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Anthocyanin- and Hydrolyzable Tannin-Rich Pomegranate Fruit Extract Modulates MAPK and NF-κB Pathways and Inhibits Skin Tumorigenesis in CD-1 Mice

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Chemoprevention has come of age as an effective cancer control modality; however, the search for novel agent(s) for the armamentarium of cancer chemoprevention continues. We argue that agents capable of intervening at more than one critical pathway in the carcinogenesis process will have greater advantage over other single-target agents. Pomegranate fruit extract (PFE) derived from the tree *Punica granatum* possesses strong antioxidant and antiinflammatory properties. Pomegranate fruit was extracted with acetone and analyzed based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and found to contain anthocyanins, ellagitannins and hydrolyzable tannins. We evaluated whether PFE possesses antitumor-promoting effects. We first determined the effect of topical application of PFE to CD-1 mice against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced conventional markers and other novel markers of skin tumor promotion. We found that topical application of PFE (2 mg/mouse) 30 min prior to TPA (3.2 nmole/mouse) application on mouse skin afforded significant inhibition, in a time-dependent manner, against TPA-mediated increase in skin edema and hyperplasia, epidermal ornithine decarboxylase (ODC) activity and protein expression of ODC and cyclooxygenase-2. We also found that topical application of PFE resulted in inhibition of TPA-induced phosphorylation of ERK1/2, p38 and JNK1/2, as well as activation of NF-κB and IKKα and phosphorylation and degradation of IκBα. We next assessed the effect of skin application of PFE on TPA-induced skin tumor promotion in 7,12-dimethylbenz(a)anthracene-initiated CD-1 mouse. The animals pretreated with PFE showed substantially reduced tumor incidence and lower tumor body burden when assessed as total number of tumors per group, percent of mice with tumors and number of tumors per animal as compared to animals that did not receive PFE. In TPA-treated group, 100% of the mice developed tumors at 16 weeks on test, whereas at this time in PFE-treated group, only 30% mice exhibited tumors. Skin application of PFE prior to TPA application also resulted in a significant delay in latency period from 9 to 14 weeks and afforded protection when tumor data were considered in terms of tumor incidence and tumor multiplicity. The results of our study provide clear evidence that PFE possesses antiskin-tumor-promoting effects in CD-1 mouse. Because PFE is capable of inhibiting conventional as well as novel biomarkers of TPA-induced tumor promotion, it may possess chemopreventive activity in a wide range of tumor models. Thus, an in-depth study to define active agent(s) in PFE capable of affording antitumor-promoting effect is warranted.

Key words: pomegranate fruit extract; chemoprevention; NF-κB; mitogen-activated protein kinase; ornithine decarboxylase

Chemoprevention, the use of naturally occurring and synthetic agents, to slow the growth, delay the onset, or reverse carcinogenesis is increasingly recognized as an important means for cancer control.[1–4] The expanded definition of chemoprevention also encompasses chemotherapy of precancerous lesions, which are called preinvasive neoplasia, dysplasia, or intraepithelial neoplasia, depending on the organ system. It is appreciated that chemoprevention can be targeted for intervention at all stages of carcinogenesis process exemplified by initiation, promotion and progression.[2] For many reasons, most notably for human acceptance, dietary substances are desirable chemopreventive agents.[5] Thus, in recent years, the search for novel agents and defining novel targets for chemoprevention have become an intense area of investigation. An ideal chemopreventive agent will be one that is acceptable to human population and has the ability to restore all dysregulated cellular and molecular pathways of multistage carcinogenesis.[5,6] This can be achieved through use of multiple agents, which is a complex undertaking. Alternatively, this could be achieved through the use of single dietary agent capable of interfering at multiple pathways in the carcinogenic process, a very desirable goal.

Pomegranate (*Punica granatum*, Punicaceae), native to Persia, is an edible fruit cultivated in Mediterranean countries, Afghanistan, India, China, Japan, Russia and the United States (California). Edible parts of pomegranate fruit (about 80% of total fruit weight) comprise 80% juice and 20% seed. Pomegranate fruit extract (PFE) is a rich source of 2 types of polyphenolic compounds: anthocyanins (such as delphinidin, cyanidin and pelargonidin), which give the fruit and juice its red color, and hydrolyzable tannins (such as punicalin, pedunculagin, punicalagin, gallagic and ellagic acid esters of glucose), which account for 92% of the antioxidant activity of the whole fruit.[7] The soluble polyphenol content in pomegranate juice varies between 0.2% and 1.0%.[8]

In recent years, health-promoting effects of PFE are being examined. Gil *et al.*[7] demonstrated that antioxidant activity of PFE, though varies depending on the variety, is higher than that of red wine and green tea. PFE has shown potent antioxidant capacity against lipid peroxidation that may be a central link for the antiatherogenic effect of PFE on lipoprotein, macrophages and platelets.[9–11] Studies have demonstrated that pretreatment of the rats with a methanolic extract of pomegranate peel followed by carbon tetrachloride resulted in restoration of catalase, peroxidase and superoxide dismutase enzyme activities.[12] Dietary supplementation of polyphenol-rich pomegranate juice to atherosclerotic mice significantly inhibited the development of atheroclerotic lesions and this may be attributed to the protection of low-density lipoprotein against oxidation.[13,14] Fermented pomegranate juice and cold-pressed PFE can reduce prostaglandin and leukotriene formation by inhibition of cyclooxygenase and lipoxygenase activities.[15] Recent studies have shown that pomegranate wine may serve as a potent inhibitor of nuclear factor kappa B (NF-κB) in vascular endothelial cells.[16]

The multistage mouse skin carcinogenesis model, although an artificial one, is an ideal system to study a number of biochemical alterations, changes in cellular functions and histologic changes that take place during the different stages of chemical carcinogenesis.[17,18] This system has also served as a useful model for initial screen for cancer chemopreventive effects of most dietary substances.[18,19] Studies have shown that skin application of tumor-

Abbreviations: COX, cyclooxygenase; DMBA, 7,12-dimethylbenz (a)anthracene; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; ODC, ornithine decarboxylase; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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promoting agents results in inflammatory responses, such as development of edema, hyperplasia, induction of proinflammatory cytokine interleukin-1α, induction of epidermal ornithine decarboxylase (ODC) and cyclooxygenase (COX) protein expression and activity, as well as activation of NF-κB.[19–22] Activation of mitogen-activated protein kinases (MAPKs)/NF-κB pathways has been shown to be involved in tumor growth and development.[23,24] In view of the antiinflammatory and antioxidative activities of PFE, as well as its inhibitory potential against COX and lipoxygenase activities,[15] in the present study, we show that topical application of PFE to CD-1 mice possesses antitumor-promoting effects and provide a mechanistic basis for such an effect.

Material and methods

ERK1/2 (phospho-p44/42, Thr202/Tyr204), JNK (phospo-p54/46, Thr183/Tyr185), p38 (phospho-p38, Thr180/Tyr204), IκBα and IκBα (phospho) antibodies were obtained from New England Biolabs (Beverly, MA). NF-κB/p65 antibody was procured from Geneka Biotechnology (Montreal, Canada). IKKα and ODC antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antimouse or antirabbit secondary antibody horseradish peroxidase conjugate was obtained from Amersham Life Science (Arlington Height, IL). 7,12-dimethyl benz(a)anthracene (DMBA) and 12-O-tetradecanoyl-phorbal acetate (TPA) were purchased from Sigma Chemicals (St. Louis, MO). The DC Bio-Rad Protein assay kit was purchased from Bio-Rad Laboratories (Herculus, CA). Novex precast tris-glycine gels were obtained from Invitrogen (Carlsbad, CA). Bradykinin, glucagon, cesium trifluoroacetate (Sigma Chemical) and *t*-IAA (Aldrich Chemical, Milwaukee, WI) were used as received.

Preparation of pomegranate fruit extract

Fresh fruit of pomegranate procured from the local supermarket was peeled, and its edible portion (seed coat and juice) was squeezed in 70% acetone-30% distilled water (1:20 by w/v). The red extract was filtered through filter paper (Whatman no. 1). The filtrate was condensed and freeze-dried. The freeze-dried extract was stored at 4°C to be used for topical treatment of animals. The biologic activity in terms of inhibition of ODC activity of 4 different PFE preparations was quite similar. Percent inhibition varied within 5%.

Sephadex LH-20 separation

Sephadex LH-20 (Pharmacia, Gaithersburg, MD) was equilibrated in water for 2 hr. PFE was applied to a 10 cm × 2.5 cm i.d. atmospheric pressure liquid chromotography column filled with Sephadex slurry. The fraction was obtained by eluting sequentially with 100 ml water and 100 ml aqueous acetone (acetone/H_2O, 4:1, v/v). The fraction containing anthocyanins, ellagitannins and other hydrolyzable oligomeric tannins was concentrated under vacuum at 35°C and reconstituted in 1 ml 80% aqueous acetone prior to matrix-assisted laser desorption/ionization analysis.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

Mass spectra were collected on a Bruker Reflex II-MALDI-TOF mass spectrometer (Billerica, MA) equipped with delayed extraction and N_2 laser (337 nm). In the positive reflectron mode, an accelerating voltage of 25.0 kV and a reflectron voltage of 26.5 kV were used. Spectra are the sum of 300 shots and spectra were calibrated with bradykinin (1,060.6 MW) and glucagon (3,483.8 MW) as external standards. In accordance with previously published results, *trans*-3-indoleacrylic acid (*t*-IAA; 5 mg/100 μL 80% aqueous acetone) was used as a matrix.[25] The ellagitannins eluted from the Sephadex column were mixed with the matrix solution at volumetric ratios of 1:2. Dowex 50X8-400 cation exchange resin (Supelco), equilibrated in 80% aqueous acetone (v/v), was used to deionize the analyte:matrix solution. Anthocyanins were detected as $[M]^+$ ions because the singly charged oxonium cation does not require cationization.[26] Deionization of the analyte:matrix solution and subsequent addition of cesium trifluoroacetate [^{133}Cs; 8 μl (0.01 M)] allowed the detection of ellagitannins and other hydrolyzable oligomeric tannins exclusively as $[M + Cs]^+$ ions.[27]

Animals and treatment for biomarker studies

Female CD-1 mice (5–6 weeks old) obtained from Charles River Laboratories (Wilmington, MA) were housed 4 per cage and were acclimatized for 1 week before use. Animals were subjected to a 12-hr light/12-hr dark cycle, housed at 24 ± 2°C and 50 ± 10% relative humidity and fed a Purnia chow diet and water *ad libitum*. For short-term biomarker studies, mice were divided into 4 groups, were shaved on the dorsal side of the skin and treated topically on the shaved area. The mice in the first group received a topical application of 200 μl acetone, and those in the second group received 2 mg PFE in 200 μl acetone/mouse. The mice in the third group received a topical application of 100 μl acetone alone, and those in the fourth group received 2 mg PFE in 100 μl acetone/mouse. Thirty minutes after these treatments, the mice in group 3 and group 4 were treated with a single topical application of TPA (3.2 nmole/100 μl acetone/mouse). At desired times after these treatments, the mice were sacrificed.

Edema and hyperplasia

To assess the inhibitory effect of preapplication of PFE on TPA-induced edema, 1 cm-diameter punches of skin from vehicle-, PFE-, TPA- or PFE- and TPA-treated animals were removed, made free of fat pads and weighed quickly. After drying for 24 hr at 50°C, the skin punches were reweighed and the loss of water content was determined. The difference in the amount of water gain between the control (vehicle-treated) and TPA-treated represented the extent of edema induced by TPA, whereas that between the control vehicle and PFE + TPA represented the inhibitory effect of PFE. For the hyperplasia study, skin was removed, fixed in 10% formalin and embedded in paraffin. Vertical sections (5 μm) were cut, mounted on a glass slide and stained with hematoxylin and eosin.

Preparation of cytosolic and nuclear lysates

Epidermis from the whole skin was separated as described earlier[28] and was homogenized in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, pH 7.4) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem, La Jolla, CA). The homgenate was then centrifuged at 14,000*g* for 25 min at 4°C and the supernatant (total cell lysate) was collected, aliquoted and stored at −80°C. For the preparation of nuclear and cytosolic lysates, 0.2 g of the epidermis was suspended in 1 ml of cold buffer (10 mM HEPES, pH 7.9, 2 mM $MgCl_2$, 10 mM KCI, 1 mM dithiothreitol, 0.1 mM EDTA and 0.1 mM PMSF) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III). After homogenization in a tight-fitting Dounce homogenizer, the homogenates were left on ice for 10 min and then centrifuged at 25,000*g* for 10 min. The supernatant was collected as cytosolic lysate and stored at −80°C. The nuclear pellet was resuspended in 0.1 ml of the buffer containing 10 mM HEPES (pH 7.9), 300 mM NaCI, 50 mM KCI, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF and 10% glycerol with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III). The suspension was gently shaken for 20 min at 4°C. After centrifugation at 25,000*g* for 10 min, the nuclear extracts (supernatants) were collected and quickly frozen at −80°C. The protein content in the lysates was measured by DC Bio-Rad assay as per the manufacturer's protocol.

ODC enzyme activity

The epidermis from the dissected skin was separated as described earlier[28] and homogenized at 4°C in a glass-to-glass homogenizer in 10 volumes of ODC buffer (50 mM Tris-HCl buffer,

pH 7.5, containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM pyridoxal-5-phosphate, 1 mM 2-mercaptoethanol and 0.1% Tween-80). The homogenate was centrifuged at 100,000*g* at 4°C and the supernatant was used for enzyme determination. ODC enzyme activity was determined in epidermal cytosolic fraction by measuring the release of $^{14}CO_2$ from the D,L-[^{14}C] ornithine by the method described earlier.[29] Briefly, 400 μl of the supernatant was added to 0.95 ml of the assay mixture (35 mM sodium phosphate, pH 7.2, 0.2 mM pyridoxal phosphate, 4 mM dithiothreitol, 1 mM EDTA, 0.4 mM L-ornithine containing 0.5 μCi of DL-[1-^{14}C] ornithine hydrochloride) in 15 ml corex centrifuge tube equipped with rubber stoppers and central well assemblies containing 0.2 ml ethanolamine and methoxyethanol in 2:1 (v/v) ratio. After incubation at 37°C for 60 min, the reaction was terminated by the addition of 1.0 ml of 2 M citric acid using a 21 G needle/syringe. The incubation was continued for 1 hr. Finally, the central well containing the ethanolamine:methoxyethanol mixture to which $^{14}CO_2$ has been trapped was transferred to a vial containing 10 ml of toluene-based scintillation fluid and 2 ml of ethanol. The radioactivity was measured in a Beckman LS 6000 SC liquid scintillation counter. Enzyme activity was expressed as picomoles CO_2 released/hr/mg protein.

Western blot analysis

For Western analysis, 25–50 μg of the protein was resolved over 8–12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot containing the transferred protein was blocked in blocking buffer for 1 hr at room temperature followed by incubation with appropriate monoclonal or polyclonal primary antibody in blocking buffer for 1.5 hr to overnight at 4°C. This was followed by incubation with antimouse, antirabbit, or antisheep secondary antibodies horseradish peroxidase for 1.5 hr and then washed 4 times with wash buffer and detected by chemiluminescence (ECL kit, Amersham Life Sciences) and autoradiography using XAR-5 film obtained from Eastman Kodak (Rochester, NY).

Immunostaining of MAPKs

At different time points posttreatment, the animals were sacrificed. The skin punch biopsies were frozen in optimal-cutting-temperature compound under liquid nitrogen immediately after removal and stored at −80°C for further use. To detect TPA-induced MAPK protein expression, immunostaining of MAPKs was performed. Briefly, 6 μm thick frozen skin sections were fixed in cold acetone for 10 min and nonspecific antibody binding was blocked using goat serum [10% phosphate-buffered saline (PBS)]. Thereafter, sections were incubated with phosphorylated form of antimouse MAPKs antibodies. Bound antimouse MAPKs was detected by incubation with biotinylated goat antimouse IgG1 followed by peroxidase-labeled streptavidin. Slides were developed with 3,3′-diaminobenzidine (DAB) as a substrate for 3–5 min. The sections were then rinsed with distilled water and counterstained with methyl green (2% for 60 min), cleared and mounted. The diaminobenzidine-peroxidase reaction gave a brown reaction product and the methyl green, a blue nuclear counterstain.

Skin tumorigenesis

Female CD-1 mice were used in DMBA- and TPA-induced 2-stage skin tumorigenesis protocol.[19] The dorsal side of the skin was shaved using electric clippers, and the mice with hair cycles in the resting phase were used for tumor studies. In each group, 20 animals were used. Tumor induction was initiated in the skin by a single topical application of 50 nmol DMBA in 0.2 ml acetone, and 1 week later, the tumor growth was promoted with twice-weekly topical applications of 3.2 nmol TPA in 0.2 ml acetone. Treatment with TPA alone or with PFE + TPA was repeated twice weekly up to the termination of the experiments at 30 weeks. Animals in both groups were watched for any apparent signs of toxicity, such as weight loss or mortality during the entire period of study. Skin tumor formation was recorded weekly, and tumors larger than 1 mm in diameter were included in the cumulative number if they persisted for 2 weeks or more.

Microscopy and photography

Images from immunostaining experiments were obtained using a Zeiss Axioplot microscope (Thornwood, NY) and Kodak Ektachrome 160T film. These images were scanned (SprintScan; Polaroid, Cambridge, MA) and formatted as tag image file format (TIFF) images in Adobe Photoshop 6.0 software to make the composite figures.

Statistical analysis

A 2-tailed Student's *t*-test was used to assess the statistical significance between the TPA-treated and PFE + TPA-treated groups. A p-value < 0.05 was considered statistically significant. In tumorigenesis experiments, the statistical significance of difference in terms of tumor incidence and multiplicity between TPA and PFE + TPA groups was evaluated by the Wilcoxon rank-sum test and chi-square analysis. An advantage of Wilcoxon rank-sum test is that its validity does not depend on any assumption about the shape of the distribution of tumor multiplicities.

Results

Chemical composition of PFE determined by MALDI-TOF MS

Positive reflectron mode MALDI-TOF MS results indicate the presence of 6 anthocyanins in PFE detected as $[M]^+$ ions: pelargonidin 3-glucoside (m/z 433.4), cyanidin 3-glucoside (m/z 449.4), delphinidin 3-glucoside (m/z 465.4), pelargonidin 3,5-diglucoside (m/z 595.5), cyanidin 3,5-diglucoside (m/z 611.5) and delphinidin 3,5-diglucoside (m/z 627.5; Table I). MALDI-TOF MS also detected an oligomeric series of ellagitannins as $[M + Cs]^+$ ions with masses between m/z 914.5 and 4,056.3 (Table II). Masses below m/z 1,217.1 correspond to known pomegranate ellagitannin structures (Table II), such as punicalin (m/z 914.5), pedunculagin (m/z 916.5) and punicalagin (m/z 1,217.1), as described by the work of Tanaka *et al*.[30–32] The higher masses correspond to structures of oligomeric ellagitannins, in which 2–5 core glucose units are crosslinked by dehydrodigalloyl units (Table II). Tentative structural assignments are based on predictive equations generated using the molecular mass of known ellagitannin monomeric units[33] as shown in Table II: glucosyl (m/z 180.2), hexahydroxydiphenoyl (m/z 302.2), gallagoyl (m/z 602.3), galloyl (m/z 152.1) and dehydrodigalloyl (m/z 302.2).

Inhibitory effect of PFE on TPA-induced cutaneous edema

Studies from our laboratory and by others have shown that TPA application to mouse skin results in cutaneous edema.[34,35] In the present study, we evaluated the protective effects of topical application of PFE in TPA-mediated cutaneous edema in CD-1 mouse. The CD-1 mice were topically treated with PFE (2 mg/mouse) and 30 min later were topically treated with TPA (3.2 nmole/mouse). As determined by the weight of 1 cm diameter punch of the dorsal skin, application of TPA to CD-1 mouse skin resulted in a significant development of skin edema at 24 and 48 hr post-TPA treatment compared to control and PFE-treated groups (Fig. 1). The skin application of PFE 30 min prior to that of TPA application showed a significant protection against TPA-induced skin

TABLE I – MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY OF THE ANTHOCYANINS IN POMEGRANATE FRUIT EXTRACT

Anthocyanins	Expected mass $[M]^+$	Observed mass $[M]^+$
Pelargonidin 3-glucoside	433.4	433.4
Cyanidin 3-glucoside	449.4	449.4
Delphinidin 3-glucoside	465.4	465.3
Pelargonidin 3,5-diglucoside	595.5	595.4
Cyanidin 3,5-diglucoside	611.5	611.4
Delphinidin 3,5-diglucoside	627.5	627.4

Data represent the observed and calculated masses of 6 anthocyanins in positive reflectron mode detected in PFE as $[M]^+$ ions.

TABLE II – MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY OF THE ELLAGITANNIN AND OTHER HYDROLYSABLE TANNINS IN POMEGRANATE FRUIT EXTRACT

Ellagitannin and other hydrolyzable tannins	Expected mass $[M + Cs]^+$	Observed mass $[M + Cs]^+$	Glucosyl	Gallagoyl	Hexahydroxydiphenoyl	Galloyl	Dehydrodigalloyl
Punicalin	914.4	914.5	1	1	0	0	0
Pedunculagin	916.4	916.5	1	0	2	0	0
	918.5	918.6	1	0	1	2	0
Punicalagin	1,216.6	1,217.1	1	1	1	0	0
	1,218.7	1,219.1	1	1	0	2	0
Dimers	1,550.9	1,551.1	2	0	2	1	1
	1,701.0	1,701.1	2	0	3	0	1
	1,703.0	1,703.1	2	0	2	2	1
	1,853.1	1,853.2	2	0	3	1	1
	1,855.2	1,855.2	2	0	2	3	1
	2,003.2	2,003.1	2	0	4	0	1
Trimers	1,881.2	1,881.3	3	0	2	0	2
	2,335.5	2,336.2	3	0	3	1	2
	2,485.6	2,485.3	3	0	4	0	2
	2,487.6	2,487.3	3	0	3	2	2
	2,639.7	2,639.3	3	0	3	3	2
	2,787.8	2,787.4	3	1	2	2	2
Tetramers	3,270.1	3,270.3	4	0	5	0	3
	3,272.1	3,272.3	4	0	4	2	3
Pentamers	4,054.7	4,054.8	5	0	6	0	4
	4,056.7	4,056.3	5	0	5	2	4

Data represent the observed and calculated masses of ellagitannin and other hydrolysable tannins in positive reflectron mode detected in PFE as $[M + Cs]^+$ ions. Masses were calculated from the number of monomeric units shown in the table.

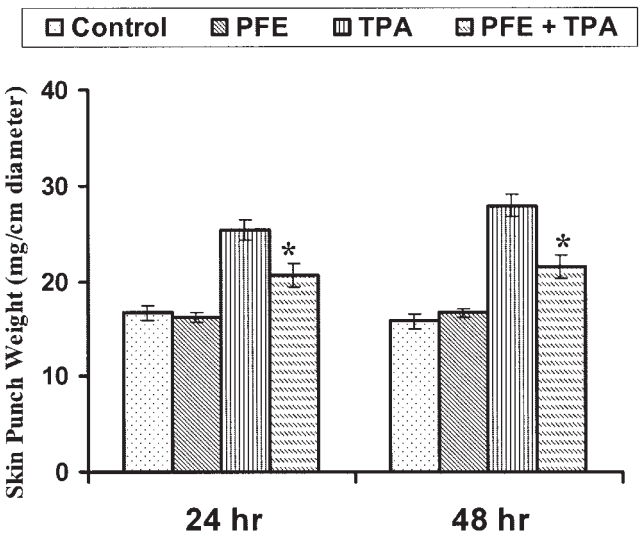

FIGURE 1 – Inhibitory effect of pomegranate fruit extract on TPA-induced skin edema in CD-1 mice. Twenty-four and 48 hr after TPA treatment, the skin edema was determined by weighing 1 cm-diameter punch skin as described in text. At least 4 determinations were made at different dorsal skin sites per mouse in each group. The data represent the mean ± SE of 8 mice (asterisk, $p < 0.01$ *vs*. TPA).

edema at 24 (54%; $p < 0.01$) and 48 (51%; $p < 0.01$) hr posttreatment. We found that topical application of PFE alone to mice did not result in an increase in skin edema at 24 and 48 hr posttreatment (Fig. 1).

Inhibitory effect of PFE on TPA-induced epidermal hyperplasia

The effect of topical application of PFE on TPA-mediated induction of epidermal hyperplasia was then assessed. As shown in Figure 2, topical application of TPA resulted in an increase in epidermal hyperplasia at 24 and 48 hr after treatment when compared to control-treated animals. The application of TPA also resulted in mixed cell infiltration in the dermis, which comprised mostly neutrophils with some mononuclear cells admixed; this effect of TPA in the dermis was also inhibited by the preapplication of PFE (Fig. 2). The topical application of PFE, however, prior to that of TPA application to mouse skin resulted in inhibition in the induction of epidermal hyperplasia (Fig. 2). Additionally, microscopic examination of the sections of skin biopsies also revealed that interstitial cell spaces were larger, and cells were spongiotic in nature in the TPA-treated group of animals compared with the vehicle- and PFE + TPA-treated groups of animals. This observation shows that topical application of PFE prior to TPA inhibited TPA-mediated induction of edema in animal skin. PFE alone, however, did not induce any epidermal hyperplasia as the histology of these animals was comparable to that of control mice (Fig. 2).

Inhibitory effect of PFE on TPA-induced epidermal ODC activity

ODC, which deacrboxylates ornithine to form putrescine, is the first and the rate-limiting enzyme in polyamine biosynthesis. Induction of ODC has been suggested to play a significant role in tumor promotion. Studies have shown that TPA-induced ODC activity is essential in mouse skin tumor promotion.[36] Therefore, we studied the effect of PFE on TPA-mediated induction of epidermal ODC activity. The effect of preapplication of PFE on TPA-mediated induction of epidermal ODC activity was studied as a function of time after the TPA application. Consistent with the previous published studies, the maximum induction of epidermal ODC after the single topical application of TPA was observed at 6 hr, which started declining after that period and reached an almost basal level by 18 hr (Fig. 3). When PFE was applied 30 min prior to each topical application of TPA, significant inhibition of epidermal ODC activity ($p < 0.001–0.01$) was observed when compared to TPA alone (Fig. 3). The application of PFE alone at the dose of 2 mg did not produce any change in epidermal ODC activity when compared with vehicle-treated control animals.

Inhibitory effect of PFE on TPA-induced epidermal ODC and COX-2 protein expression

We next assessed the effect of skin application of PFE on TPA-induced epidermal ODC and COX-2 protein expression. Western blot analysis revealed that topical application of TPA to CD-1 mice resulted in a marked increase in epidermal ODC protein expression at 6, 12 and 24 hr post-TPA treatment compared to control (Fig. 4). Topical application of PFE 30 min prior to TPA

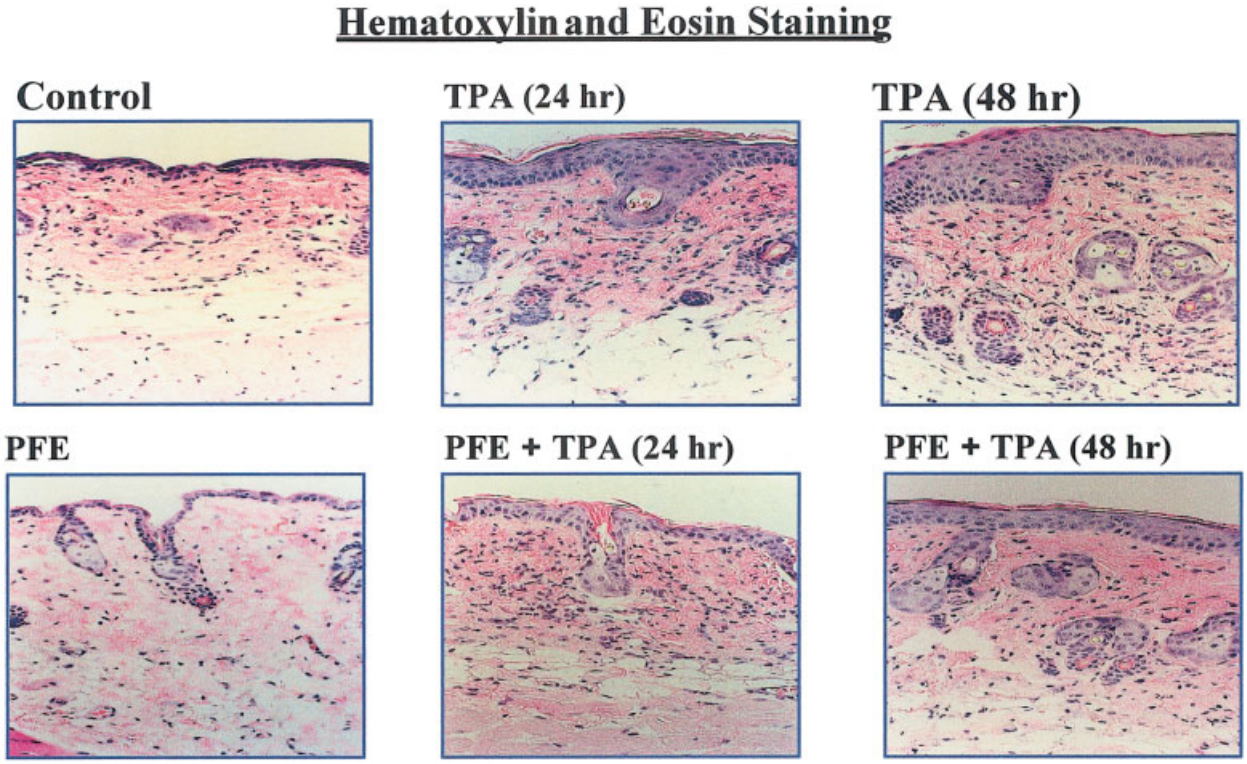

FIGURE 2 – Inhibitory effect of pomegranate fruit extract on TPA-induced hyperplasia in CD-1 mice. Twenty-four and 48 hr after treatment, the animals were sacrificed; skin biopsies were processed for hematoxylin and eosin staining. Representative pictures are shown. Sections were photographed using an 80× objective.

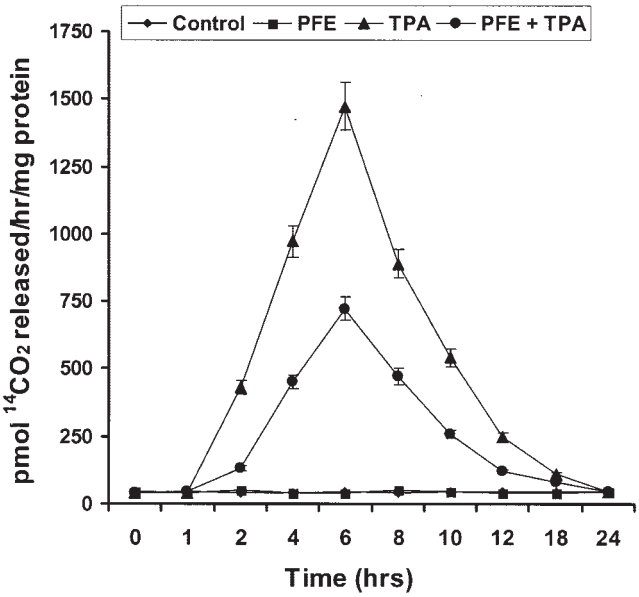

FIGURE 3 – Inhibitory effect of pomegranate fruit extract on TPA-induced epidermal ODC activity in CD-1 mice. The animals were sacrificed at different time points after TPA treatment, epidermal cytosolic fraction was prepared and ODC activity was determined. The data are shown as ODC activity (pmol/hr/mg protein) and represent the mean ± SE of 8 mice; each assay was performed in duplicate ($p < 0.001$–0.01 *vs.* TPA).

application resulted in inhibition in ODC protein expression. We also found that topical application of TPA resulted in an increased expression of COX-2 in a time-dependent manner in mouse skin.

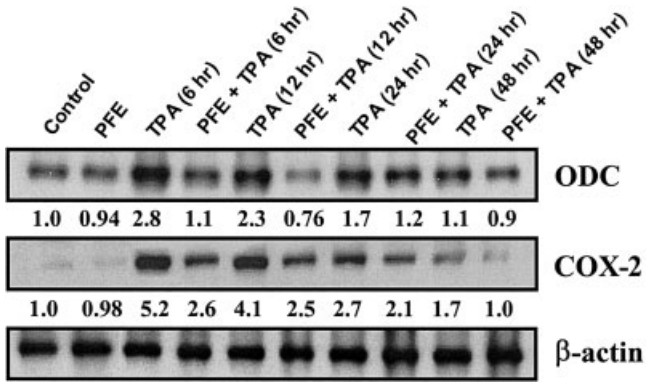

FIGURE 4 – Inhibitory effect of pomegranate fruit extract on TPA-induced epidermal ODC and COX-2 protein expression in CD-1 mice. At different time after treatment, the animals were sacrificed, epidermal protein lysate was prepared and ODC and COX-2 protein expression were determined as described in text. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for β-actin. The immunoblots shown here are representative of 3 independent experiments with similar results. The values below the figures represent relative density of the band.

The affect of TPA application on COX-2 protein expression was more pronounced at 6 hr post-TPA application; then it gradually subsides but was still higher when compared to control. Topical application of PFE prior to TPA application resulted in inhibition of COX-2 protein expression when compared to TPA-alone group at all time points (Fig. 4). The application of PFE alone at the dose of 2 mg did not produce any change in epidermal ODC and COX-2 protein expression when compared with vehicle-treated control animals.

Inhibitory effect of PFE on TPA-mediated phosphorylation of MAPKs

To determine whether TPA could induce activation of MAPKs under *in vivo* condition in CD-1 mice, Western blot analysis and immunohistochemistry were performed using phospho-specific MAPKs antibodies. In the present study, as evident from Western blot analysis, we found that topical application of TPA resulted in an increased phosphorylation of ERK1/2 (p44 and p42), JNK1/2 (p54 and p46) and p38 (Fig. 5). We found that topical application of TPA resulted in an increased phosphorylation of ERK1/2 (p44 and p42) at 6 hr post-TPA application and then it gradually subsides. The effect of TPA application on phosphorylation of p38 was more pronounced at 12 hr and for JNK was more pronounced at 6 hr post-TPA application; then it gradually subsides but was still higher when compared to control. Topical application of PFE prior to TPA application resulted in inhibition TPA-mediated phosphorylation of MAPKs protein (Fig. 5). To confirm our result, we performed immunohistochemistry in skin biopsies of these mice to examine the expression of the phosphorylated form of MAPKs (such as ERK1/2, JNK1/2 and p38). We found that topical application of TPA to CD-1 mice resulted in a marked expression of the phosphorylated form of ERK in the epidermis and basal layer of the epidermis at 6 hr. However, topical application of PFE prior to TPA application was found to result in a significant inhibition in the phosphorylated form of ERK (Fig. 6). We also found that topical application of TPA resulted in an increased expression of the phosphorylated form of JNK and p38. Preapplication PFE significantly inhibited TPA-induced phosphorylation of JNK and p38 (Figs. 7 and 8). These results further confirm our Western blotting data (Fig. 5).

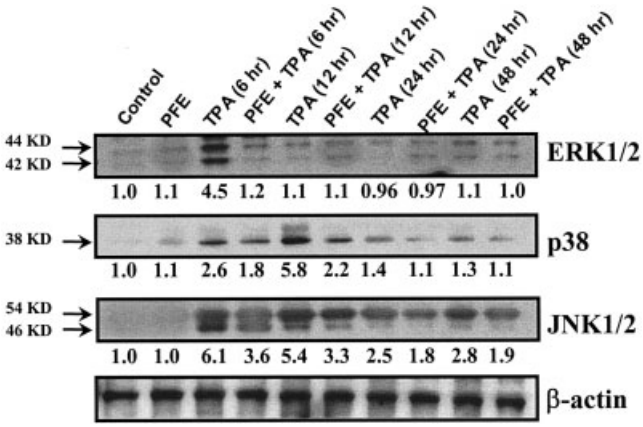

FIGURE 5 – Inhibitory effect of pomegranate fruit extract on TPA-induced phosphorylation of ERK1/2, JNK1/2 and p38 in CD-1 mice. At different time after treatment, the animals were sacrificed; epidermal protein lysate was prepared and Western blot analysis for protein expression was performed as described in text. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for β-actin. The immunoblots shown here are representative of 3 independent experiments with similar results. The values below the figures represent relative density of the band.

Inhibitory effect of PFE on TPA-induced activation of NF-κB and IKKα and phosphorylation and degradation of IκBα protein expression

Studies have shown that one of the critical events in NF-κB activation is its dissociation with subsequent degradation of inhibitory protein IκBα via phosphorylation and ubiquination.[37–39] Activation and nuclear translocation of NF-κB is preceded by the phosphorylation and proteolytic degradation of IκBα.[39,40] To determine whether the inhibitory effect of PFE was attributable to an effect on IκBα degradation, we examined the cytoplasmic level of IκBα protein expression by Western blot analysis. We found that TPA application to mouse skin resulted in the degradation of IκBα protein expression at 12 and 24 hr after treatment. However, topical application of PFE 30 min prior to TPA application resulted in inhibition of TPA-induced degradation of IκBα protein (Fig. 9). We next assessed whether TPA application affects the phosphorylation of IκBα protein. As shown by Western blot, TPA-induced a marked increase in the phosphorylation level of

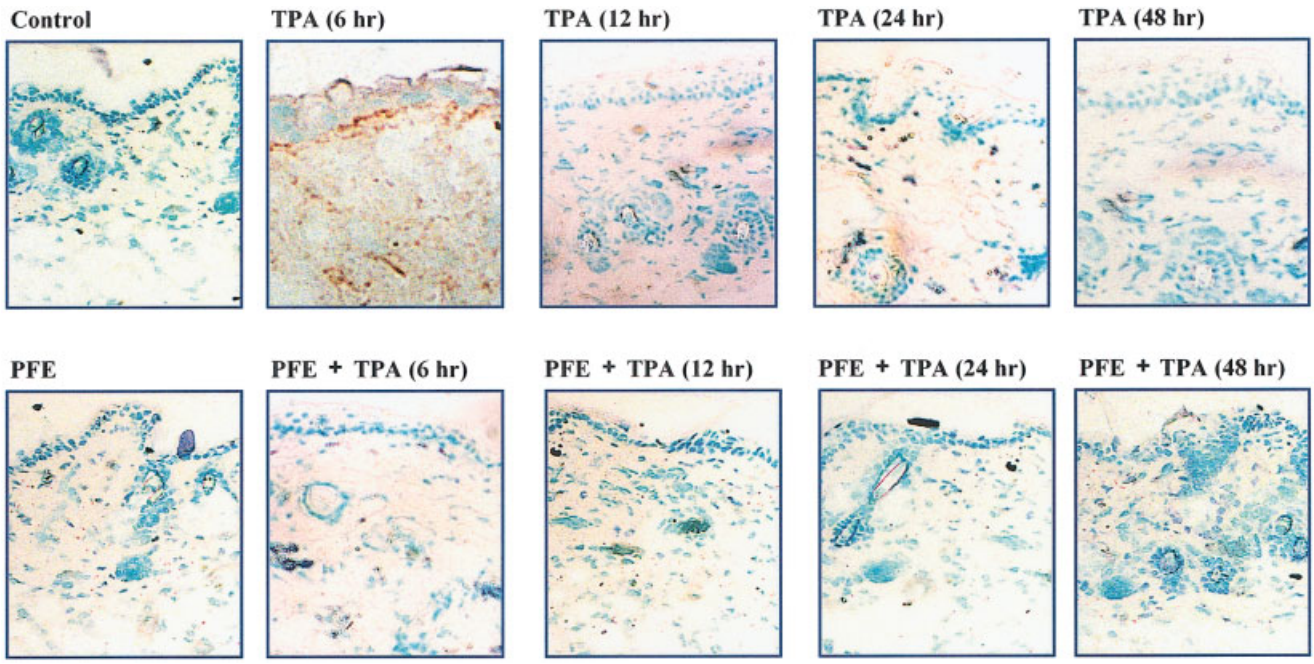

FIGURE 6 – Inhibitory effect of pomegranate fruit extract on TPA-induced phosphorylation of ERK in CD-1 mice. At different time after treatment, the animals were sacrificed, the skin punch biopsies were frozen in optimal-cutting-temperature compound under liquid nitrogen immediately after removal and immunostaining of phosphorylated form of ERK was performed as detailed in text. A representative picture from 3 independent immunostaining is shown. Scale bar = 50 μm.

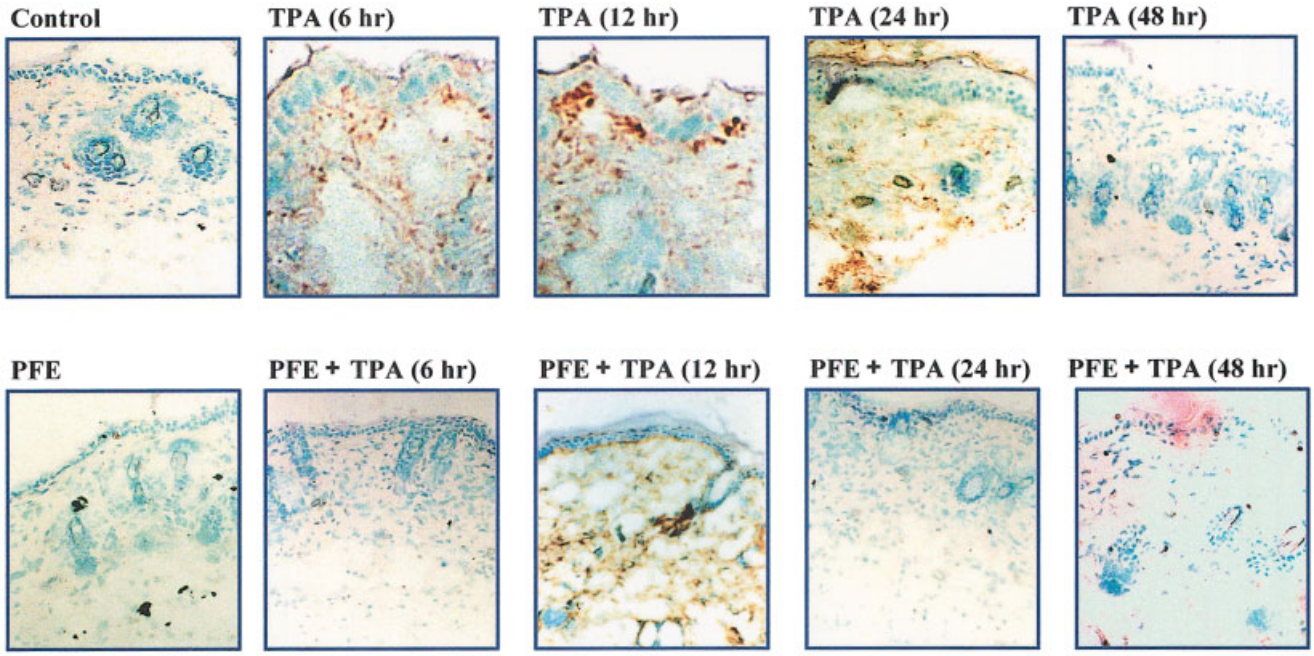

FIGURE 7 – Inhibitory effect of pomegranate fruit extract on TPA-induced phosphorylation of p38 in CD-1 mice. At different time after treatment, the animals were sacrificed, the skin punch biopsies were frozen in optimal-cutting-temperature compound under liquid nitrogen immediately after removal and immunostaining of phosphorylated form of p38 was performed as detailed in text. A representative picture from 3 independent immunostaining is shown. Scale bar = 50 μm.

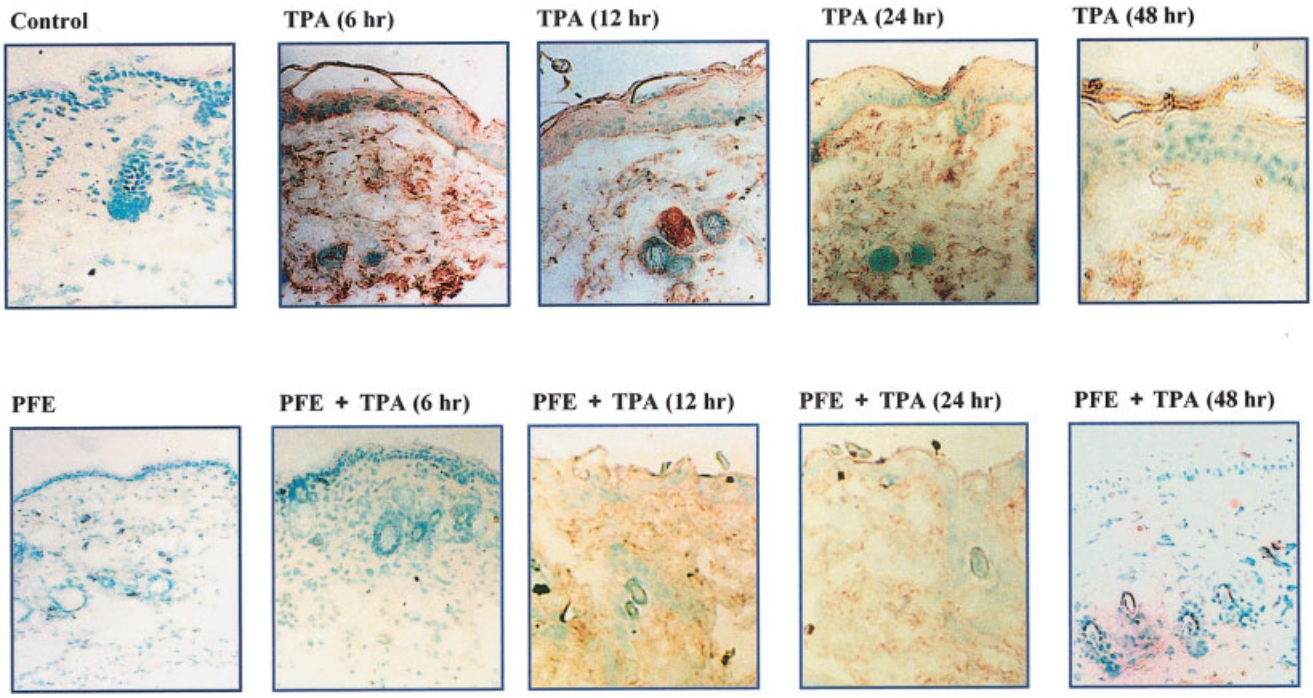

FIGURE 8 – Inhibitory effect of pomegranate fruit extract on TPA-induced phosphorylation of JNK in CD-1 mice. At different time after treatment, the animals were sacrificed, the skin punch biopsies were frozen in optimal-cutting-temperature compound under liquid nitrogen immediately after removal and immunostaining of phosphorylated form of JNK was performed as detailed in text. A representative picture from 3 independent immunostaining is shown. Scale bar = 50 μm.

IκBα protein at Ser^{32} after treatment, which was inhibited by topical application of PFE prior to TPA application (Fig. 9). Studies have shown that IKKα activity is necessary for IκBα protein phosphorylation/degradation.[41,42] To determine whether inhibition of TPA-induced IKKα activation by PFE is attributable to suppression of IκBα phosphorylation/degradation, we also measured IKKα protein level. We found that TPA application resulted in the activation of IKKα protein that in turn phosphorylate and

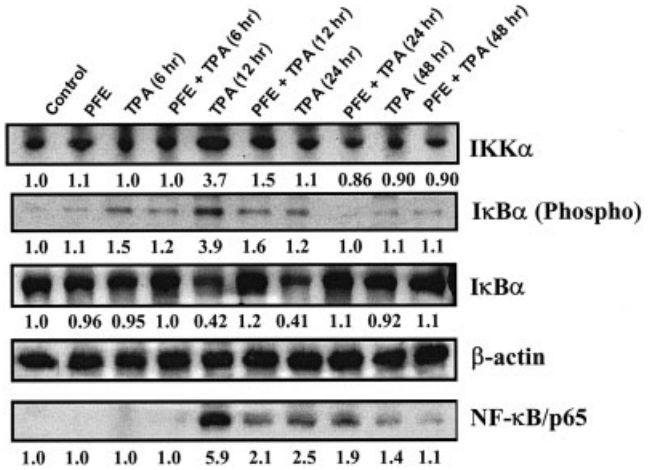

FIGURE 9 – Inhibitory effect of pomegranate fruit extract on TPA-induced activation of NF-κB, IKKα and phosphorylation and degradation of IκBα in CD-1 mice. At different time after treatment, the animals were sacrificed, epidermal cytosolic and nuclear lysates were prepared and protein expression was determined as described in text. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The immunoblots shown here are representative of 3 independent experiments with similar results. The values below the figures represent relative density of the band.

degrade IκBα protein. Topical application of PFE prior to TPA application inhibited TPA-induced activation of IKKα (Fig. 9). Next, we investigated whether topical application of PFE inhibits TPA-induced activation and nuclear translocation of p65, the functionally active subunit of NF-κB in mouse skin. In the nuclear fraction, we found that TPA application onto the skin of CD-1 mice resulted in the activation and nuclear translocation of NF-κB/p65. However, topical application of PFE prior to TPA application inhibited TPA-induced NF-κB/p65 activation and nuclear translocation (Fig. 9).

Inhibitory effect of PFE on TPA-induced skin tumor promotion

We next assessed the effect of skin application of PFE on TPA-induced skin tumor promotion in 7,12-dimethylbenz(a)anthracene-initiated CD-1 mouse. As shown by data in Figure 10, topical application of PFE prior to that of TPA in DMBA-initiated CD-1 mouse skin resulted in an inhibition of skin tumorigenesis. This inhibition was evident when tumor data were considered as the percentage of mice with tumors (Fig. 10*a*), the number of tumors per group (Fig. 10*b*) and the number of tumors per mouse (Fig. 10*c*). The animals pretreated with PFE showed substantially reduced tumor incidence and lower tumor body burden when assessed as total number of tumors per group, percent of mice with tumors and number of tumors per animal as compared to animals that did not receive PFE (Fig. 10). In TPA-treated group, 100% of the mice developed tumors at 16 weeks on test, whereas at this time in PFE-treated group, only 30% of mice exhibited tumors (Fig. 10*a*). Skin application of PFE prior to TPA application resulted in a delay in latency period from 9 to 14 weeks and afforded protection when tumor data were considered in terms of tumor incidence and tumor multiplicity throughout the treatment period ($p < 0.05$, chi-square test). At the termination of the experiment at 30 weeks, 20% of the mice were tumor-free in the group that received skin application of PFE prior to each TPA application. At the termination of the experiment at 30 weeks on test, compared with a total of 105 tumors in non-PFE-treated group of animals, only 38 tumors in PFE-treated group were recorded (Fig. 10*b*). Compared with the non-PFE-treated group, such decrease in the total number of tumor in the PFE-treated group corresponds to 64% inhibition. When these tumor data were considered in terms of number of tumors per mice, at the termination

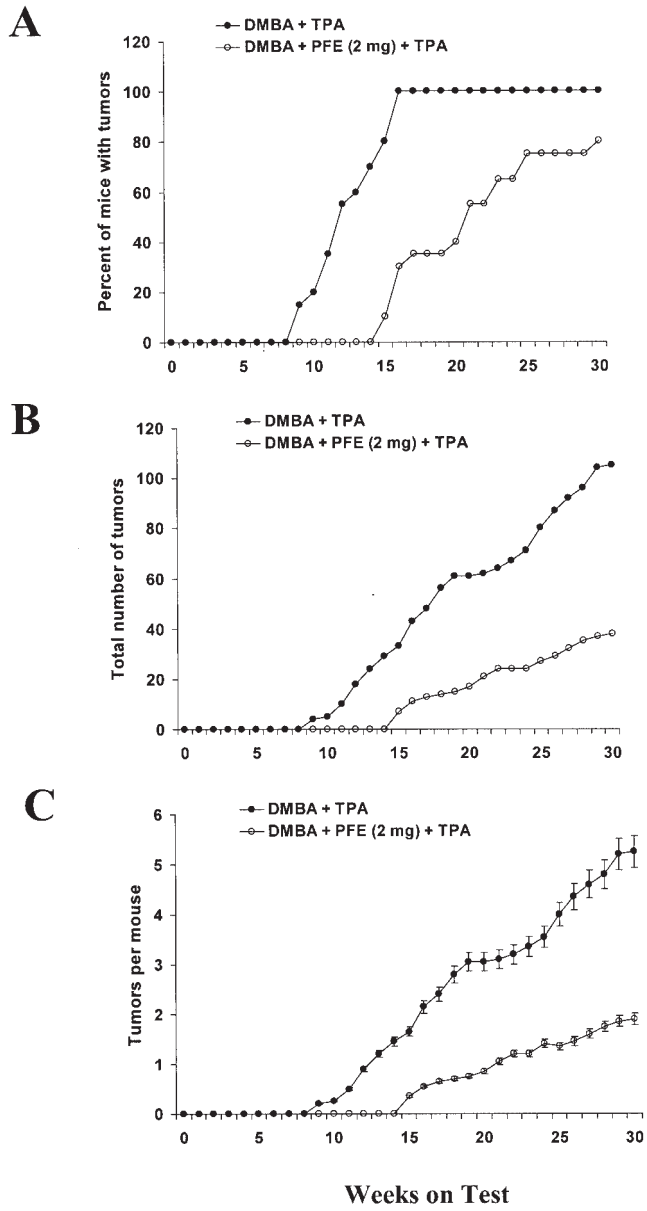

FIGURE 10 – Inhibitory effect of pomegranate fruit extract on DMBA-initiated and TPA-promoted tumor formation in CD-1 mice. In each group, 20 animals were used. Tumorigenesis was initiated in the animals by a single topical application of 50 nmol DMBA in 0.2 ml vehicle on the dorsal shaved skin, and 1 week later, the tumor growth was promoted with twice-weekly applications of 3.2 nmol TPA in 0.2 ml vehicle. To assess its antiskin-tumor-promoting effect, PFE at a dose of 2 mg/animal was applied topically 30 min prior to each TPA application in different groups. Treatment with TPA alone or PFE + TPA was repeated twice weekly up to the termination of the experiments at 30 weeks. Animals in all the groups were watched for any apparent signs of toxicity, such as weight loss or mortality during the entire period of study. Skin tumor formation was recorded weekly, and tumors larger than 1 mm in diameter were included in the cumulative number only if they persisted for 2 weeks or more. The tumor data are represented as the percentage of mice with tumors (*a*), the number of tumors per mouse (*b*) and the number of tumors per group (*c*). The data were analyzed by Wilcoxon rank-sum test and chi-square analysis.

of the experiment at 30 weeks on test, compared with 5.2 tumors per mouse in non-PFE-treated group of animals, only 1.9 tumors per mouse in PFE-treated group were recorded (Fig. 10*c*). Compared with the non-PFE-treated group, such decrease in the num-

ber of tumor per mouse in the PFE-treated group correspond to 64% inhibition.

Discussion

Cancer chemoprevention has become an important area of cancer research, which, in addition to providing a practical approach to identifying potentially useful inhibitors of cancer development, also affords excellent opportunities to study the mechanisms of carcinogenesis.[2,6,43,44] One excitement of chemoprevention is that agents can be targeted for intervention either at the initiation, promotion, or progression stage of multistage carcinogenesis. The intervention of cancer at the promotion stage appears to be most appropriate and practical. The major reason for this relates to the fact that tumor promotion is a reversible event at least in early stages and requires repeated and prolonged exposure of a promoting agent.[1,45] For this reason, it is important to identify mechanism-based effective novel antitumor-promoting agents. It is appreciated that those agents, which have the ability to intervene at more than one critical pathway in the carcinogenic process, will have greater advantage over other single-target agents. This study was designed to show the chemopreventive potential of PFE by using carcinogenesis-associated biochemical endpoints in a mouse skin tumorigenesis model. The topical application of TPA to mouse skin or its treatment in certain epidermal cells is known to result in a number of biochemical alterations, changes in cellular functions and histologic changes leading to skin tumor promotion.[21,22,46] Our data clearly demonstrate that topical application of PFE prior to TPA application affords significant inhibition of TPA-induced skin edema and hyperplasia (Figs. 1 and 2).

ODC, the first and the rate-limiting enzyme in the biosynthesis of polyamines, plays an important role in the regulation of cell proliferation and development of cancer.[47] Studies with the mouse skin model showed an excellent correlation between the induction of ODC activity and the tumor-promoting ability of a variety of substances.[48,49] Several lines of evidence indicate that aberrations in ODC regulation, and subsequent polyamine accumulation, are intimately associated with neoplastic transformation.[50,51] Elevated levels of ODC gene products are consistently detected in transformed cell lines, virtually all animal tumors and in certain tissues predisposed to tumorigenesis.[50] Agents that block induction of ODC can prevent tumor formation; therefore, its inhibition was shown to be a promising tool for screening inhibitors of tumorigenesis.[52,53] In the present study, topical application of PFE prior to that of TPA resulted in a significant inhibition of TPA-mediated induction of epidermal ODC activity and ODC protein expression (Figs. 3 and 4). It is reasonable to believe that PFE application inhibited the action of the tumor promoter and/or the enzymatic pathway(s) that regulates the ODC induction rather than interacting directly with the enzyme. Extensive data implicating that ODC overexpression is a necessary condition for tumor promotion derive from the studies with retinoic acid[54] and difluoromethylornithine,[55] which are potent inhibitors of ODC induction and ODC enzyme activity, respectively.

COX, an important enzyme involved in mediating the inflammatory process, catalyzes the rate-limiting step in the synthesis of prostaglandins (PGs) from arachidonic acid.[56,57] There are 2 isoform of COX, designated as COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and appears to be responsible for maintaining normal physiologic functions. COX-2 plays an important role in cutaneous inflammation, cell proliferation and skin tumor promotion.[58,59] COX-2 has been implicated in carcinogenesis process and its overexpression has been shown to enhance cellular invasion, induce angiogenesis, regulate antiapoptotic cellular defenses and augment immunologic response through production of PGE_2.[58–60] Tumor promotion is closely linked to inflammation and oxidative stress, and it is likely that compounds that have antiinflammatory and antioxidative properties act as antitumor promoters as well.[61] There is a considerable body of compelling evidence that inhibition of COX-2 expression or activity is important for not only alleviating inflammation, but also for prevention of cancer. Topical application of TPA produces epidermal hyperproliferation, including COX-2 induction, associated with epidermal hyperplasia.[21,34] The results of the present study demonstrate the inhibitory effect of PFE against TPA-mediated induction of epidermal COX-2 protein expression (Fig. 4). This inhibition of COX-2 also correlates with the inhibitory effect of PFE against TPA-mediated induction of skin edema (Fig. 1) and hyperplasia (Fig. 2). These studies suggest that the primary effect of PFE may be against inflammatory responses, which may then result in inhibition of tumor promotion.

MAPKs constitute a superfamily of proteins that include ERK1/2, JNK1/2 and P38 kinase.[23,44] The involvement of MAPKs pathway in tumor proliferation is well documented. Activation of the MAPKs pathway occurs in response to integrin-mediated cellular adhesion to the extracellular matrix, which plays a critical role in both tumor metastasis and angiogenesis.[62,63] In the present study, employing Western blot analysis and immunohistochemistry, we found that topical application of TPA resulted in a marked increase in the phosphorylated form of ERK1/2, JNK1/2 and p38 protein expression (Figs. 5–8). Importantly, topical application of PFE prior to TPA application was found to inhibit TPA-mediated phosphorylation of MAPKs (Figs. 5–8). Several studies have shown that JNK pathway plays a major role in cellular function, such as cell proliferation and transformation,[64] whereas the ERK pathway suppresses apoptosis and enhances cell survival or tumorigenesis.[65] Studies have shown that ERK1/2 and p38 are involved in the transcriptional activation of NF-κB.[66,67]

In the present study, we further investigated the effect of PFE on the pattern of NF-κB activation and its nuclear translocation by TPA in CD-1 mouse skin. NF-κB has emerged as one of the most promising molecular targets in the prevention of cancer. NF-κB resides in the inactive state in the cytoplasm as a heterotrimer consisting of p50, p65 and IκBα subunits. An IκBα kinase, IKK, phosphorylates serine residues in IκBα at position 32 and 36.[68] Upon phosphorylation and subsequent degradation of IκBα, NF-κB activates and translocates to the nucleus, where it binds to DNA and activates the transcription of various genes.[68,69] Several lines of evidence suggest that proteins from the NF-κB and IκB families are involved in carcinogenesis. In the present study, we have demonstrated that topical application of TPA to mouse skin resulted in activation and nuclear translocation of NF-κB (Fig. 9). We also found that TPA application to mouse skin resulted in an increased expression of IKKα and phosphorylation and degradation of IκBα protein (Fig. 9). Interestingly, we observed that topical application of PFE prior to TPA application to mouse skin inhibited TPA-induced NF-κB and IKKα activation and phosphorylation and degradation of IκBα protein (Fig. 9). Because PFE inhibited IκBα phosphorylation and degradation, this study suggests that the effect of PFE on NF-κB/p65 is through inhibition of phosphorylation and subsequent proteolysis of IκBα. Studies have also shown that NF-κB activity affects cell survival and determines the sensitivity of cancer cells to cytotoxic agents as well as ionizing radiation.[70]

The preapplication of PFE to that of TPA showed substantially reduced tumor incidence and lower tumor body burden when assessed as total number of tumors per group, percent of mice with tumors and number of tumors per animal, as compared to animals that did not receive PFE (Fig. 10). These chemopreventive and antitumor promotion observations in murine skin by PFE can be explained by the biochemical mechanisms examined in the present study. These data provide highly useful information for further study to elucidate the exact nature of the compounds present in PFE responsible for chemopreventive effects.

In summary, our results suggest that topical application of PFE prior to TPA application to CD-1 mice resulted in a significant decrease in skin edema, hyperplasia, epidermal ODC activity and protein expression of ODC and COX-2, classical markers of inflammation and tumor promotion. In addition, our results also

suggest that topical application of PFE prior to TPA application also resulted in inhibition of phosphorylation of MAPKs, activation of NF-κB/p65 and IKKα and degradation and phosphorylation of IκBα. Our data clearly demonstrate that PFE could be a potent antitumor-promoting agent because it inhibits several biomarkers of TPA-induced tumor promotion in an *in vivo* animal model. One might envision the use of chemopreventive agents such as PFE in an emollient or patch for chemoprevention or treatment of skin cancer. Because PFE is capable of inhibiting conventional as well as novel biomarkers of TPA-induced tumor promotion, it may possess chemopreventive activity in a wide range of tumor models. Therefore, an in-depth study to define active agent(s) in PFE capable of affording antitumor-promoting effect in various tumor model systems is warranted.

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