Inhibitory Effect of Perilla Leaf Extract and Luteolin on Mouse Skin Tumor Promotion

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In the present study, the effects of perilla leaf extract (PLE) and luteolin on 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin papillomas in mice were investigated. Topical application of PLE prior to TPA treatment in DMBA-initiated mouse skin resulted in a significant reduction in tumor incidence and multiplicity. An even more potent preventive effect was observed with topical application of luteolin, which we previously identified as an antiinflammatory constituent. PLE was dissolved in drinking water at a 0.05% dose and mice ingested it ad libitum; no significant difference was observed in tumor incidence or multiplicity but there was a significant reduction in tumor volume between the PLE-treated and untreated groups. These results suggest that PLE has potent antipromotion activity and ingesting it as a daily food may provide a beneficial chemopreventive effect.

Key words Perilla frutescens; luteolin; chemoprevention; 12-O-tetradecanoylphorbol-13-acetate

The leaves of Perilla frutescens are used as a garnish with raw fish in Japan. The aim of this use is not only as a flavoring but also as an antidote to food poisoning.1) We previously reported that oral administration of a perilla leaf extract (PLE) to mice inhibits the overproduction of tumor necrosis factor-α (TNF-α)2) and inflammatory and allergic ear edema.3) PLE has been reported to suppress anti-DNP IgE production,4) Th2-type cytokine production,5) systemic allergic reaction induced by compound 48/80,6) and IgA nephropathy.7) We further identified the main active component of perilla as luteolin, an ordinary flavonoid that is ubiquitous in nature.8) Flavonoids have demonstrated a variety of biological effects including antioxidation, antiinflammation, and antiallergic effects, and antiplatelet and antithrombotic action.9—12) Some flavonoids, especially quercetin, are reported to protect against cancer.13) Therefore the actual preventive effect of flavonoids ingested as a food against cancer are not completely known in spite of many reports that various flavonoids provide chemoprevention when administered via a parenteral route. Luteolin is reported to inhibit NO production,14) active oxygen species,15) TNF-α-induced ICAM-1 expression,16) and metallopeptidases.17) There are few reports, however, showing that luteolin is effective in cancer prevention in spite of its many biological activities.

We reported that oral administration of PLE and luteolin to mice clearly inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema.18) TPA is not only an inflammation inducer but also the most frequently used skin tumor promotion agent.19) It is believed that PLE and luteolin which can inhibit TPA-induced inflammation, also inhibit TPA-induced tumor promotion. We therefore investigated the effects of PLE and luteolin on 7,12-dimethylbenz[a]anthracene (DMBA)- and TPA-induced skin papillomas in mice.

MATERIALS AND METHODS

Mice Female ICR mice (5 weeks old) were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The animals were given a standard laboratory diet and water ad libitum. The experiments were done under the Guidelines for Animal Experiments under the Law (No. 105) and Notification (No. 6) of the government.

PLE Dried leaves of a green type of perilla (P. frutescens (L.) Britton var. acuta Kudo forma viridis Makino; 5 g) were soaked in 5 ml of distilled water for 1 h and then homogenized for 10 min with Polytron equipment (Kinematik, Switzerland) at a power setting of 5. The homogenate was filtered through nylon mesh and centrifuged at 7000×g for 10 min at 4 °C. The resulting supernatant was passed through a membrane filter with a 0.45 μm pore size (Millipore, Tokyo, Japan) and the yield of PLE was 4.4 ml. This process was repeated and 253 ml of decoction was obtained. It was dried with a rotary evaporator and gave a final amount of 5.92 g of exsiccated PLE.

Chemicals DMBA and TPA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and luteolin from Funakoshi Co. (Tokyo, Japan).

Topical Application for Tumor Promotion The dorsal skin of mice was shaved using electric clippers, and mice with the hair cycle in the resting phase were used in all experiments. The mice were randomly divided into 5 groups of 20 animals each. The mice in group I were left untreated and served as a negative control for any spontaneous tumor induction. To assess whether PLE alone produces tumor promoting effects, a single 50 μg dose of DMBA in 0.2 ml of acetone/mouse as a tumor initiator was applied topically on the dorsal shaved skin of the mice in group II, and 1 week later the mice were treated with PLE 1 mg in 0.2 ml of acetone/mouse/application twice a week until the end of the experiment. The dorsal shaved skin of mice in groups III, IV, and V received a single 2.5 μg topical application of DMBA in 0.2 ml of acetone/mouse as a tumor initiator. One week later, the animals in group III were treated topically with 0.2 ml of acetone, and groups IV and V with PLE or luteolin 1 mg in 0.2 ml of acetone/mouse/application, respectively. Thirty minutes following these treatments, animals in each of the latter three groups received a topical application of TPA 1 μg in 0.2 ml of acetone/mouse/application. Treatment with
the tumor promoter alone (group III) or PLE (group IV) or luteolin (group V) plus tumor promoter were repeated twice weekly until the termination of the experiment 20 weeks after the start of DMBA administration. All groups were checked for food and water consumption and any apparent signs of toxicity such as weight loss or mortality throughout the study. Skin tumor formation was recorded weekly and tumors greater than 1 mm in diameter were included in the cumulative total count. The percentage of mice bearing papillomas and the average number of papillomas/mouse were recorded. The statistical significance of difference between tumor incidence in sample-treated and untreated groups was determined using the Dunnett test.

**Oral Administration for Tumor Promotion**  Similar to the topical application test, the oral effect of PLE on two-stage mouse skin carcinogenesis was also evaluated using DMBA/TPA. The dorsal skin of mice was shaved using electric clippers, and mice with the hair cycle in the resting phase were used in all experiments. They were randomly divided into 2 groups of 20 animals each. The mice in group VI drank regular water and the mice in group VII drank 0.5% PLE-containing water *ad libitum* during the entire experimental period. The volume of water consumed by both groups was approximately 7.8 ml/mouse/day and no significant difference was found between groups VI and VII. Body weight was checked every 4 weeks, and no apparent signs of toxicity were detected. Mice were topically administered on the dorsal shaved skin a single 2.5 μg dose of DMBA in 0.2 ml of acetone/mouse as a tumor initiator. One week later, animals in both groups received topical TPA 1 μg in 0.2 ml of acetone/mouse/application. Tumor promoter treatments were repeated twice weekly until termination of the experiment. Skin tumor formation was recorded weekly, and tumors greater than 1 mm in diameter were included in the cumulative total if they persisted for 2 weeks or more. The percentage of mice bearing papillomas and the average number of papillomas/mouse were recorded. The statistical significance of difference between tumor incidences in sample-treated and untreated groups was determined using the Dunnett test.

**RESULTS**

**Effect of Topical PLE and Luteolin on Tumor Promotion**  We first investigated the toxic effects of PLE applied topically, as monitored by weight gain profile. No noticeable difference was observed between PLE-treated and untreated groups of animals throughout the experiment (data not shown). Nor did topical application of PLE alone twice weekly to DMBA-initiated mice result in any tumorigenicity (Fig. 1). These observations suggest that topical application of PLE 1 mg was devoid of any apparent toxicity as well as any tumor promoting effect during the entire experimental period.

The data were analyzed to determine the preventive effects of PLE on TPA-induced tumor promotion (Fig. 1). Topical application of PLE or TPA to DMBA-initiated mouse skin resulted in a significant reduction in tumor incidence throughout the experiment. The first skin tumor appeared at 9 weeks in each group, but the incidence gradually differentiated. At the termination of the experiment at 20 weeks, as shown in Fig. 1A, compared with 85% mice with skin tumors in the non-PLE-treated group, only 55% in the PLE-treated group had developed tumors (*p*<0.0001). Similarly, when the tumor data were evaluated in this protocol for tumor multiplicity (cumulative number of tumors/group or number of tumors/mouse), beginning with the appearance of the first tumor until termination of the experiment, topical application of PLE exhibited highly significant protection against TPA-induced tumor promotion in mouse skin (Fig. 1B). Upon conclusion of the experiment, compared with 5.5 tumors/mouse in the non-PLE-treated group, there were only 2.6 tumors/mouse in the PLE-treated group (*p*<0.0001).

Luteolin was identified as the antiinflammatory constituent of PLE in our previous report, and its topical application at a dose of 1 mg/mouse/application prior to that of each TPA application afforded exceptionally high protection against TPA-induced tumor promotion in DMBA-initiated mouse skin. At the termination of the experiment at 20 weeks, tumor incidence in the luteolin-treated group had decreased to less than 29% (Fig. 1A), and the average number of papillomas was also significantly lower at only 0.9 tumor/mouse (Fig. 1B).

**Antitumor Promotion with Oral Administration of PLE**

*Fig. 1.* Protective Effect of Topical Application of Perilla Leaf Extract (PLE) and Luteolin against Tumor Incidence and Tumor Multiplicity during TPA-Induced Tumor Promotion in DMBA-Initiated Mouse Skin
To determine the antitumor promotion activity of PLE when administered orally to mice, it was dissolved in drinking water at a 0.05% dose and ingested it ad libitum. Drinking water consumed was checked twice weekly, and that both groups of mice ingested 7.8 ml/animal/d throughout the experiment; no significant difference between the PLE-treated and untreated groups was detected. No toxic effect of PLE ingestion was detected because no noticeable weight gain profile difference was observed between the treated and untreated groups (data not shown).

The oral effect of PLE on TPA-induced tumor promotion differed with its parenteral effect. The first skin tumor appeared at 8 weeks in both groups, as shown in Fig. 2A. Twenty weeks later at the termination of the experiment, the tumor incidence in the PLE group was 85%, compared with 100% mice with skin tumors in the water group. However, no significant difference was observed in tumor multiplicity at 20 weeks: 8.3 tumors/mouse in the water group and 8.6 tumors/mouse in the PLE-treated group (Fig. 2B).

Ingestion of PLE, however, significantly reduced tumor volume in the dorsal skin of mice (Fig. 3A). To determine the inhibition of papilloma formation, two mice were chosen from each group at the termination of the experiment and their tumors carefully cut and weighed (Fig. 3B). The papilloma weights in the water group were 4.4 to 247.8 mg, but those in the PLE group were only 0.3 to 24.2 mg.

**DISCUSSION**

Topical application of PLE 1 mg applied twice weekly resulted in skin neoplasms accounting for 30% protection in tumor incidence. We therefore consider that the antipromotion effect is a notable function of PLE and luteolin in addition to their antiinflammatory and cytokine inhibitory activities reported previously.2,3) This activity is probably based on
the effect of luteolin, which has been identified as an anti-inflammatory constituent of perilla, because topical application of luteolin also resulted in a significant antipromotion.

The phenotypes of antipromotion are fundamentally different between topical application and oral administration. The topical application of both substances inhibited tumor development; the direct effect on TP A may be a potent chemopreventive activity. A contribution to the potent effect may be that topical application disturbs the association of TP A with the skin. There are numerous reports of antipromotion activities of various topically applied molecules, making it difficult to exclude this possibility. Although the phenotype of antitumor promotion is growth inhibition, PLE demonstrates antitumor activity when administered orally, and therefore it is usually effective. The effect of luteolin administered orally could not be investigated because the substance is difficult to dissolve in drinking water and to give *ad libitum*.

TP A, typically used as a tumor promoter, also causes experimental acute dermatitis. We reported earlier that oral administration of PLE to mice inhibits TP A-induced ear edema.3) It was also shown that interleukin (IL)-1α, IL-1β, IL-6, interferon (IFN)-γ, and TNF-α were locally produced in TP A-induced ear edema. Oral administration of PLE also inhibited TP A-induced ear edema and simultaneously inhibited the local production of IL-6, IFN-γ, and TNF-α. We therefore consider that the essential aspect of tumor promotion is the chronic inflammation that accompanies the production of inflammatory cytokines. This hypothesis is compatible with the report that TNF-α is the essential cytokine in tumor promotion because mouse skin papilloma formation was not seen in TNF-α-deficient mice.21) The inhibition of local cytokine production, especially TNF-α inhibition, may contribute to cancer prevention.

REFERENCES