Inhibitory Effects of Flavonol Glycosides on 12-*O*-Tetradecanoylphorbol-13-acetate-Induced Tumor Promotion[1)]

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The two-stage carcinogenesis by 7,12-dimethylbenz[*a*]anthracene and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mice was inhibited by kaempferol and flavonol glycosides, whereas naringenin, a flavanone, had no effect. The induction of epidermal ornithine decarboxylase activity by TPA was also inhibited by kaempferol, whereas mauritianin, a kaempferol glycoside, failed to inhibit it. In addition, the effect of the flavonol glycosides on cell-mediated immunosuppression in the two-stage carcinogenesis, observed in terms of initiation after 14 weeks, was antagonized by mauritianin and myricitrin. Cell-mediated immunosuppression in the two-stage carcinogenesis was unaffected by kaempferol and naringenin. These results suggest that the inhibitory effects of flavonol glycosides may have been at least partly due to activation of immune responses against tumors.

Keywords 12-*O*-tetradecanoylphorbol-13-acetate; mauritianin; flavonoid; flavonol glycoside; anti-tumor promoter; ornithine decarboxylase; delayed-type hypersensitivity; two-stage carcinogenesis

Introduction

Many 3-hydroxyflavones (flavonols) are known to possess mutagenic activity, but their 3-glycosides have no such activity.[2-5)] So far, all flavonols have been found to be non-carcinogenic, except in one case.[6)] The reason why flavonols do not have carcinogenic activity, in spite of their mutagenicity, is unknown. Quercetin, a flavonol derivative, inhibit a number of the *in vitro* and *in vivo* effects of the tumor promoters 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and teleocidin, including stimulation of ^{32}Pi incorporation into phospholipids,[7)] stimulation of 2-deoxy-D-glucose uptake, aggregation of human platelets,[8)] induction of alkaline phosphatase activity in mouse skin,[9)] induction of ornithine decarboxylase (ODC) in mouse skin,[10)] inflammation in mouse skin,[12)] and activation of Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C).[13)] The inhibitory effects of flavonoids are considered to be related to their anti-tumor-promoting activity.[14,15)] To examine this possibility, we tested the anti-tumor promotion activity of flavonols *in vivo*. This paper reports studies on the effect of flavonol glycosides, one of the most ubiquitous types of flavonoid, on the tumor-promoting effect of TPA. Delayed-type hypersensitivity (DTH) in the skin during the two-stage carcinogenesis in mice was suppressed by tumor promotion.[16)] This suppression was cancelled by treatment with flavonol glycosides at 14 weeks. Since flavonoids are widely distributed in the plant kingdom in fruits and vegetables, it is important to determine their potential carcinogenicity.

Materials and Methods

Animals Female ICR mice were obtained from Shizuoka Laboratory Center, Shizuoka, Japan. The animals were housed in an air-conditioned room (22—23 °C) lit from 08:00 to 20:00. Food and water were available *ad libitum*.

Chemicals 7,12-Dimethylbenz[*a*]anthracene was purchased from Sigma Chemical Co., St. Louis, U.S.A.; 12-*O*-tetradecanoylphorbol-13-acetate was from Chemicals for Cancer Research Inc., Chicago, U.S.A. Kaempferol, rutin and dinitrofluorobenzene were purchased from Tokyo Chemical Industries Co., Tokyo, Japan. Apigenin and naringenin were from Carl Roth KG, Karlsruhe, West Germany. DL-[1-^{14}C]Ornithine (49.1 mCi/mmol) was obtained from New England Nuclear, Boston, MA. Mauritianin and myricitrin were isolated from plant materials in our laboratory.

Assay of ODC Activity[17)] Naringenin, apigenin, kaempferol or mauritianin (5 μmol) or the vehicle alone (200 μl), was applied topically 30 min before TPA treatment. TPA was dissolved in acetone, and flavonoids were dissolved in acetone–dimethyl sulfoxide (DMSO); these solutions were applied to the shaved area in a volume of 200 μl using a micropipette. Four hours after the application of TPA (5 μg), the mice were killed by cervical dislocation. The epidermis was separated by brief heat treatment and ODC activities in the soluble epidermal supernatants were determined by measuring the release of $^{14}CO_2$ from [1-^{14}C]ornithine, as described previously. The results were expressed as nmol CO_2/30 min/mg protein. The protein concentration in the epidermal extract was measured using a Bio-rad protein assay kit.[18)]

Cell-Mediated Immune Response in Mice during the Two-Stage Carcinogenesis Primary sensitization was induced by dinitrofluorobenzene (DNFB) or sheep red blood cells (SRBC) as follows: DNFB was dissolved in acetone at 0.25% (w/v) and 100 μl was applied to the dorsal skin 24 h after TPA treatment. After 4 d, delayed-type hypersensitivity was elicited by painting 100 μl of 0.25% DNFB solution in acetone onto the right hind paw. SRBC were suspended in phosphate-buffered saline (PBS) at a density of 8×10^7 cells/ml and 0.25 ml was injected intraperitoneally. After 4 d, 1×10^8 SRBC suspended in 0.05 ml of PBS were injected into the left hind paw for elicitation. After 24 h, the edema resulting from elicitation in the hind paw was measured with a dial caliper. The thickness of the footpad swelling due to delayed-type hypersensitivity minus that of the opposite footpad was expressed in 0.1 mm units. Mice in each of the 2 subgroups were sensitized first with DNFB and, 1 week later, with SRBC at 7 or 14 and 8 or 15 weeks, respectively. Each subgroup consisted of 10 mice.

Two-Stage Carcinogenesis Experiments The backs of mice (7 weeks old) were shaved with electric clippers. Initiation was accomplished by a single topical application of 50 μg of 7,12-dimethylbenzanthracene (DMBA). Promotion with 2.5 μg of TPA, applied twice weekly, was begun 1 week after initiation. Flavonoids (5 μmol), or their vehicle, acetone–DMSO (9 : 1), as a control, were applied topically 30—40 min before each TPA treatment. DMBA and TPA were dissolved in acetone, and flavonoids were dissolved in acetone–DMSO; the solutions were applied to the shaved area in a volume of 100 μl using a micropipette. The backs of the animals were shaved once a week to remove growing hair. The numbers and diameter of skin tumors were measured every other week, and the experiment was continued for 18 weeks. Experimental and appropriate control groups each consisted of 20 mice.

Results

Effects of Flavonoids on TPA-Mediated Epidermal ODC Induction in Mice A single application of TPA (5 μg) resulted in substantial but transient ODC activity in mouse epidermal supernatant with a peak at about 4 h after the

TABLE I. Inhibition of TPA-Induced Epidermal ODC by Flavonoids

Treatment	ODC activity[a] (nmol CO_2/mg protein/30 min)	Inhibition (%)
Vehicle	2.8±0.23	—
Naringenin	2.2±0.27[b]	21
Apigenin	1.9±0.25[c]	32
Kaempferol	1.6±0.21[c]	43
Mauritianin	2.9±0.33	ND[d]

Mice were topically treated with vehicle or one of the above agents (5 μmol) 30 min prior to the application of TPA (5 μg). Four hours after the TPA treatment, mice were killed for determinations of ODC activity. *a*) Mean±S.E. of individual determinations from 5 mice. *b*) $p<0.01$, *c*) $p<0.001$ by Student's *t* test as compared with the control group. *d*) ND, not determined.

TABLE II. Effect of Flavonoids on Immunological Activity Induced by DNFB in ICR Mice During the Two-Stage Carcinogenesis

Treatment	Footpad swelling caused by DTH[b] 7 weeks[c]		14 weeks[c]	
Normal mice	16.9±3.50[f]	(100)[d]	11.4±4.44[f]	(100)[d]
Naringenin[a]	17.0±3.22[f]	(100)	10.5±4.21	(92)
Kaempferol[a]	17.2±4.22[f]	(102)	10.2±6.81	(90)
Mauritianin[a]	16.5±5.57[f]	(95)	12.5±5.33[e]	(110)
Rutin[a]	17.0±4.01[f]	(100)	7.7±4.34	(68)
Myricitrin[a]	19.0±7.32[f]	(112)	16.3±6.42[f]	(144)
DMBA+TPA	2.7±2.01	(16)	5.4±4.32	(47)
+Naringenin	2.0±1.04	(12)	2.9±3.01	(25)
+Kaempferol	3.3±2.54	(19)	8.5±4.34	(74)
+Mauritianin	4.3±3.12	(25)	12.0±5.38[f]	(106)
+Rutin	1.8±1.36	(10)	7.8±3.78	(69)
+Myricitrin	5.5±4.52	(32)	11.3±3.57[f]	(100)

Each value is the average of readings from 10 mice (*a*) 5 mice). *b*) Mean± S.E. *c*) Weeks after initiation. *d*) % of normal mice. *e*) $p<0.05$, *f*) $p<0.01$ by Student's *t* test as compared with the DMBA+TPA group.

treatment. ODC induction by TPA was potently inhibited by treatment of the mouse skin with kaempferol and apigenin, as in the case of quercetin. However, naringenin and mauritianin were found to have no effect (Table I).

Effects of Flavonoid Glycosides on Cell-Mediated Immune Response in Mice During the Two-Stage Carcinogenesis The group treated with DMBA plus TPA showed a strongly suppressed DNFB-delayed-type hypersensitivity (DTH), 16—47% that of the non-treated group through weeks 7 and 14. None of the flavonoids had any effect at week 7. The DTH in the mauritianin- and myricitrin-treated groups were restored to the level of the non-treated group at week 14. However, the groups treated with kaempferol and rutin were only slightly affected as revealed by this assay (Table II). SRBC-DTH was not significantly suppressed during two-stage carcinogenesis (Table III).

Inhibitory Effect of Flavonoids on the Tumor-Promoting Activity of TPA The effect of flavonols on the incidence of TPA-induced skin papilloma formation was examined. As shown in Figs. 1 and 2, the treatment of initiated mice with 2.5 μg of TPA alone resulted in 21.2 papillomas/mouse 18 weeks after the start of promotion. The application of 5 μmol of flavonol and its glycoside 30—40 min before TPA resulted in 45—58% inhibition of the number of papillomas per mouse compared with mice that received only TPA. The percentages of tumor-bearing mice were also markedly reduced by treatment with flavonol glycosides (Fig. 1). Treatment of mouse skin with flavonol

TABLE III. Effect of Flavonoids on Immunological Activity Induced by SRBC in ICR Mice During the Two-Stage Carcinogenesis

Treatment	Footpad swelling caused by DTH[b] 8 weeks[c]		15 weeks[c]	
Normal mice	6.6±2.61	(100)[d]	7.7±2.54	(100)[d]
Naringenin[a]	6.2±2.03	(94)	5.1±2.07	(66)
Kaempferol[a]	7.2±3.60	(109)	8.6±2.98	(111)
Mauritianin[a]	6.4±2.88	(97)	5.5±1.96	(71)
Rutin[a]	6.5±2.74	(98)	4.4±2.04[e]	(57)
Myricitrin[a]	9.7±2.20[e]	(147)	6.5±2.03	(84)
DMBA+TPA	5.1±2.84	(77)	4.8±1.52[f]	(62)
+Naringenin	5.3±2.35	(80)	5.5±2.01	(71)
+Kaempferol	7.2±3.60	(109)	8.6±2.01	(112)
+Mauritianin	6.9±3.50	(105)	10.0±3.13	(130)
+Rutin	5.9±3.84	(90)	6.7±2.74	(87)
+Myricitrin	5.7±2.70	(86)	7.4±3.22	(96)

Each value is the average of readings from 10 mice (*a*) 5 mice). *b*) Mean± S.E. *c*) Weeks after initiation. *d*) % of normal mice. *e*) $p<0.05$, *f*) $p<0.01$ by Student's *t* test as compared with the DMBA+TPA group.

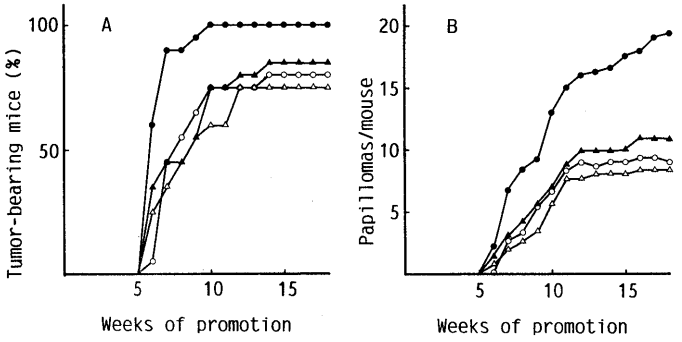

Fig. 1. Inhibitory Effect of Flavonol Glycosides on the Promotion of Skin Papillomas by TPA in DMBA-Initiated Mice

From one week after initiation by a single topical application of 50 μg of DMBA, 2.5 μg of TPA was applied twice weekly. Topical application of mauritianin (5 μmol), rutin (5 μmol), myricitrin (5 μmol) and vehicle was performed 30 min before each TPA treatment. Data are expressed (A) as percentage of mice bearing papillomas, and (B) as average numbers of papillomas per mouse. ●, +TPA with vehicle alone; ○, +TPA with mauritianin; ▲, +TPA with rutin; △, +TPA with myricitrin.

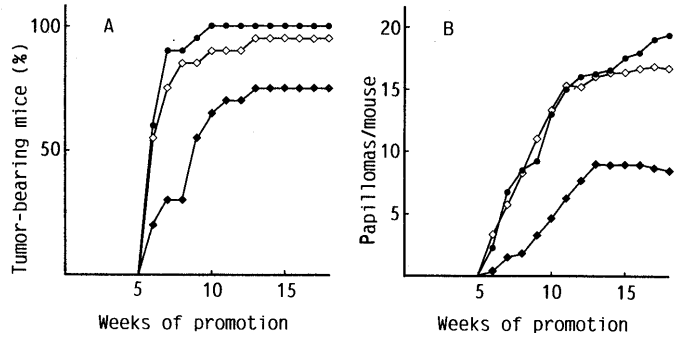

Fig. 2. Inhibitory Effect of Naringenin and Kaempferol on the Promotion of Skin Papillomas by TPA in DMBA-Initiated Mice

From one week after initiation by a single topical application of 50 μg of DMBA, 2.5 μg of TPA was applied twice weekly. Topical application of naringenin (5 μmol), kaempferol (5 μmol) and vehicle was performed 30 min before each TPA treatment. Data are expressed (A) as percentage of mice bearing papillomas, and (B) as average numbers of papillomas per mouse. ●, +TPA with vehicle alone; ◇, +TPA with naringenin; ◆, +TPA with kaempferol.

derivatives markedly suppressed TPA-induced tumor promotion (Figs. 1 and 2). Naringenin, a flavanone derivative, was found to have no effect on TPA-induced tumor-promoting activity in two-stage carcinogenesis (Fig. 2).

Discussion

Quercetin[1,10,14] and morin[15] among the flavonoids have been shown to have anti-tumor-promoting activity in mouse skin in the two-stage carcinogenesis experiments. In the present study, we found that kaempferol and the flavonol glycosides, mauritianin, rutin and myricitrin, all had anti-tumor-promoting activity in mice. The aglycones of flavonoids inhibited the following *in vitro* and *in vivo* effects of TPA and teleocidin: stimulation of ^{32}Pi incorporation into phospholipids,[7] stimulation of 2-deoxy-D-glucose uptake, aggregation of human platelets,[8] induction of alkaline phosphatase activity in mouse skin,[9] induction of ODC in mouse skin,[10] inflammatory activity in mouse skin,[11,12] and activation of protein kinase C,[13] but do not inhibit TPA-induced [^{3}H]thymidine incorporation into epidermal deoxyribonucleic acid (DNA)[15] or modulation of phorbol ester receptors in mouse skin.[19] Flavonol glycosides have no effect on stimulation of ^{32}Pi incorporation into phospholipids.[7] Also, the flavonol glycoside, mauritianin has no effect on the induction of ODC in mouse skin. Flavonol glycosides inhibited TPA-induced inflammation in mice.[12]

These data indicate that the effects of flavonoid glycosides differ from those of the aglycones in the inhibition of tumor-promoting activity. The SRBC-DTH was not suppressed by DMBA plus TPA in the mouse. The DNFB-DTH was suppressed during tumor promotion to 30% of that of normal mice in TPA-treated skin areas.[16] We found that the DNFB-DTH was suppressed to 16% of that of normal mice at 7 weeks, and this suppression did not respond to treatment with flavonoids. It was as low as 47% of that of normal mice at 14 weeks, but this suppression was cancelled by treatment with mauritianin and myricitrin (flavonol glycosides). DMBA is also known to deplete Langerhans cells (LC) in treated skin[20]; LC are associated with local cutaneous DTH.[21] However, a part of the effect of flavonol glycosides is suggested to be due to activation of immune responses against tumors, in which LC are involved, suggesting the reappearance of LC associated with tumor regression. Flavanones have previously been found to have no effect on TPA-induced inflammation.[12] Our present results also suggest that naringenin, a flavanone derivative, does not inhibit tumor promotion by TPA. Thus, the effects of flavonoid derivatives on TPA-induced inflammation[12] seem to be roughly in parallel with their inhibitory activities on tumor promotion in mice.

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