Carcinogenesis vol.28 no.6 pp.1224–1231, 2007
doi:10.1093/carcin/bgm005
Advance Access publication January 18, 2007

Xanthorrhizol inhibits 12-*O*-tetradecanoylphorbol-13-acetate-induced acute inflammation and two-stage mouse skin carcinogenesis by blocking the expression of ornithine decarboxylase, cyclooxygenase-2 and inducible nitric oxide synthase through mitogen-activated protein kinases and/or the nuclear factor-κB

Won Yoon Chung[1,2,3], Jae Hee Park[1], Mi Jeong Kim[1,3], Heui Ok Kim[1], Jae Kwan Hwang[4], Sang Kook Lee[5] and Kwang Kyun Park[1,2,3,*]

[1]Department of Oral Biology, [2]Oral Cancer Research Institute and Oral Science Research Institute and, [3]Brain Korea 21 Project, Yonsei University College of Dentistry, Shinchon-Dong 134, Seodaemoon-Ku, Seoul 120-752, Korea, [4]Department of Biotechnology, Yonsei University, Seoul 120-749, Korea and [5]College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea

[*]To whom correspondence should be addressed. Tel: +82 2 2228 3056; Fax: +82 2 364 7113;
Email: biochelab@yumc.yonsei.ac.kr

Xanthorrhizol is an active component isolated from *Curcuma xanthorrhiza* Roxb. (Zingiberaceae) that is traditionally used in Indonesia for medicinal purposes. In the present study, we found that the topical application of xanthorrhizol before 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment significantly inhibits TPA-induced mouse ear edema and TPA-induced tumor promotion in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated ICR mouse skin. The topical application of xanthorrhizol following the induction of papillomas with TPA-induced hyperplasia and dysplasia also reduced tumor multiplicity and incidence in DMBA-initiated mouse skin. To further elucidate the molecular mechanisms underlying the antitumor-promoting activity of xanthorrhizol, its effect on the TPA-induced expression of ornithine decarboxylase (ODC), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) and the upstream signaling molecules controlling these proteins were explored in mouse skin. The pre-treatment with xanthorrhizol inhibited the expression of ODC, iNOS and COX-2 proteins and nuclear factor-κB (NF-κB) activation in both mouse skin with TPA-induced acute inflammation and DMBA-initiated mouse skin promoted by TPA for 19 weeks. When mouse skin was treated after TPA-induced production of papillomas, xanthorrhizol remarkably suppressed the expression of ODC, iNOS and COX-2 and inhibited the activation of NF-κB. Furthermore, western blot analysis showed that xanthorrhizol suppressed the activation of extracellular signal-regulated protein kinase, p38, c-Jun-N-terminal kinase and Akt in mice after topical application for 6 weeks following the induction of papillomas. Taken together, the present study demonstrates that xanthorrhizol not only delays or inhibits tumor formation, but also reverses the carcinogenic process at pre-malignant stages by reducing the protein levels of ODC, iNOS and COX-2 regulated by the NF-κB, mitogen-activated protein kinases and/or Akt.

Introduction

Cancer chemoprevention is defined as the administration of agents to prevent the induction of or to inhibit or delay the progression of cancer (1). A chemopreventive agent may also inhibit or reverse carcinogenesis at a pre-malignant stage (2). All stages of carcinogenesis, termed initiation, promotion and progression, can be targeted for chemopreventive intervention (3). Chemoprevention is recognized as an important approach to control malignancy and recent studies have focused on the search for desirable chemopreventive agents. Plant-derived phytochemicals are a promising and important group of potential cancer chemopreventive agents because of their low toxicity and apparent benefit in other chronic diseases (4). Particularly, phytochemicals with antioxidative and anti-inflammatory activities have demonstrated potent chemopreventive and anticancer activity in multi-stage carcinogenesis (5,6).

Previous reports suggest that 12-*O*-tetradecanoylphorbol-13-acetate (TPA) promotes 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated mouse skin carcinogenesis, which is closely related to the inflammatory responses. These responses include the development of edema, hyperplasia, induction of pro-inflammatory cytokine interleukin-1α, enhanced release of reactive oxygen species, induction of epidermal ornithine decarboxylase (ODC) and over-expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) proteins (7,8). In addition, increased expression of COX-2 and/or iNOS was observed in many different types of tumors and transformed cells (9,10). The COX-2 protein catalyzes the biosynthesis of prostaglandins from arachidonic acid (11). Unlike the constitutively expressed COX-1 important for maintaining normal physiological functions, COX-2 is undetectable in most normal tissues and is induced transiently by pro-inflammatory cytokines, growth factors, oncogenes and tumor promoters (12). Over-expression of COX-2 inhibits apoptosis and increases the proliferation, invasiveness and metastasis of malignant cells (13,14). Another inducible enzyme, iNOS causes the overproduction of nitric oxide during inflammation and tumor development (15,16). Besides its ability to act as an initiator of carcinogenesis, nitric oxide is also involved in the promotional stage of tumorigenesis or neoplastic transformation (17) and in tumor progression by regulating angiogenesis (18). Therefore, suppressing the induction and activity of COX-2 and/or iNOS is a new paradigm for preventing carcinogenesis in several organs. In fact, the molecular mechanism underlying the chemopreventive activities of some compounds derived from edible or traditional medicinal plants is to inhibit COX-2 and iNOS expression (19,20).

Xanthorrhizol is a sesquiterpenoid isolated from the rhizomes of *Curcuma xanthorrhiza* Roxb. (Zingiberaceae) which is known as temu lawak or Javanese tumeric, and is structurally related with curcumin from *Curcuma longa* Linn. (21). It is traditionally used in Indonesia for dietary and medicinal purpose as cholagogues, aromatic stomachics, analgesics and as a rheumatic remedy (22). Although the anti-inflammatory (23) and antitumor activity (24) of the extract of *C.xanthorrhiza* has been reported, little attention has been paid to the activity of xanthorrhizol, one of its principle active ingredients. Recently, our group reported that xanthorrhizol suppressed COX-2 and iNOS activity in lipopolysaccharide-treated mouse macrophages (25) and inhibited the formation of tumor nodules in a spontaneous mouse lung metastasis model (26).

In the present study to assess the cancer chemopreventive activity of xanthorrhizol, we examined the anti-inflammatory and antitumor-promoting activity of xanthorrhizol in TPA-induced acute inflammation and in a multi-stage mouse skin carcinogenesis model, an ideal system

Abbreviations: BCA, bicinchoninic acid; COX-2, cyclooxygenase-2; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMSO, dimethylsulfoxide; EI, electron ionization; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal-regulated protein kinase; iNOS, inducible nitric oxide synthase; JNK, c-jun-N-terminal kinase; MEK, MAPK kinase; MAPK, mitogen-activated protein kinase; NMR, nuclear magnetic resonance; NF-κB, nuclear factor-κB; ODC, ornithine decarboxylase; PBST, phosphate-buffered saline containing 0.1% Tween 20; PTEN, phosphatase and tensin homologue deleted on chromosome 10; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

to study a number of biochemical alterations in the different stages of chemical carcinogenesis (27). To further elucidate the molecular mechanisms underlying the antitumor activity of xanthorrhizol, its effect on the TPA-induced activation of ODC, COX-2 and iNOS and the upstream signaling molecules controlling these proteins were also explored in mouse skin with inflammation, and at the different promotional stages during multi-stage skin carcinogenesis.

Materials and methods

Materials

The DMBA, TPA and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO). All other chemicals and solvents used were commercial products of analytical grade. Rabbit polyclonal COX-2 antibody was purchased from Cayman Chemical Co. (Ann Arbor, MI). Primary antibodies for ODC, extracellular signal-regulated protein kinase (ERK)-1/2, pERK1/2, p38, pp38, c-jun-N-terminal kinase (JNK)-1/2, pJNK1, pJNK2 and p65 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IκBα was from Cell Signaling Technology (Beverly, MA). Anti-rabbit and anti-goat horseradish peroxidase-conjugated secondary antibodies were products of Santa Cruz Biotechnology. An oligonucleotide probe containing the nuclear factor-κB (NF-κB) consensus sequence from the mouse COX-2 promoter region was obtained from Bionics (Seoul, Korea). Enhanced chemiluminescence detection kit and [γ-^{32}P]ATP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Bicinchoninic acid (BCA) protein assay kit was obtained from Pierce (Rockford, IL).

Isolation of xanthorrhizol

Xanthorrhizol was isolated from the rhizomes of *C.xanthorrhiza* as described previously (21). Briefly, the dried rhizomes (100 g) of *C.xanthorrhiza* purchased from the market in Yokjakarta, Indonesia (voucher specimen no. YS98015) were extracted with 75% MeOH (v/v, 400 ml). The concentrated methanol extract was fractioned successively with ethyl acetate, *n*-butanol and water. Xanthorrhizol was isolated from the ethyl acetate fraction using silica gel column chromatography (Merck, Seoul, Korea; 70–230 meshes, 5 × 43 cm, *n*-hexane–ethyl acetate = 10:1, v/v). Xanthorrhizol was identified by direct comparison of the ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR and electron ionization (EI)-mass spectral results with previously published data (28).

Animals

Female ICR mice (5–6 weeks of age) were supplied from the Dae-Han Biolink Experimental Animal Center (Daejeon, Korea). The animals were fed with a standard laboratory diet (Daejong, Seoul, Korea) and water *ad libitum*, and kept on a 12 h light–dark cycle at a temperature of 22 ± 2°C. Animal studies were performed after the experimental protocols approved by animal ethics committee of Yonsei University College of Dentistry.

TPA-induced mouse ear edema

The right ear of each female ICR mouse (6 weeks of age, six animals per group) was topically treated with various concentrations of xanthorrhizol in 50 μl of vehicle (DMSO–acetone = 15:85, v/v) 30 min prior to the application of 5 nmol TPA in 50 μl vehicle. The left ears were treated with vehicle alone. The control group received vehicle only on both ears. Four hours later, the mice were killed by cervical dislocation and the ears were removed. Right and left ear punches 6 mm in diameter were taken from each mouse. Edema was measured as the difference between the weight of the right ear punch and that of the left. The increase in edema was directly proportional to the degree of inflammation.

TPA-induced acute inflammation in mouse skin

Female ICR mice (6 weeks of age, six per group) were topically treated with various concentrations of xanthorrhizol in 0.2 ml vehicle (DMSO–acetone = 15:85, v/v) 30 min prior to the application of 10 nmol TPA in 0.2 ml vehicle on a shaved area of their back. Control mice were treated with vehicle alone. One (for NF-κB), 2 (for iNOS) and 4 h (for ODC and COX-2) later, the mice were killed and the excised dorsal skin was immediately placed at −80°C until analysis.

Two-stage mouse skin carcinogenesis

The dorsal region of 6-week-old female ICR mice (30 per group) was shaved with an electric clipper. To evaluate the antitumor-promoting activity of xanthorrhizol, the mice were treated topically with a single dose of 0.2 μmol DMBA dissolved in 0.2 ml acetone. One week later, the DMBA-initiated mice were topically treated with 7.5 nmol TPA in 0.2 ml vehicle (DMSO–acetone = 15:85, v/v) three times weekly for 19 weeks. Two and 6 μmol xanthorrhizol in 0.2 ml vehicle was topically applied 30 min before each TPA treatment. Control mice were pre-treated with vehicle alone.

The inhibitory activity of xanthorrhizol on papillomagenesis was examined at different stages of tumor promotion. One week after a single topical application of 0.2 μmol DMBA in 0.2 ml acetone, the mice were treated with 7.5 nmol TPA in 0.2 ml vehicle three times weekly for 6, 18 and 24 weeks to induce papillomas with hyperplasia, mild dysplasia and moderate dysplasia as described previously (29). Two weeks later, the mice were topically treated with 6 μmol xanthorrhizol in 0.2 ml vehicle for 6 weeks. Control mice were treated with vehicle alone.

Tumors that were at least 1 mm in diameter were counted and recorded biweekly and the results were expressed as the average number of tumors per mouse (tumor multiplicity) and as the percentage of tumor-bearing mice (tumor incidence). After the termination of the experiments, the mice were killed and the excised dorsal skins were immediately stored at −80°C until analysis.

Protein extraction

For the isolation of protein, the dorsal skins of mice derived from TPA-induced acute inflammation and the two-stage skin carcinogenesis experiments were excised. After the fat from the whole skin was removed on ice, the epidermis was immediately pulverized in liquid nitrogen. The pulverized skin was homogenized for 1 h in 800 μl of ice-cold lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM ethyleneglycol-*bis*(aminoethylether)-tetraacetic acid, 0.5% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1μg/ml leupeptin] containing a protease inhibitor cocktail tablet (Boehringer-Mannheim, Mannheim, Germany). The lysate was centrifuged at 14 000 r.p.m. for 30 min at 4°C. The supernatant was collected, aliquoted and used as total protein extracts. The protein concentration was determined by BCA protein assay.

To prepare cytosolic and nuclear extracts from mouse skin, the fat-free mouse dorsal skin was homogenized in 1 ml of hypotonic buffer [10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.9), 2 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonylfluoride]. A volume of 125 μl of 10% Nonidet P-40 solution was added to the homogenates and the mixture centrifuged at 12 000 r.p.m. for 30 sec. The supernatant was collected as cytosolic extract. The pelleted nuclei were washed with 400 μl of the above buffer plus 25 μl of 10% Nonidet P-40, centrifuged and re-suspended in 50 μl of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.9) containing 10% glycerol, 400 mM NaCl, 50 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulfonylfluoride. The mixture was incubated on ice for 20 min and centrifuged at 12 000 r.p.m. for 5 min at 4°C. The supernatants were collected and used as nuclear extracts after determination of protein concentrations.

Western blotting

Whole extract and cytosolic or nuclear extract samples containing 30 μg protein were boiled in sodium dodesylsulfate sample buffer for 5 min and resolved on 12% sodium dodesylsulfate–polyacrylamide gels. After electrophoresis, the proteins in the gel were transferred to polyvinylidene fluoride membranes (Amersham, Arlington Heights, IL). The blots were blocked with 5% non-fat dry milk in phosphate-buffered saline containing 0.1% Tween 20 (PBST) for 2 h at room temperature and then washed in PBST buffer. The membranes were incubated for 4 h at room temperature with 1:1000 dilutions of the primary antibodies against ODC, COX-2, IκBα, ERK1/2, pERK1/2, p38 or pp38, for 2 h with 1:2000 dilutions of the primary antibodies against iNOS or NF-κB (p65) or for 12 h at 4°C with a 1:500 dilution of primary antibodies against JNK1/2, pJNK1 or JNK2. Blots were washed three times with PBST at 5 min intervals followed by incubation with a 1:5000 dilution of the respective horseradish peroxidase-conjugated secondary antibodies (rabbit or goat) for 1 h, and again washed three times with PBST. The targeted proteins were visualized with an enhanced chemiluminescence detection kit according to the manufacturer's protocol.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was performed using a DNA protein-binding detection kit (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol. Briefly, the NF-κB oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech). The binding reaction was carried out in a total volume of 25 μl containing 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 4% (v/v) glycerol, 0.1 mg/ml sonicated salmon sperm DNA, 10 μg nuclear extracts and 100 000 c.p.m. of the labeled probe. After 50 min incubation at room temperature, samples were separated by electrophoresis on 6% non-denaturing polyacrylamide gels at 150 V in 0.25× Tris–Borate–EDTA buffer. Finally, the gel was then dried and exposed to X-ray film.

Statistical analysis

Data were expressed as the mean ± SE of at least three independent experiments. One-way analysis of variance was conducted using SAS software (8.1, SAS Institute, Cary, NC) to assess the anti-inflammatory activity of xanthorrhizol using a mouse ear edema test. Two-way analysis of variance was performed to determine antitumor activity of xanthorrhizol in a two-stage mouse skin carcinogenesis model. Repeated-measured analysis was used for tumor multiplicity of each time point. P values < 0.05 were considered to be statistically significant.

Results

Inhibitory effect of xanthorrhizol on TPA-induced acute inflammation

The anti-inflammatory effect of xanthorrhizol was evaluated by determining its inhibitory effect on TPA-induced ear edema in mice. As shown in Figure 1, ear edema induced by the topical application of TPA was suppressed by pre-treatment with xanthorrhizol in a dose-related manner ($P < 0.005$). Topical application of xanthorrhizol alone did not induce ear edema in mice.

Inhibitory effect of xanthorrhizol on TPA-induced tumor formation in mice

To examine the antitumor-promoting activity of xanthorrhizol, we examined the inhibitory effects of xanthorrhizol on TPA-induced tumor formation in DMBA-initiated mouse skin. All the mice treated with 7.5 nmol TPA for 19 weeks after initiation by DMBA developed an average of 15.5 ± 2.3 skin tumors per mouse (tumor multiplicity). Pre-treatment with 2 and 6 μmol xanthorrhizol reduced tumor multiplicity to 6.9 ± 1.1 ($P < 0.005$) and 4.0 ± 1.1 ($P < 0.005$), respectively, at 19 weeks. In addition, xanthorrhizol at 2 and 6 μmol dose dependently lowered the percentage of tumor-bearing mice (tumor incidence) to 80 and 57%, respectively, at the termination of the experiments (Figure 2).

Furthermore, the tumor multiplicity ($P < 0.05$) and incidence were reduced in the DMBA-initiated mice that were topically treated with xanthorrhizol for 6 weeks after the induction of papillomas with hyperplasia, mild dysplasia and moderate dysplasia by topical TPA application for 6, 18 and 24 weeks, respectively, as summarized in Table I.

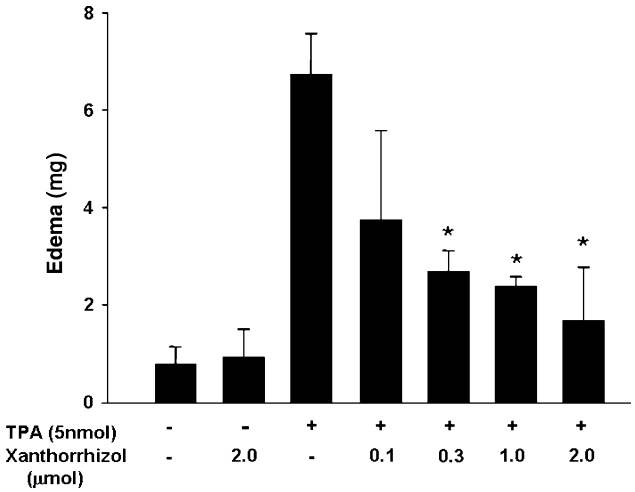

Fig. 1. Inhibitory effect of xanthorrhizol on TPA-induced mouse ear edema. The right ear of each female ICR mouse was topically treated with various concentrations of xanthorrhizol in 50 μl of vehicle (DMSO–acetone = 15:85, v/v) 30 min prior to the application of 5 nmol TPA in 50 μl vehicle. The left ears of each mouse were treated with vehicle alone. The control group received vehicle only on both ears. Four hours later, the mice were killed by cervical dislocation and the ears were removed. Right and left ear punches 6 mm in diameter were taken from each mouse. Edema was measured as the difference between the weight of the right ear punch and that of the left. Data are expressed as mean ± SE obtained from 6 mice per group. Experiments were repeated twice independently. ∗Significantly different from the TPA alone treated group ($P < 0.005$).

Inhibitory effect of xanthorrhizol on TPA-induced ODC expression in mouse skin

The ODC protein is a rate-limiting enzyme in the synthesis of polyamines that play pivotal roles in cell growth and proliferation, and induced expression of ODC is recognized as a biochemical hallmark of tumor promotion (30). The expression of ODC was increased markedly in mouse epidermis with TPA-induced acute inflammation (Figure 3A), in mouse epidermis treated with TPA for 19 weeks after DMBA initiation (Figure 3B) and in mouse epidermis at the different promotional stages induced by TPA treatment for 6, 18 and 24 weeks, respectively, after DMBA initiation (Figure 3C). The increased ODC expression in mouse epidermis with acute inflammation and tumor promotion induced by TPA was inhibited by pre-treatment with xanthorrhizol in a dose-dependent manner (Figure 3A and B). The topical application of xanthorrhizol after the induction of papillomas with hyperplasia and dysplasia also potently inhibited ODC expression (Figure 3C).

Inhibitory effect of xanthorrhizol on TPA-induced COX-2 and iNOS expression in mouse skin

Previous studies demonstrated transiently increased iNOS and COX-2 protein levels with maximal expression at 2 and 4 h, respectively, after the topical application of 10 nmol TPA onto shaved areas on the backs of female ICR mice (31). In the present study, we first confirmed that iNOS and COX-2 expression was elevated in mouse epidermis treated with TPA for 2 and 4 h, respectively (Figure 3A), in TPA-promoted mouse epidermis for 19 weeks after DMBA initiation (Figure 3B), and in the epidermis of DMBA-initiated mice at the different promotional stages induced by TPA treatment for 6, 18 and 24 weeks, respectively (Figure 3C). Pre-treatment with xanthorrhizol 30 min prior to the topical application of TPA resulted in a dose-dependent decrease in the level of iNOS and COX-2 protein in mouse skin with TPA-induced acute inflammation (Figure 3A) as well as in the DMBA-initiated mouse skin promoted by TPA for 19 weeks (Figure 3B). In addition, TPA-induced up-regulation of iNOS and COX-2 was significantly suppressed by xanthorrhizol treatment at the different promotional stages with hyperplasia, mild dysplasia and moderate dysplasia, respectively (Figure 3C).

Inhibitory effect of xanthorrhizol on TPA-induced NF-κB activation in mouse skin

Recent studies demonstrated that NF-κB is involved in the regulation of iNOS and COX-2 expression (32). NF-κB is sequestered in the cytoplasm by its association with the inhibitory binding protein IκBα. The phosphorylation, ubiquitination and subsequent degradation of IκBα release NF-κB and allow it to enter the nucleus where it induces the expression of iNOS and COX-2. To determine the effect of xanthorrhizol on TPA-stimulated NF-κB activation in mouse skin, we examined the cytosolic level of IκBα and the nuclear translocation of NF-κB by western blot analysis. The topical application of xanthorrhizol 30 min prior to TPA treatment dose dependently inhibited the TPA-induced degradation of IκBα protein in mouse skin. In addition, xanthorrhizol treatment caused a significant reduction of TPA-induced nuclear translocation of p65, the functionally active subunit of NF-κB protein and TPA-induced DNA binding of NF-κB. Interestingly, xanthorrhizol treatment alone appeared to induce NF-κB nuclear translocation and DNA binding (Figure 4A). However, xanthorrhizol inhibited the TPA-induced activation of NF-κB by blocking the degradation of IκBα protein in mouse skin pre-treated with xanthorrhizol before tumor promotion with TPA (Figure 4B), and also in mouse skin topically applied by xanthorrhizol for 6 weeks after the induction of papillomas with hyperplasia and dysplasia (Figure 4C).

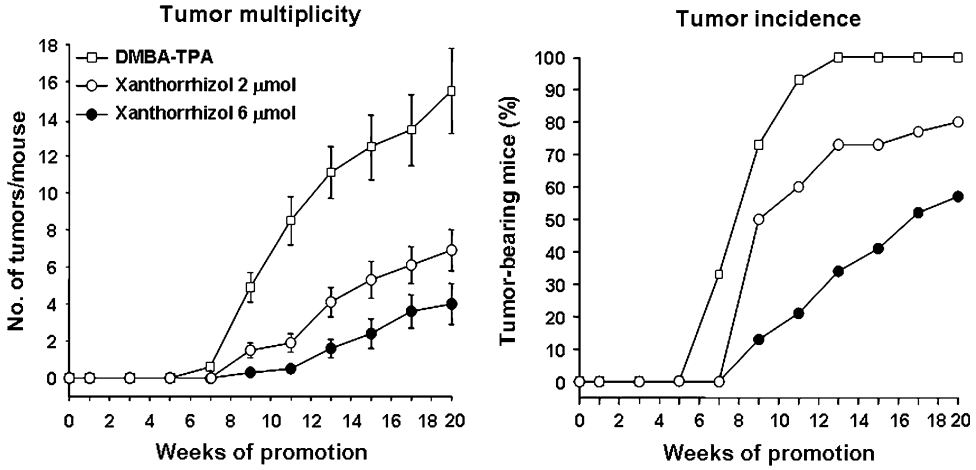

Fig. 2. Inhibitory effect of xanthorrhizol on mouse skin tumor promotion. Female ICR mice received vehicle (DMSO–acetone = 15:85, v/v) or 2 and 6 μmol xanthorrhizol 30 min prior to each topical application of 7.5 nmol TPA three times weekly for 19 weeks following DMBA initiation, as described in Materials and methods. Control animals received vehicle alone and did not produced papillomas. Tumors of at least 1 mm in diameter were counted and recorded biweekly, and the results were expressed as the average number of tumors per mouse (tumor multiplicity) and the percentage of tumor-bearing mice (tumor incidence).

Table I. Inhibitory effects of xanthorrhizol at different promotional stages of two-stage mouse skin carcinogenesis

Treatment	The average number of tumors per mouse (tumor multiplicity)	The percentage of tumor-bearing mice (tumor incidence)
DMBA–TPA^{6wk}–Vehicle^{6wk}	6.0 ± 2.1	32
DMBA–TPA^{6wk}–XT^{6wk}	3.8 ± 0.8	23
DMBA–TPA^{18wk}–Vehicle^{6wk}	14.4 ± 3.3	76
DMBA–TPA^{18wk}–XT^{6wk}	11.2 ± 1.0	59
DMBA–TPA^{24wk}–Vehicle^{6wk}	14.4 ± 1.3	80
DMBA–TPA^{24wk}–XT^{6wk}	11.6 ± 1.3	61

One week after a single topical application of 0.2 μmol DMBA in 0.2 ml acetone, the mice were treated with 7.5 nmol TPA in 0.2 ml vehicle (DMSO–acetone = 15:85) three times weekly for 6, 18 and 24 weeks to induce papillomas with hyperplasia, mild dysplasia and moderate dysplasia, respectively. Two weeks later, the mice were topically treated with 6 μmol xanthorrhizol (XT) in 0.2 ml vehicle for 6 weeks. Control mice were treated with vehicle alone. Tumors of at least 1 mm in diameter were counted and recorded biweekly, and the results were expressed as the average number of tumors per mouse (tumor multiplicity) and the percentage of tumor-bearing mice (tumor incidence). The superscripts indicate the periods of TPA or XT application on the dorsal skins of mice.

Inhibitory effect of xanthorrhizol on TPA-induced activation of mitogen-activated protein kinases and Akt in mouse skin

It has been reported that topical application of TPA in mouse skin results in a marked increase in the phosphorylated form of ERK, JNK and p38 kinase (33–35). These mitogen-activated protein kinases (MAPKs) regulate, at least in part, the induction of COX-2 through NF-κB activation in mouse skin with TPA-induced inflammation (33–35). In the present study, western blot analysis showed that the activation of MAPKs was gradually increased during progression of TPA-induced papillomagenesis in DMBA-initiated mouse skin. Akt activation was highly increased in mouse skin with mild dysplasia rather than in mouse skin with moderate hyperplasia. The phosphorylation of these MAPKs and Akt as well as the expression of JNK, p38 kinase and Akt in mouse skin was significantly suppressed by the topical application of xanthorrhizol for 6 weeks following the induction of papillomas with hyperplasia, mild dysplasia and moderate dysplasia (Figure 5).

Discussion

Carcinogenesis is a multi-step process exemplified by initiation, promotion and progression steps in which genetic and epigenetic events determine the neoplastic conversion of normal cells (36). Tumor promotion is a reversible event, at least in early stages, and repeated and prolonged applications of a promoting agent are required to promote tumorigenesis. Dietary phytochemicals may intervene to alter the expression or activity of intracellular signaling proteins involved in the process of tumor promotion and to block or reverse deleterious changes in cellular signaling, thereby preventing cancer (4).

The mouse skin carcinogenesis model represents one of the best means to study malignant transformation, including the multi-stage nature of tumor development and the molecular mechanisms that contribute to the development of human epithelial cancers. The DMBA–TPA mouse skin tumorigenesis protocol predominantly generates an A-T DNA base transversion at codon 61 of the H-*ras* gene that results in a constitutively activated Ras protein (37). Thus, activation of *ras* is an essential step in tumor initiation. Ras activates several downstream signal transduction pathways including the Raf–MAPK Kinase (MAPK) and the phosphatidylinositol 3-kinase–phosphatase and tensin homologue deleted on chromosome 10 (PTEN)–Akt pathways. These two pathways are highly relevant in the process of tumor promotion and progression in mouse skin carcinogenesis, affecting not only cell proliferation, but also apoptosis by promoting the survival of the tumor cells (38).

On the other hand, tumor promotion is closely linked to inflammation and the induction of ODC. ODC, the rate-limiting enzyme in the biosynthesis of intracellular polyamines, plays an important role in the regulation of cell proliferation and development of cancer. Induction of ODC is known to occur in response to TPA treatment in DMBA-initiated mouse skin, and ODC is constitutively elevated in the resulting skin tumors (37). Transgenic mice that over-express ODC in hair follicle keratinocytes were much more sensitive to DMBA-induced carcinogenesis than littermate controls, and did not require treatment with a tumor promoter to develop tumors. These results suggest that ODC over-expression is a sufficient condition for tumor promotion in this model (39). The proteins iNOS and COX-2 have been reported to be closely associated with cutaneous inflammation, cell proliferation and skin tumor promotion (19). Like other early-response gene products, iNOS and COX-2 can be rapidly and transiently induced by pro-inflammatory mediators and mitogenic stimuli including cytokines, endotoxins, growth factors, oncogenes and phorbol esters (12,31). Inappropriate up-regulation of iNOS and COX-2 has frequently been observed in various pre-malignant and

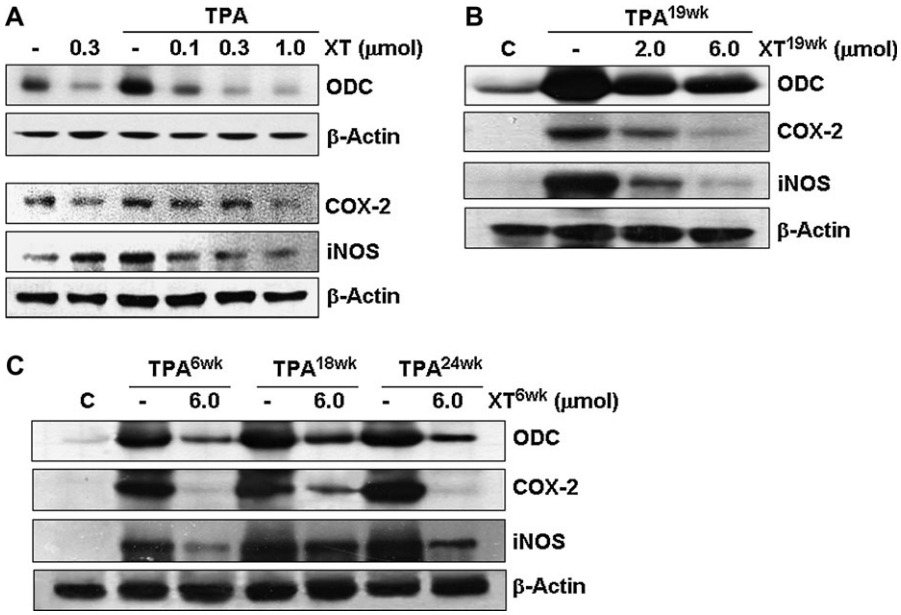

Fig. 3. Inhibitory effect of xanthorrhizol (XT) on the TPA-induced expression of ODC, COX-2 and iNOS proteins in mouse skin. (**A**) Female ICR mice were topically treated with various concentrations of XT in 0.2 ml vehicle (DMSO–acetone = 15:85, v/v) 30 min prior to the application of 10 nmol TPA in 0.2 ml vehicle. Control mice were treated with vehicle alone. Two hours (for iNOS) or 4 h (for ODC and COX-2) later, the mice were killed. (**B**) The DMBA-initiated mice received vehicle (DMSO–acetone = 15:85, v/v) or 2 and 6 μmol XT 30 min prior to each topical application of 7.5 nmol TPA three times weekly for 19 weeks. (**C**) The DMBA-initiated mice were treated with 7.5 nmol TPA three times weekly for 6, 18 and 24 weeks to induce papillomas with hyperplasia, mild dysplasia and moderate dysplasia, respectively. Two weeks later, the mice were topically treated with 6 μmol XT in 0.2 ml vehicle for 6 weeks. Control mice were treated with vehicle alone. At the termination of each experiment, the mice were killed. Protein expression was measured by western blot analysis. The western blot shown is representative of results obtained from more than three animals. The superscripts indicate the periods of TPA or XT application on the dorsal skins of mice.

malignant tissues (9,40). A considerable amount of compelling evidence suggests that the inhibition of iNOS and COX-2 expression or activity is important not only for alleviating inflammation, but also for the prevention of cancer (26,33,41). Therefore, suppressing of ODC, iNOS and COX-2 induction during carcinogenic progression is recognized as an important and commonly accepted approach to effectively inhibit tumor promotion.

The current study was designed to establish the chemopreventive potential of xanthorrhizol and to provide a comprehensive evaluation of the molecular mechanisms underlying its activity in TPA-induced mouse acute inflammation model, and in the DMBA-initiated and TPA-promoted mouse skin carcinogenesis model. Our data show that the topical application of xanthorrhizol before TPA treatment significantly inhibits TPA-induced mouse ear edema and TPA-induced tumor promotion in DMBA-initiated ICR mouse skin. In addition, we assessed the effect of xanthorrhizol on papillomagenesis at different stages of tumor promotion. TPA treatment for 6, 18 and 24 weeks produced papillomas with hyperplasia, mild dysplasia and moderate dysplasia, not yet any invasive growth, in DMBA-initiated mouse skin, as shown in our previous study (29). The topical application of xanthorrhizol, following the generation of papillomas with TPA-induced epithelial hyperplasia, mild dysplasia and moderate dysplasia in mouse skin, reduced the number of tumors per mouse (tumor multiplicity) and the percentage of tumor-bearing mice (tumor incidence) in DMBA-initiated mice. These results indicate that the repeated application of xanthorrhizol not only delays or inhibits tumor formation, but also reverses the carcinogenic process in pre-malignant tumors.

To explain the molecular mechanisms underlying the anti-inflammatory effect of xanthorrhizol and its protective effect on TPA-caused tumor promotion in DMBA-initiated ICR mouse skin, we examined the expression of well-known biomarkers of inflammation and tumor promotion; ODC, iNOS and COX-2. The ODC, iNOS and COX-2 proteins were highly expressed in mouse skin with hyperplasia compared with control mice treated with vehicle alone. The increased expression of these biomarkers was maintained during the development of papillomas with mild dysplasia and moderate dysplasia. Pretreatment with xanthorrhizol inhibited the induction of ODC, iNOS and COX-2 proteins in mouse skin with TPA-induced inflammation, and in DMBA-initiated mouse skin treated with TPA for 19 weeks. When applied to the mouse skin after TPA-induced tumor formation, xanthorrhizol remarkably suppressed the expression of ODC, iNOS and COX-2.

The significant inhibition of induction of ODC, iNOS and COX-2 by xanthorrhizol led us to investigate the effects of xanthorrhizol on the signaling molecules that regulate these proteins. Recent studies demonstrate that ODC is dramatically induced in response to *ras* activation in a variety of *in vitro* models and in the DMBA–TPA-treated mouse skin model (37,42). These results suggest that ODC expression is controlled both transcriptionally and translationally by the Ras effector pathways Raf–MEK–ERK and phosphatidylinositol 3-kinase/Akt (43,44). Pharmacologic inhibition of ERK by specific MEK1/2 blockers, such as U0126 and PD98059, prevented the induction of ODC (45). In a transgenic mouse line showing induction of MEK protein and ERK1/2 phosphorylation but no change in Akt-1, an examination of tumors revealed high levels of ODC protein and activity (46). On the other hand, elevated levels of ODC may cooperate with Raf–ERK via activation of Akt to convert normal keratinocytes into invasive malignant cells (47). These previous findings suggest that the induction of ODC is downstream of ERK activation, whereas the association of Akt with ODC is not clear.

The expression of iNOS and COX-2 appear to be regulated by NF-κB in cultured cell lines and in mouse skin with TPA-induced cutaneous inflammation (19,33). The eukaryotic transcription factor NF-κB plays a central role in general inflammation as well as tumorigenesis (19). Rapid phosphorylation of IκBα and its subsequent degradation after cells are exposed to external stimuli such as mitogens, inflammatory cytokines and reactive oxygen species leads to increased nuclear translocation and DNA binding of NF-κB. NF-κB induces the transcription of several target genes including COX-2 and iNOS. The transcriptional activity of NF-κB is regulated via an

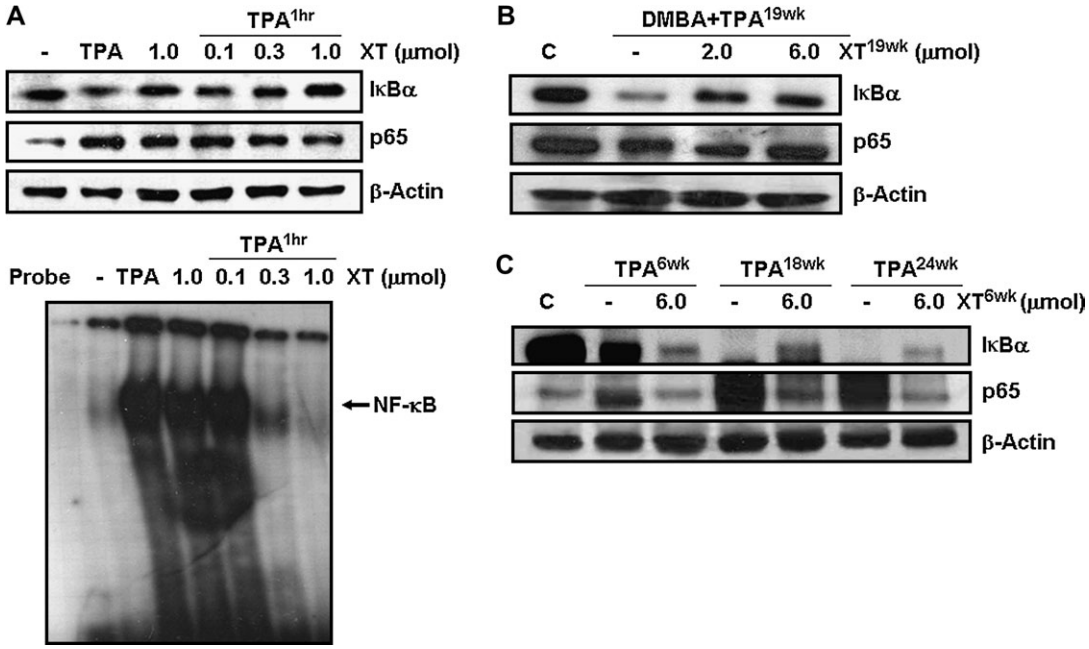

Fig. 4. Inhibitory effect of xanthorrhizol (XT) on TPA-induced NF-κB activation in mouse skin. (**A**) Female ICR mice were topically treated with various concentrations of XT in 0.2 ml vehicle (DMSO–acetone = 15:85, v/v) 30 min prior to the application of 10 nmol TPA in 0.2 ml vehicle. Control mice were treated with vehicle alone. The mice were killed 1 h after the TPA treatment. Nuclear and cytosolic extracts were prepared from the mouse skin for western blot analysis. For analysis by the electrophoretic mobility shift assay, epidermal nuclear extracts were prepared and incubated with radiolabeled oligonucleotides containing the NF-κB consensus sequence. (**B**) The DMBA-initiated mice received vehicle (DMSO–acetone = 15:85, v/v) or 2 and 6 μmol XT 30 min prior to each topical application of 7.5 nmol TPA three times weekly for 19 weeks. (**C**) The DMBA-initiated mice were treated with 7.5 nmol TPA three times weekly for 6, 18 and 24 weeks. Two weeks later, the mice were topically treated with 6 μmol XT in 0.2 ml vehicle for 6 weeks. Control mice were treated with vehicle alone. The IκBα level in cytosolic extracts and the p65 level in nuclear extracts were measured by western blot analysis. The western blot shown is representative of results obtained from more than three animals. The superscripts indicate the periods of TPA or XT application on the dorsal skins of mice.

elaborate series of intracellular signal transduction events. Studies show that ERK (33), p38 (34) and Akt (41) are involved in the transcriptional activation of NF-κB in mouse skin with TPA-induced inflammation. Moreover, a recent study using ERK1−/− mice proved the importance of ERK activity in the development of mouse skin papillomas by DMBA and TPA (48). In the present study, xanthorrhizol suppressed the nuclear accumulation and DNA binding of NF-κB by blocking TPA-induced IκBα degradation in mouse skin with TPA-induced inflammation. Repeated pre-treatment or post-treatment following the induction of papilloma with xanthorrhizol also inhibited IκBα degradation in the cytosol and the translocation of NF-κB into nucleus in DMBA-initiated mouse skin.

Furthermore, we found that ERK, p38 and JNK phosphorylation and the level of p38 protein were persistently increased with the progression of mouse skin papillomagenesis. In mouse skin with mild dysplasia, Akt activation was highly expressed, but was reduced in mouse skin with moderate dysplasia compared with mild dysplasia. The expression of JNK was also remarkably elevated in mouse skin with hyperplasia and dysplasia induced by TPA. These data were in accordance with a previous report showing that the content of JNK1 and JNK2 isoforms and JNK activity was increased, and that ERK was preferentially activated in advanced tumor stages in malignant cell lines derived from the DMBA–TPA-treated mouse skin carcinogenesis model (36). Topical application of xanthorrhizol following the induction of papillomas with hyperplasia and dysplasia blocked the activation of the upstream molecules ERK, p38 and Akt associated with NF-κB signaling, as well as that of JNK. The JNK is activated by oncogenic Ras proteins and results in the activation of activated protein-1 transcription factor (49). Binding sites for activated protein-1 are present on the COX-2 promoter, as are sites for NF-κB (50). Thus, JNK may regulate COX-2 expression through activated protein-1 rather than through NF-κB in our experimental system.

In conclusion, the present study demonstrates that pre-treatment with xanthorrhizol inhibits TPA-induced acute inflammation in mice

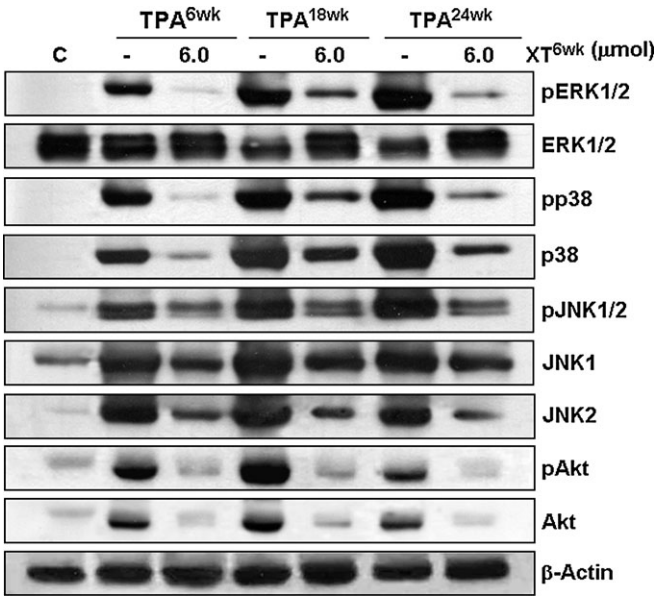

Fig. 5. Inhibitory effect of xanthorrhizol (XT) on TPA-induced expression of MAPKs and Akt in DMBA-initiated mouse skin at different stages of tumor promotion. One week after a single topical application of 0.2 μmol DMBA, the mice were treated with 7.5 nmol TPA three times weekly for 6, 18 and 24 weeks to induce papillomas with hyperplasia, mild dysplasia and moderate dysplasia, respectively. Two weeks later, the mice were topically treated with 6 μmol XT in 0.2 ml vehicle for 6 weeks. Control mice were treated with vehicle alone. At the termination of each experiment, the mice were killed and the dorsal skins were excised. Protein expression was measured by western blot analysis. The western blot shown is representative of results obtained from more than three animals. The superscripts indicate the periods of TPA or XT application on the dorsal skins of mice.

and TPA-induced tumor promotion in DMBA-initiated mice by reducing the protein levels of ODC, iNOS and COX-2, which are regulated by the NF-κB pathway, MAPKs and/or Akt. Moreover, xanthorrhizol treatment led to the reduction of existing skin tumors in mouse skin carcinogenesis induced by DMBA–TPA. These results suggest that xanthorrhizol is an attractive candidate for a chemopreventive agent. Above all, this is the first investigation of the changes that occur in biomarkers closely associated with carcinogenesis at different stages of tumor promotion in animal model.

Acknowledgements

This research was supported by the Korea Ministry of Health and Welfare, Grant No. 02-PJ1-PG10-20802-0006, by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2005-005-J05902) and in part by Yonsei University College of Dentistry Research Fund of 2002.

Conflict of Interest Statement: None declared.

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Received September 25, 2006; revised January 6, 2007; accepted January 9, 2007