, 6- and 8-MITCs. The anti-proliferative activity

of 8-MITC was the greatest, followed by that of 4- and 6-MITCs; 2-MITC exhibited the lowest activity. On the other hand Figure 3 shows the cytotoxicity of 2-, 4-, 6- and 8-MITCs to mouse peritoneal exudate macrophages (non-proliferating cells). All ITCs were cytotoxic above 10 μ M, and 8-MITC again was the most toxic. This indicates that the cellular targets of ITCs may not be dependent upon the cell cycle. In addition, these results suggest that 4- and 6-MITCs can directly kill non-mutated and mutated skin cells when applied topically.

Inhibition of nitric oxide production

TPA has been reported to induce nitric oxide (Gilbert *et al.*, 1993; Hortelano *et al.*, 1993) which is a potent radical carcinogen (Esumi *et al.*, 1995; Jackson *et al.*, 1996). Therefore, it is likely that the anti-carcinogenic activity of 4- and 6-MITCs could result from the inhibition of TPA-induced nitric oxide production. Indeed, 2-, 4-, 6- and 8-MITCs did inhibit the production of nitric oxide by mouse peritoneal macrophages (Fig-

ure 4). Whether TPA induce the production of nitric oxide in mouse skin cells is an open question. Although 4-MITC has been reported to have potent suppressive activity with respect to the induction of mammary tumors in rat (Zhang *et al.*, 1994), the presently observed anti-carcinogenic activity of 4- and 6- MITCs was not so potent. This discrepancy could be due to the differences in the method of application of the carcinogens and ITCs, that is, the administration by Zhang *et al.* (1994) was performed orally, whereas the current study involved topical application to the skin. Further investigation concerning to this difference is necessary in order to clarify the anti-carcinogenic mechanism of ITC.

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