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Inhibitory effects of oligonol on phorbol ester-induced tumor promotion and COX-2 expression in mouse skin: NF-κB and C/EBP as potential targets

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ABSTRACT

Plant polyphenols possess anti-oxidant and anti-inflammatory activities and are hence potential candidates for preventing cancer. The present study was aimed at evaluating the anti-inflammatory and anti-tumor promoting activity of oligonol, a formulation of catechin-type oligomers, in mouse skin stimulated with a proto-type tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Pretreatment of mouse skin with oligonol significantly inhibited TPA-induced expression of cyclooxygenase-2 (COX-2). Oligonol diminished nuclear translocation and DNA binding of nuclear factor-kappaB (NF-κB) via blockade of phosphorylation and subsequent degradation of IκBα in TPA-treated mouse skin. Moreover, oligonol suppressed TPA-induced DNA binding of CCAAT/enhancer-binding protein (C/EBP) in mouse skin. Oligonol pretreatment also attenuated the phosphorylation and/or catalytic activities of extracellular signal-regulated protein kinase-1/2 (ERK1/2) and p38 mitogen-activated protein (MAP) kinase. Moreover, p38 MAP kinase inhibitor SB203580, but not the MEK inhibitor U0126, negated TPA-induced DNA binding of C/EBP. In addition, oligonol reduced the incidence and the multiplicity of papillomas and squamous cell carcinomas in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated and TPA-promoted mouse skin, and prolonged the survival of tumor-bearing mice. Pretreatment with oligonol diminished the levels of proliferating cell nuclear antigen and expression of COX-2 in papillomas and carcinomas, respectively, as compared to DMBA plus TPA treatment alone. Taken together, the above findings suggest that oligonol inhibits TPA-induced COX-2 expression by blocking the activation of NF-κB and C/EBP via modulation of MAP kinases and suppresses chemically induced mouse skin tumorigenesis.

1. Introduction

Inflammation plays a critical role in the promotional stage of carcinogenesis [1,2]. Intracellular signaling network, especially those involved in mediating inflammatory response, often functions abnormally during carcinogenesis. Therefore, the normalization of inappropriately amplified inflammatory signaling cascades might be a rational approach for achieving chemoprevention [3,4]. Accumulating data from preclinical studies suggest that a wide variety of anti-inflammatory phytochemicals present in our regular diet can exert anti-tumor promoting activity [5,6].

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One of the key molecules in the inflammatory signaling is cyclooxygenase-2 (COX-2), which catalyzes the synthesis of a series of prostaglandins (PG) from arachidonic acid [4,7]. Aberrant up-regulation of COX-2 has been frequently observed in various premalignant and malignant tissues [7,8]. It has been shown that *cox-2* overexpressing transgenic mice are highly susceptible to spontaneous skin tumor formation [9], while *cox-2* knock out animals are less prone to experimentally induced tumorigenesis [10]. We have previously reported that topical application of a prototype tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) activates intracellular signal transduction pathways mediated by proline-directed serine/threonine kinases, and their downstream transcription factors, thereby inducing aberrant expression of COX-2 in mouse skin [11].

A wide variety of plant polyphenols have anti-oxidative, anti-inflammatory and chemopreventive properties [12,13]. Several studies have demonstrated that polyphenolic fractions obtained from grape seeds can significantly inhibit the mouse skin tumorigenesis [14,15]. Grape seed-derived polyphenols have also been shown to inhibit the growth of human breast cancer (MDA-MB468) cells in culture [16]. Oligonol, a mixture of catechin-type monomers and proanthocyanidin oligomers derived from grape seeds, possesses anti-oxidant [17,18] and anti-inflammatory properties [19]. Moreover, oligonol protects against β-amyloid-induced oxidative cell death in rat pheochromocytoma (PC-12) cells [20]. We have recently reported that topically applied oligonol inhibited the activation of upstream kinases, the DNA binding of CCAAT/enhancer-binding protein (C/EBP) and activator protein-1 (AP-1), and subsequent expression of COX-2 in HR-1 hairless mouse skin irradiated with ultraviolet B (UVB) radiation [21]. As part of the program evaluating the health beneficial effects of oligonol, we have sought to study the anti-inflammatory and anti-tumor promoting potential of this oligomeric polyphenol in mouse skin. Here, we report that topical application of oligonol suppresses TPA-induced COX-2 expression in mouse skin by blocking the activation of nuclear factor-kappaB (NF-κB) and C/EBP via modulation of extracellular signal-regulated protein kinase-1/2 (ERK1/2) and p38 mitogen-activated protein (MAP) kinase. In addition, oligonol reduces the incidence and the multiplicity of papillomas as well as carcinomas in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated and TPA-promoted mouse skin carcinogenesis, and prolongs survival time of tumor-bearing mice.

2. Materials and methods

2.1. Materials

Oligonol® (>95% purity) was obtained from Amino Up Chemical Co. Ltd. (Sapporo, Japan). DMBA and primary antibody for β-actin were procured from Sigma Chemical Company (St. Louis, MO, USA). TPA was purchased from Alexis Biochemicals (San Diego, CA, USA). Rabbit polyclonal COX-2 antibody was procured from Cayman Chemical Co. (Ann Arbor, MI, USA). Primary antibodies for ERK1/2, phospho-ERK1/2, p38, p50, p65, proliferating cell nuclear antigen (PCNA) and inhibitory kappaB-α (IκBα) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-phospho-p38 MAP kinase antibody was obtained from BD Biosciences (San Jose, CA, USA). Phospho-IκBα antibody was procured from Cell Signaling Technology (Beverly, MA, USA). Anti-rabbit and anti-mouse horseradish peroxidase conjugated-secondary antibodies were products of Zymed Laboratories (San Francisco, CA, USA). Enhanced chemiluminescence (ECL) detection kit and [γ-^{32}P]ATP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

2.2. Animal treatment

Female ICR mice (6–7 weeks) were purchased from Sankyo Laboservice Corporation Inc. (SLC, Tokyo, Japan). The animals were housed in climate-controlled quarters (24 °C at 50% humidity) with a 12 h light/12 h dark cycle. The dorsal side of skin was shaved using an electric clipper, and only those animals in the resting phase of the hair cycle were used in all experiments. Oligonol (1 or 10 mg) was dissolved in 0.2 ml acetone:ethanol (2:1 vol/vol). DMBA (0.2 μmol), SB203580 (4 μmol), and U0126 (4 μmol) were dissolved in acetone:dimethylsulfoxide (85:15 vol/vol). TPA (10 nmol) was dissolved in 0.2 ml of acetone. All materials were applied topically to the dorsal shaved area. The experimental protocols were approved by the Animal Care and Use Committee (ACUC) of Seoul National University, South Korea.

2.3. Western blot analysis

Shaven backs of female ICR mice were topically treated with oligonol (1 or 10 mg/mouse) 30 min before TPA (10 nmol) application, and killed by cervical dislocation either 1 or 4 h later. In other experiments, dorsal skins of mice were treated with TPA (10 nmol) after 30-min of treatment with SB203580 or U0126. For the isolation of epidermal protein from mouse skin, the dorsal skin was excised, fat and dermis were removed on ice, and the collected epidermis was immediately placed in liquid nitrogen and pulverized with mortar and pestle. The pulverized skin was homogenized on ice for 20 s with a Polytron tissue homogenizer in 800 μl of ice-cold lysis buffer [150 mM NaCl, 0.5% Triton X-100, 50 mM Tris–HCl (pH 7.4), 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, and protease inhibitor cocktail tablet]. Lysates were centrifuged at 14,800*g* for 15 min. Supernatant was collected and total protein concentration was quantified by using the bichinconinic acid (BCA) protein assay kit. Cell lysates (30–50 μg protein) were boiled in sodium dodecylsulfate (SDS) sample buffer for 5 min before electrophoresis on 10–12% SDS–polyacrylamide gel. After transfer to polyvinylidene fluoride membrane (Gelman Laboratory, Ann Arbor, MI), the blots were blocked with 5% fat-free dry milk–PBST buffer (phosphate-buffered saline containing 0.1% Tween 20) or 1% bovine serum albumin in TBST (Tris-buffered saline containing 0.1% Tween 20) for 1 h at room temperature. Membranes were incubated for 4 h at room temperature with 1:1000 dilutions of primary anti-

bodies for COX-2, ERK, phospho-ERK, and p38, and for 12 h at 4 C with 1:1000 dilutions of primary antibodies for phospho-p38, IκBα, phospho-IκBα, p65, and p50. Each membrane was probed with primary antibody for β-actin with 1:4000 dilutions for 2 h at room temperature. Blots were washed three times with 1× PBST or 1× TBST at 5 min intervals followed by incubation with 1:5000 dilution of horseradish peroxidase conjugated rabbit or mouse secondary antibodies for 1 h, and again washed in PBST or TBST buffer for three times. The transferred proteins were visualized with an ECL detection kit according to the manufacturer's instructions.

2.4. Preparation of cytosolic and nuclear extracts from mouse skin

The cytoplasmic and nuclear extracts from mouse skin were prepared as described previously [22]. In brief, scraped dorsal skin was homogenized in 800 μl of hypotonic buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. To the homogenates was added 80 μl of 10% Nonidet P-40 (NP-40) solution, and the mixture was then centrifuged for 2 min at 14,000*g*. The supernatant was collected as cytosolic fraction. The precipitated nuclei were washed once with 500 μl of buffer A plus 40 μl of 10% NP-40, centrifuged, resuspended in 200 μl of buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 20% glycerol) and centrifuged for 5 min at 14,800*g*. The supernatant containing nuclear proteins was collected and stored at −70° C after determination of protein concentrations.

2.5. Electrophoretic mobility shift assay (EMSA)

The EMSA for measurement of DNA binding of different transcription factors was performed using a DNA–protein binding detection kit according to the manufacturer's protocol (GIBCO BRL, Grand Island, NY, USA). Briefly, oligonucleotides harboring binding sites for NF-κB (5′-GATCGAG GGGGACTTTCCCAGC-3′) and C/EBP (5′-AGA-GATTGCCTGACGTC AGAGAGCTAG-3′) were labeled with [γ-^{32}P] ATP by T_4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech, Buckinghamshire, UK). The binding reaction was carried out in 25 μl of the mixture containing 5 μl of incubation buffer [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol, and 0.1 mg/ml sonicated salmon sperm DNA], 10 μg of nuclear extracts, and 100,000 cpm of [γ-^{32}P]ATP-end labeled oligonucleotide. After 50 min incubation at room temperature, 2 μl of 0.1% bromophenol blue was added, and samples were electrophoresed through 6% non-denaturing polyacrylamide gel at 150 V in a cold room for 2 h. Finally, the gel was dried and exposed to X-ray film.

2.6. In vitro MAP kinase activity assay

In vitro kinase assays for determining the catalytic activities of ERK1/2 and p38 MAP kinase were performed by using non-radioactive kinase assay kits (Cell Signaling Technology, Inc., Beverly, MA) as described by the protocol provided by the manufacturer. Total epidermal protein extracts from mice treated with TPA in presence or absence of oligonol were prepared as described in Section 2.3. Epidermal tissue lysates (containing 200 μg protein) were incubated with specific immobilized phospho-ERK or phospho-p38 monoclonal antibodies with gentle rocking overnight at 4° C. The beads were washed twice each with 500 μl of lysis buffer and the same volume of kinase buffer [25 mM Tris–HCl (pH 7.5), 5 mM glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$]. The kinase reaction was performed in the presence of 100 μM ATP and 2 μg of Elk-1 (a substrate for ERK kinase assay) or ATF-2 (a substrate for p38 MAP kinase assay) fusion proteins at 30° C for 30 min. The phosphorylation of Elk-1 and ATF-2 was measured by immunoblotting with phospho-Elk1 and phospho-ATF-2 antibodies, respectively.

2.7. Two-stage mouse skin carcinogenesis

Female ICR mice were randomly divided into five groups, each consisting of 25 animals. Mice were treated on their shaven backs with a single topical dose of DMBA (0.2 μmol) dissolved in 0.2 ml acetone:DMSO (85:15 v/v) (group II, III, and IV) or the same volume of solvent (group I and V) alone. One week after initiation with DMBA, animals in group II, III, and IV were topically treated with TPA (10 nmol/0.2 ml acetone) twice a week until termination of the experiment. Oligonol was topically applied 30 min before each TPA treatment to animals in group III and IV at a dose of 1 and 10 mg/mouse, respectively. Animals in group V were treated only with oligonol (10 mg/mouse) twice a week for 40 weeks. Mice in group I served as control, and were treated with vehicle alone. Starting one week following TPA treatment, tumors of at least 1 mm diameter were counted every week till 20 weeks. Ten mice from each treatment group were sacrificed after 20 weeks, and papillomas and surrounding skin tissues were collected for biochemical analysis. Remaining 15 animals in each treatment group received TPA and oligonol till 40 weeks. The incidence and the number of cancerous lesions in each group were monitored and counted on a weekly basis. Animals were sacrificed at 40 weeks later, and skin carcinomas were collected for further biochemical analysis. The results were expressed as the percentage of papilloma- or carcinoma-bearing mice (incidence) and the average number of papillomas or carcinomas per mouse (multiplicity).

2.8. Histology and immunohistochemical analysis

The dissected skin was prepared for immunohistochemical analysis for the expression pattern of COX-2 in mouse skin treated with TPA in presence or absence of oligonol. Collected mouse skin papillomas and carcinomas from different treatment groups were also processed for histological and immunohistochemical analysis. For histological analysis, 4 μm sections of papillomas and carcinomas from the group treated with DMBA plus TPA only and those from the group pretreated with oligonol (10 mg/mouse) were fixed in 10% buffered formalin,

embedded in paraffin, and stained with hematoxylin and eosin. For immunohistochemistry, 4 μm sections of 10% formalin-fixed, paraffin-embedded mouse skin, and papillomas were cut on salinized glass slides and deparaffinized three times with xylene and rehydrated through graded alcohol bath. The deparaffinized sections were heated and boiled twice for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To diminish non-specific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 min. For the detection of PCNA and COX-2, slides were incubated separately with affinity purified rabbit polyclonal PCNA or COX-2 antibody (Santa Cruz, CA, USA) at room temperature for 40 min in Tris-buffered saline containing 0.05% Tween 20

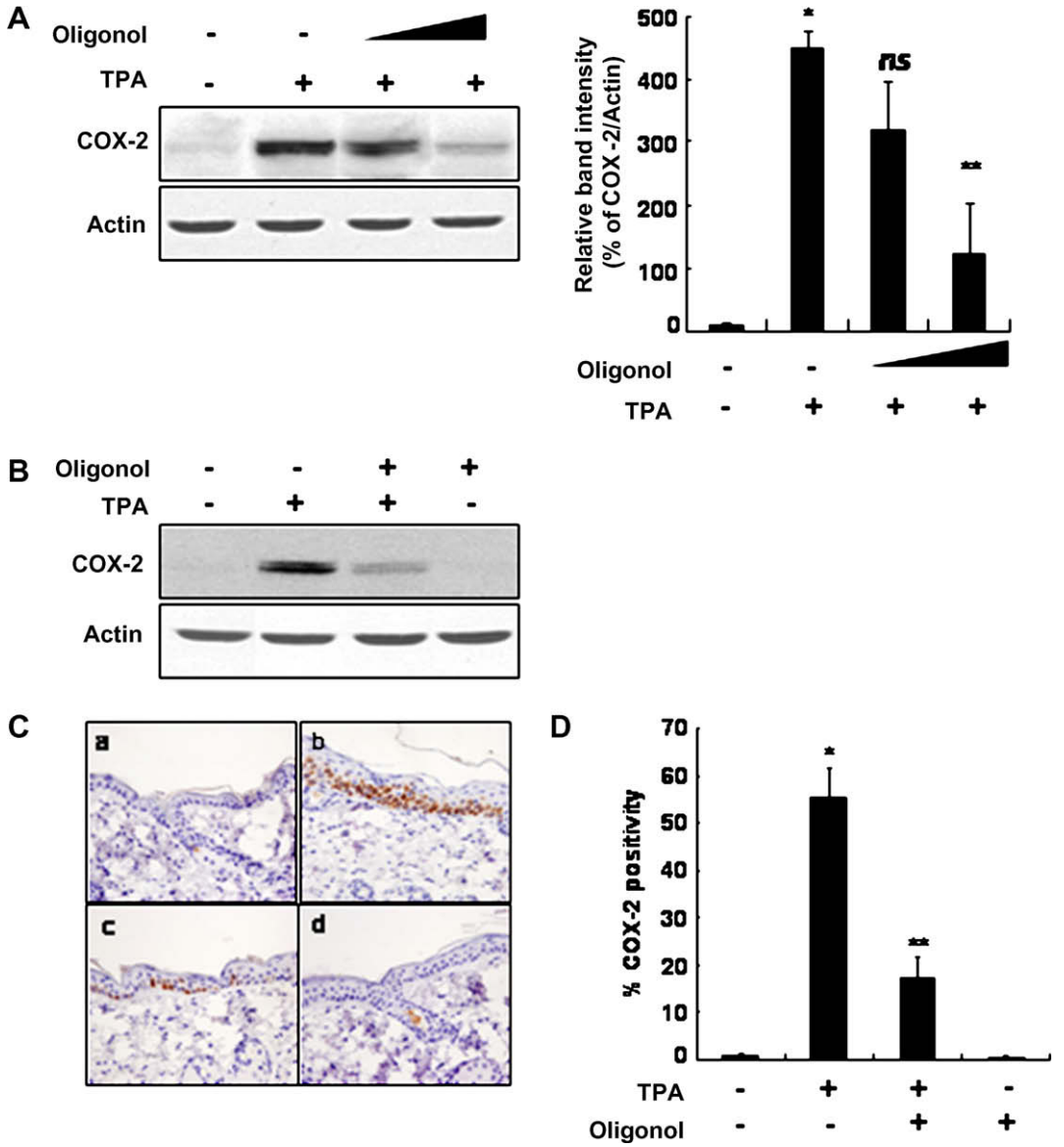

Fig. 1. Inhibitory effects of oligonol on TPA -induced expression of COX-2 in mouse skin. Shaven backs of female ICR mice ($n = 3$ per treatment group) were treated topically with oligonol (1 or 10 mg/mouse). After 30 min, mice were treated topically with TPA (10 nmol). Control animals were treated with acetone in lieu of TPA. (A) Animals were sacrificed after 4 h of TPA treatment and total cell lysates were analyzed for COX-2 expression by immunoblotting. Quantification of COX-2 immunoblot was normalized to that of actin followed by statistical analysis of relative image density. $^{*}p < 0.001$ (control vs. TPA alone), *ns*, not significant (TPA alone vs. 1 mg oligonol plus TPA), $^{**}p < 0.001$ (TPA alone vs. 10 mg oligonol plus TPA). (B) Dorsal skins of female ICR mice were pretreated with oligonol (10 mg/mouse) in the presence or absence of TPA. Control animals were treated only with acetone. Epidermal lysates were subjected to Western blot analysis to examine the expression of COX-2. Data presented are representative of three independent experiments. (C) Mice ($n = 3$ per treatment group) were treated as described in Fig. 1B and immunohistochemical analysis of epidermal COX-2 expression was done. Representative photomicrographs showing positive epidermal COX-2 staining appeared as brown in different treatment groups: (a) acetone alone, (b) TPA alone, (c) oligonol 10 mg/mouse plus TPA, and (d) oligonol 10 mg/mouse only. (D) Quantitative analysis of immunohistochemical data showing percent COX-2 positivity in mouse skin treated with oligonol in the presence or absence of TPA. $^{*}p < 0.001$ (acetone control vs. TPA alone), $^{**}p < 0.001$ (TPA alone vs. oligonol 10 mg/mouse plus TPA).

and then developed using anti-rabbit HRP EnVision™ System (Dako, Glostrup, Denmark). The peroxidase binding sites were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark). Finally, counterstaining was performed using Mayer's hematoxylin.

2.9. Statistical evaluation

Values were expressed as the mean ± SEM of at least three independent experiments. Statistical significance was determined by Student's *t*-test and a *p*-value of less than 0.05 was considered to be statistically significant.

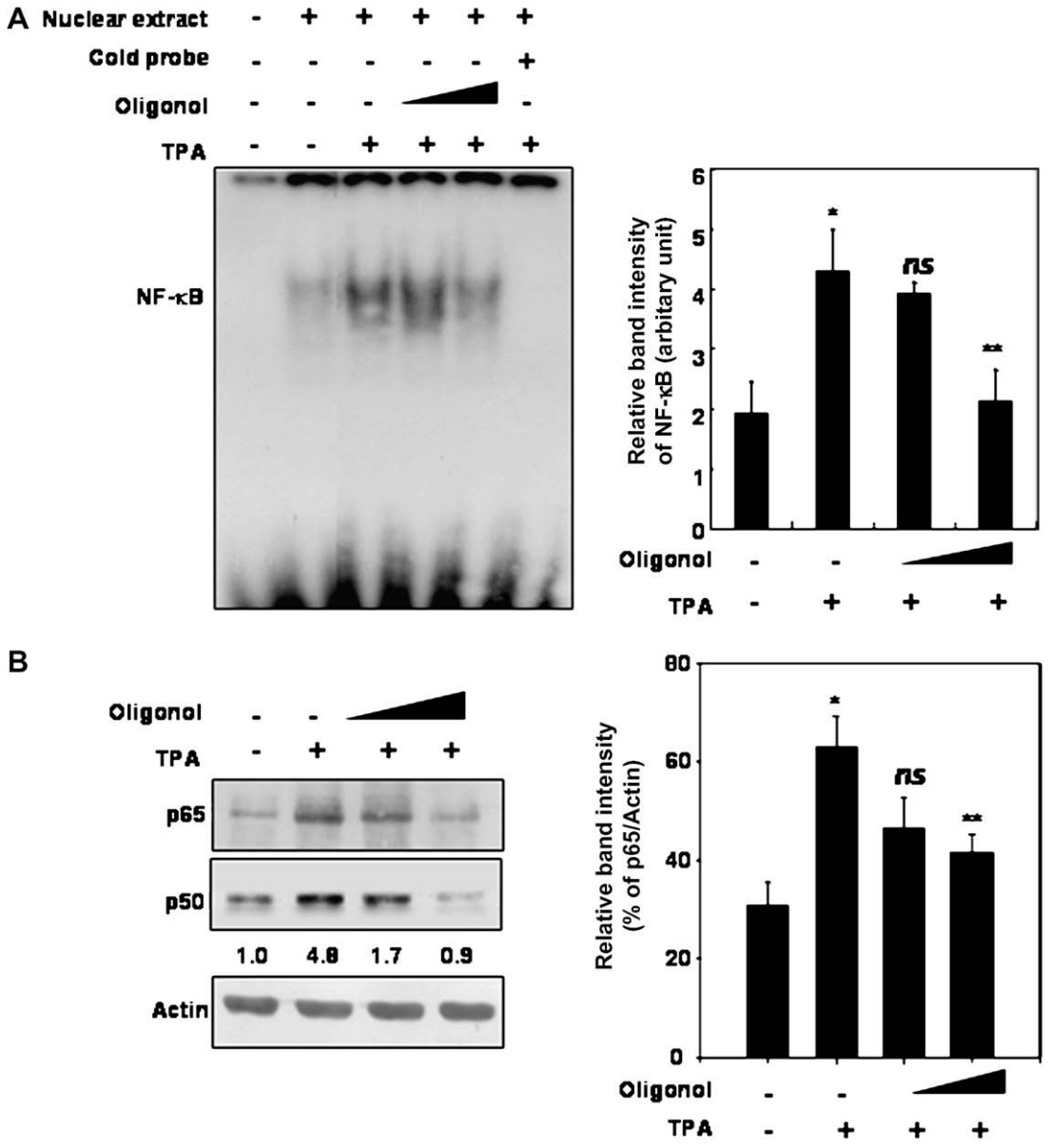

Fig. 2. Inhibitory effects of oligonol on TPA-induced activation of NF-κB and C/EBP in mouse skin. Mice ($n = 3$ per treatment group) were treated as described in Fig. 1 and sacrificed 1 h after TPA treatment. The epidermal cytosolic and nuclear extracts were prepared as described in Section 2. (A) Nuclear extract (10 μg) were incubated with radio-labeled NF-κB oligonucleotide and the DNA-binding assay was done by EMSA. Relative band intensity of NF-κB was quantified and statistical analysis was done. $^{*}p < 0.05$ (control vs. TPA alone), $^{**}p < 0.05$ (TPA alone vs. 10 mg oligonol plus TPA), *ns*, not significant. (B) Nuclear protein (50 μg) was separated by 10% SDS–polyacrylamide gel and immunoblot was performed by using primary antibodies specific to detect p65 and p50 proteins. Quantification of p65 immunoblot was normalized to that of actin followed by statistical analysis of relative image density. $^{*}p < 0.01$ (control vs. TPA alone), *ns*, not significant (TPA alone vs. 1 mg oligonol plus TPA), $^{**}p < 0.01$ (TPA alone vs. 10 mg oligonol plus TPA). (C) Cytosolic extracts from mice treated with acetone, TPA alone, and oligonol (1 or 10 mg/mouse) plus TPA were subjected to western blot analysis to examine the expression of plκBα and IκBα using specific antibodies. Quantification of IκBα immunoblot was normalized to that of actin followed by statistical analysis of relative image density. $^{*}p < 0.01$ (acetone control vs. TPA alone), $^{**}p < 0.05$ (TPA alone vs. 10 mg oligonol plus TPA), *ns*, not significant. (D) Nuclear extract (10 μg) was incubated with radio-labeled C/EBP oligonucleotide and the DNA-binding assay was performed. Data are representative of three independent experiments showing a similar trend.

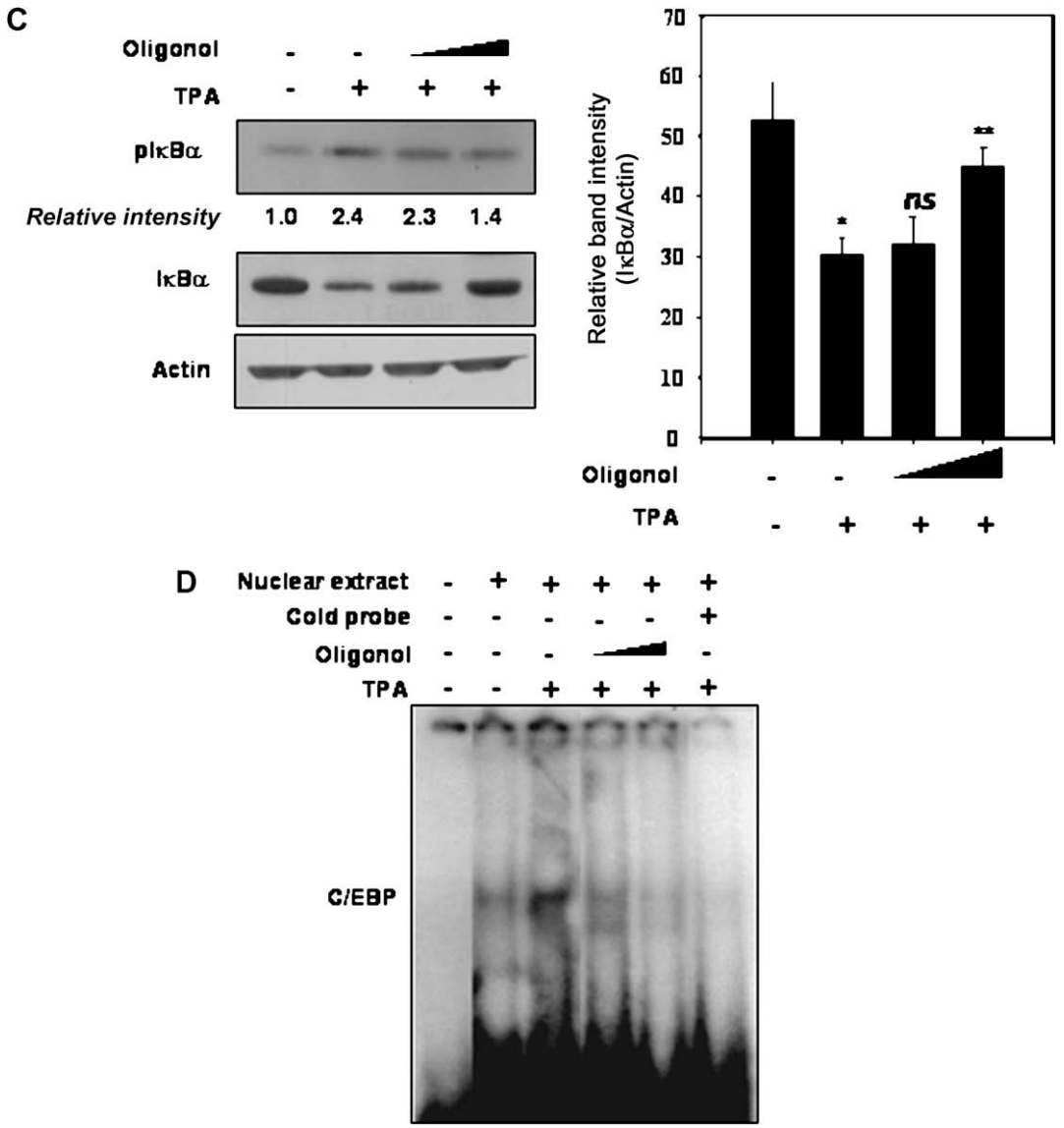

Fig. 2 (*continued*)

3. Results

3.1. Oligonol suppressed TPA-induced expression of COX-2 in mouse skin in vivo

We have previously reported that a single topical application of TPA (10 nmol) onto shaven backs of female ICR mice induces the expression of COX-2 protein maximally at 4 h [22]. In the present study, topical application of oligonol (10 mg/mouse), 30 min prior to TPA application resulted in a statistically significant ($p < 0.001$) inhibition of COX-2 protein expression in mouse skin after 4 h of TPA treatment (Fig. 1A). However, treatment with oligonol at a dose of 10 mg/mouse alone did not induce the expression of COX-2 in mouse skin (Fig. 1B). These findings were confirmed by immunohistochemical analysis, which revealed that TPA-induced epidermal COX-2 expression was diminished by pretreatment of with oligonol (Fig. 1C). Immunohistochemical analysis also revealed that treatment of mouse skin with oligonol alone did not influence constitutive COX-2 expression. As shown in Fig. 1D, pretreatment with oligonol reduced the number of COX-2-positive cells by 69% as compared to TPA treatment alone.

3.2. Oligonol attenuated TPA-induced activation of NF-κB and C/EBP in mouse skin

Since the 5′-flanking region of COX-2 gene promoter contains binding sequences for various transcription factors including NF-κB and C/EBP [23,24], we first attempted to examine the effects of oligonol on TPA-induced DNA binding of NF-κB in mouse skin *in vivo*. Nuclear extracts obtained from TPA-treated mouse skin, with or without oligonol pretreatment, were subjected to EMSA using the oligonucleotide harboring the NF-κB binding sequence. As shown in Fig. 2A, oligonol at a dose of 10 mg/mouse significantly inhibited TPA-induced DNA binding of NF-κB. Moreover, oligonol attenuated nuclear translocation of p65/RelA and p50 proteins, which are the active subunits of NF-κB (Fig. 2B). In addition, the TPA-induced phosphorylation and subsequent degradation of IκBα was blunted by pretreatment with oligonol (Fig. 2C).

Besides NF-κB, the promoter region of COX-2 gene contains binding sequences for another transcription factor C/EBP, which is also known to regulate COX-2 expression [23,24]. As shown in Fig. 2D, pretreatment of mouse skin with oligonol inhibited TPA-induced DNA binding of C/EBP.

3.3. Oligonol suppressed TPA-induced activation of ERK1/2 and p38 MAP kinase in mouse skin

Members of the MAP kinase family proteins are involved in the regulation of TPA-induced activation of transcription factors, such as NF-κB and AP-1, and the expression of COX-2 in mouse skin [22,25,26]. ERK1/2 and p38 MAP kinase have also been reported to activate C/EBP, another transcription factor involved in COX-2 up-regulation, in response to diverse stimuli including bacterial lipopolysaccharides (LPS) in various cultured cell lines [27–29]. We, therefore, examined the effect of oligonol on TPA-induced activation of ERK1/2 and p38 MAP kinase. Pretreatment with oligonol suppressed TPA-induced phosphorylation of p38 MAP kinase, while that of ERK1/2 remained unaffected (Fig. 3A). Topical application of oligonol alone to mouse skin did not affect activation of ERK1/2 or p38 MAP kinase (Fig. 3B). Besides inducing the activation of ERK1/2 and p38 MAP kinase via phosphorylation, topical application of TPA was reported to stimulate the catalytic activities of these enzymes in mouse skin [22]. The non-radioactive kinase assay showed that pretreatment of mouse skin with oligonol suppressed TPA-induced catalytic activities of ERK1/2 and p38 MAP kinase (Fig. 3C).

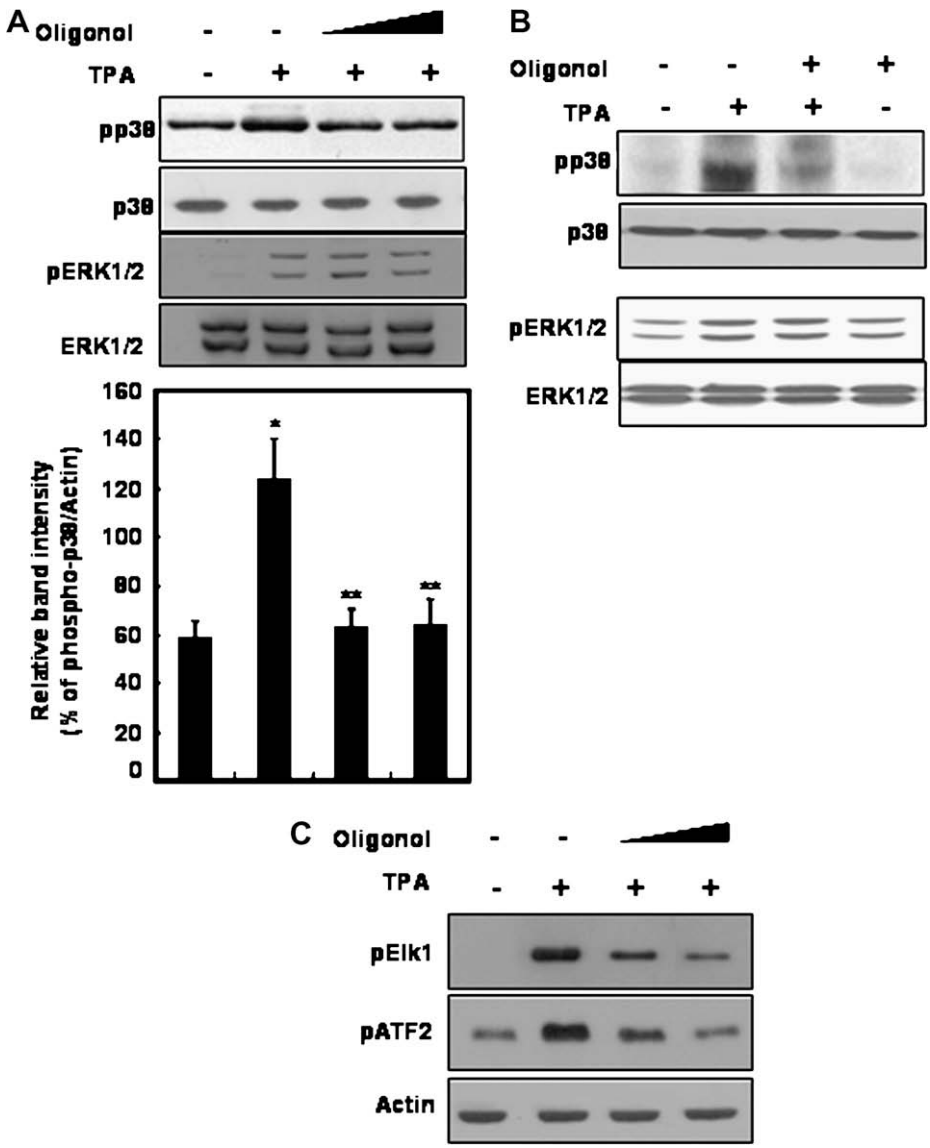

Fig. 3. Effects of oligonol on TPA-induced activation of ERK1/2 and p38 MAP kinase in mouse skin. Shaved backs of female ICR mice ($n = 3$ per treatment group) were treated as described in Fig. 1 and sacrificed 1 or 4 h after TPA treatment. (A) Whole epidermal tissue lysates prepared after 1 h of TPA treatment were analyzed for the expression of ERK1/2, phospho-ERK1/2, p38, and phospho-p38 MAP kinase by immunoblotting. Quantification of phospho-p38 MAP kinase immunoblot was normalized to that of p38 MAP kinase followed by statistical analysis of relative image density. $^{*}p < 0.01$ (control vs. TPA alone), $^{**}p < 0.01$ (TPA alone vs. 1 or 10 mg oligonol plus TPA). (B) Mice ($n = 3$ per treatment group) were pretreated with oligonol (10 mg/mouse) with or without TPA. Control animals were treated with acetone only. Mice were sacrificed 4 h after TPA treatment. Epidermal lysates were subjected to Western blot analysis to detect the expression levels of pERK1/2 and phospho-p38 MAP kinase. (C) Epidermal tissue lysates prepared after 1 h of TPA treatment were subjected to *in vitro* kinase assay for measuring the activities of ERK1/2 and p38 MAP kinase following the protocol described in Section 2.6. Expression levels of pElk1 and pATF2 in different treatment groups indicate the ERK1/2 and p38 MAP kinase activity, respectively. Data are representative of two independent experiments, which gave rise to a similar trend.

3.4. The p38 MAP kinase inhibitor SB203580, but not the ERK inhibitor U0126, attenuated TPA-induced DNA binding of C/EBP in mouse skin

Since the upstream ERK1/2 and p38 MAP kinases have been shown to regulate the activation of C/EBP in cells exposed to LPS [27–29], we attempted to examine whether these enzymes play a critical role in regulating TPA-induced DNA binding of C/EBP in mouse skin *in vivo*. Our study revealed that pretreatment of mouse skin with SB203580 (p38 MAP kinase inhibitor), but not U0126 (ERK inhibitor), abrogated TPA-induced DNA binding of C/EBP (Fig. 4), suggesting a regulatory role of p38 MAP kinase in C/EBP activation in TPA-stimulated mouse skin *in vivo*.

3.5. Oligonol suppressed DMBA-initiated and TPA-promoted mouse skin tumorigenesis and reduced the expression of PCNA in papillomas

The finding that oligonol suppressed TPA-induced COX-2 expression led us to examine its effect on mouse skin tumor promotion. Oligonol (1 or 10 mg/mouse) was applied to the DMBA-initiated dorsal skin of female ICR mice 30 min prior to each topical application of TPA twice a week for 20 weeks. The onset of papillomas in DMBA-initiated mouse skin was evident 7 weeks after TPA treatment, which resulted in a 100% incidence with 20.56 ± 2.38 papillomas/mouse at the 20th week (Figs. 5A and 5C). Pretreatment with oligonol (10 mg/mouse) lowered the multiplicity of papillomas (Fig. 5A). Hematoxylin and eosin staining of papillomas from groups treated with DMBA plus TPA only (Fig. 5B, left) and that from animals pretreated with oligonol (Fig. 5B, right) showed hypertropic squamous epithelium forming papillary fronds. Pretreatment with oligonol (10 mg/mouse) for 20 weeks also lowered the tumor incidence by 20% (Fig. 5C). Mice pretreated with oligonol at a dose of 1 mg/mouse for 20 weeks exhibited a similar incidence of papillomas as that observed in the DMBA plus TPA-treated group (Fig. 5C), but showed a statistically insignificant reduction in the multiplicity (14.96 ± 2.23 papillomas/mouse) (Fig. 5A). It is interesting to note that topical application of oligonol alone at a dose of 10 mg/mouse twice a week for 20 weeks did not produce any visible signs of adverse skin reactions.

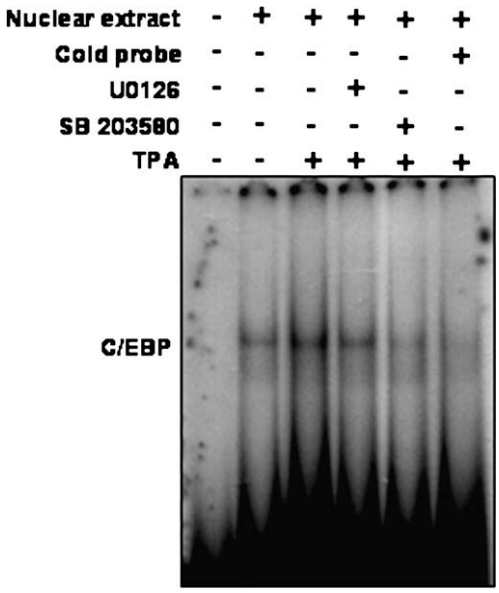

Fig. 4. Effects of pharmacological inhibitors of ERK1/2 and p38 MAP kinase on TPA-induced DNA binding of C/EBP in mouse skin. Mice ($n = 3$ per treatment group) were treated topically with the p38 MAP kinase inhibitor SB203580 (4 μmol) or the MEK inhibitor U0126 (4 μmol) 30 min prior to the topical application of TPA. Control animals were treated with acetone lieu of TPA. Animals were killed 1 h after TPA treatment and epidermal nuclear extract was prepared as described in Section 2. Nuclear extract (10 μg) was incubated with radio-labeled C/EBP oligonucleotide and the DNA-binding assay was done by EMSA. Data are representative of three different experiments showing a similar pattern of C/EBP DNA binding.

One of the biochemical hallmarks of tumor promotion is PCNA, which is overexpressed in mouse skin chronically exposed to diverse stimuli including UVB [30], sodium arsenate [31], and TPA [32]. To examine the effect of oligonol on PCNA expression, papillomas from DMBA plus TPA-treated group and those from oligonol (10 mg/mouse) pretreated mice as well as skin tissues treated with vehicle only were subjected to immunohistochemical analysis for PCNA. As illustrated in Fig. 5D, papillomas from DMBA plus TPA-treated animals showed a marked increase in PCNA expression, which was abrogated by pretreatment with oligonol.

3.6. Oligonol reduced the formation of chemically induced mouse skin carcinomas and prolonged the survival of tumor-bearing mice

The progression of benign tumors into malignant cancer often leads to metastatic spread and eventual death of the afflicted animals. The malignant conversion of DMBA-initiated and TPA-promoted benign papillomas requires further exposure to the tumor promoter [33]. Continued treatment of papilloma-bearing mice with TPA twice a week from 21st week to 40th week resulted in about the 60% incidence of squamous cell carcinomas. Pretreatment with oligonol 1 and 10 mg/mouse delayed the onset of carcinomas by 1 and 3 weeks, respectively. The incidence of carcinomas was lowered by 37% in mice pretreated with oligonol (10 mg/mouse) (Fig. 6A). Skin carcinomas from mice treated with DMBA plus TPA only and those from the group pretreated with oligonol were subjected to histopathological analysis to verify the malignant conversion of the papillomas into carcinomas (Fig. 6B). After 40 weeks, the cumulative number of carcinomas was reduced by 50% in tumor-bearing mice pretreated with oligonol as compared to the DMBA plus TPA treatment alone (data not shown). Immunoblot analysis revealed that the expression of COX-2 was diminished in carcinomas from oligonol pretreated mice as compared to those from the DMBA plus TPA-treated group (Fig. 6C). The survival rate among the papilloma-bearing mice from different treatment groups were also lowered as assessed by Kaplan–Meyer analysis, indicative of a survival benefit conferred by oligonol. While 53% animals belonging to DMBA plus TPA treatment group survived after 40 weeks, 73% and 100% animals in groups pretreated with oligonol at doses of 1 and 10 mg/mouse, respectively, were alive (Fig. 6D).

4. Discussion

Compelling evidence from population- and laboratory-based studies supports that numerous phytochemicals present in our daily diet can prevent various degenerative diseases including cancer [3,6,34]. Recently, food-derived polyphenols have received considerable attention as potential candidates for cancer chemoprevention [35]. The term ‘chemoprevention’ refers to the use of natural or synthetic agents to delay, reverse or retard the multistage carcinogenesis process. Numerous polyphenolic compounds present in edible and medicinal plants have been shown to possess chemopreventive properties. Oligonol is a formulation containing catechin-type monomers and oligomers derived from grape seeds or lychee fruit. Several recent studies have demonstrated the antioxidative and anti-inflammatory potential of oligonol [17–19,21,36]. In the present study, we investigated the anti-inflammatory and anti-tumor effects of oligonol on TPA-stimulated mouse skin carcinogenesis and explored the underlying molecular mechanisms.

Since an elevated expression of COX-2 is causally linked to tumor promotion [7,37], the inhibition of TPA-induced COX-2 expression by oligonol suggests that this oligomeric polyphenol formulation exerts not only anti-inflammatory but also anti-tumor promoting effects in mouse skin *in vivo*. The inhibitory effect of oligonol on the expression of COX-2 in TPA-stimulated mouse skin is in agreement with the results of our recent study demonstrating that

topical application of oligonol inhibits UVB-induced COX-2 expression in HR-1 hairless mouse skin [21].

The TPA-induced COX-2 expression is regulated by different transcription factors including NF-κB [4], AP-1 [38], and C/EBP [23]. Therefore, we examined the effect of oligonol on TPA-induced activation of these transcription factors in mouse skin. Our study revealed that pretreatment with oligonol inhibited TPA-induced DNA-binding of NF-κB and C/EBP. However, oligonol failed to inhibit the AP-1 DNA binding in TPA-stimulated mouse skin (data not shown). The transcription factor NF-κB, predominantly a heterodimer of p50 and p65 proteins, is normally kept

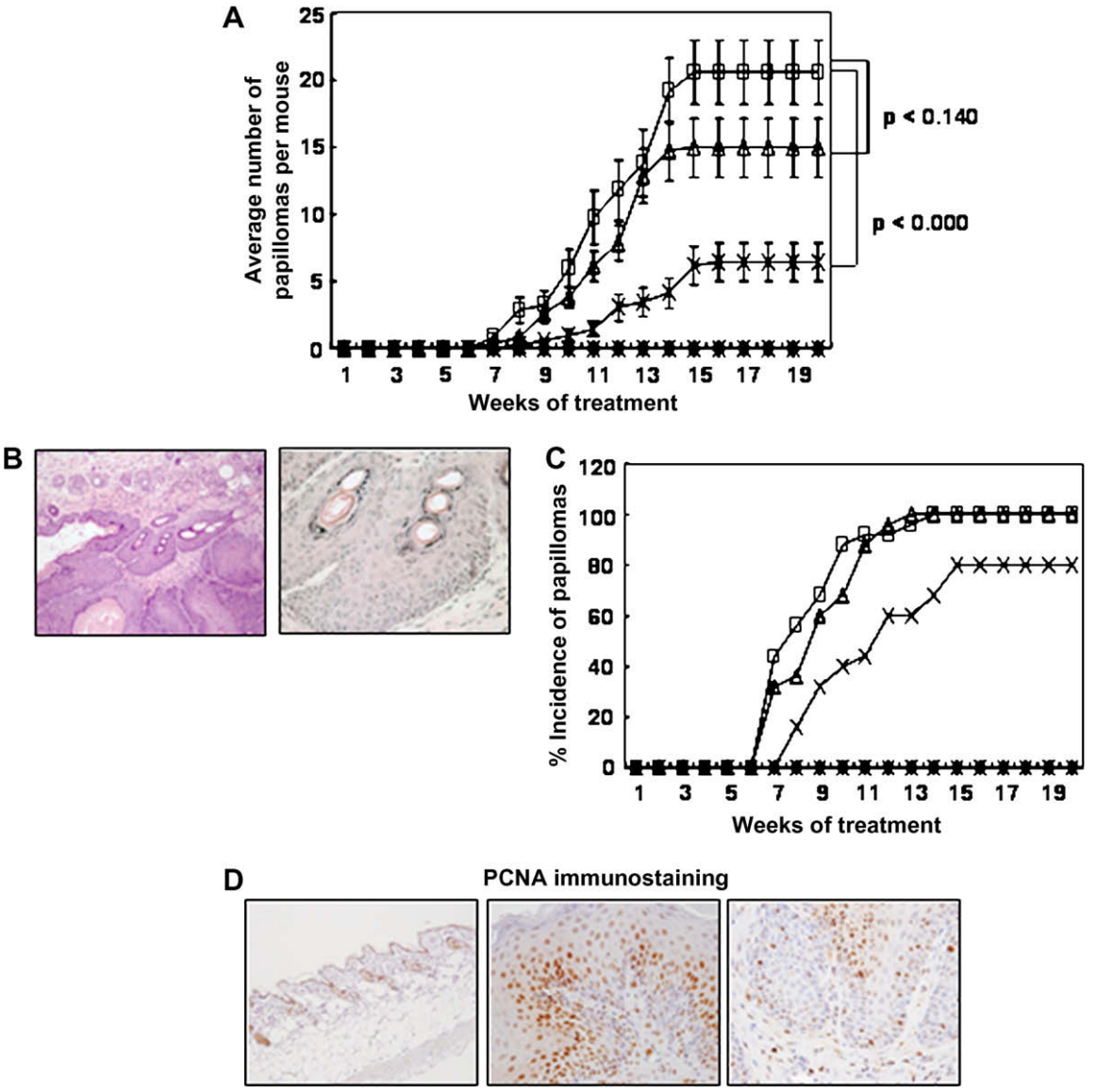

Fig. 5. Inhibitory effects of oligonol on mouse skin tumor promotion. Female ICR mice (n = 25 per treatment group) were first treated with DMBA (0.2 μmol) except those in group I (control group) and V (oligonol only treated group). After one week of DMBA initiation, animals in group II were treated with acetone, and those in group III and IV were treated with 1 and 10 mg oligonol, respectively, 30 min prior to each topical application of TPA (10 nmol) twice a week for 20 weeks. Animals in group I (control animals) were treated with acetone alone, while mice in group V were treated with acetone plus oligonol 10 mg/mouse. Treatment groups: (◇) Acetone only, (□) DMBA plus TPA, (△) DMBA plus 1 mg oligonol plus TPA, (×) DMBA plus 10 mg oligonol plus TPA, and (*) Acetone plus 10 mg oligonol. (A) Comparison of average numbers of papillomas per mouse in different treatment groups. The multiplicity of papillomas in oligonol (10 mg/mouse) pretreated group is significantly lower than that observed in the DMBA plus TPA-treated group ($p < 0.000$). (B) Histopathology of papillomas from DMBA plus TPA treatment group (left) and oligonol (10 mg/mouse) pretreated group (right). Hematoxylin and Eosin staining of respective papillomas showed hypertrophic squamous epithelium forming papillary fronds. (C) The percent incidence of papillomas in different treatment groups: (◇) Acetone only, (□) DMBA plus TPA, (△) DMBA plus 1 mg oligonol plus TPA, (×) DMBA plus 10 mg oligonol plus TPA, and (*) Acetone plus 10 mg oligonol. (D) Effects of oligonol on the expression of PCNA in mouse skin papillomas. After 20 weeks of treatment, sections of normal skin and papillomas (n = 5 for each) were subjected to immunohistochemical analysis using a specific antibody to detect PCNA levels in mouse skin treated with acetone only (left), in papillomas from the DMBA plus TPA-treated group (center) and in oligonol (10 mg/mouse) pretreated group (right). Data are representative of 5 mice from each treatment group.

sequestered in cytoplasm as an inactive complex with its inhibitory counterpart IκBα. In response to diverse pro-oxidative and pro-inflammatory stimuli, IκBα is phosphorylated and subsequently degraded by the ubiquitin-proteasome system, thereby releasing the functionally active NF-κB [39]. Topical application of TPA has been reported to enhance nuclear translocation and the DNA binding of NF-κB in mouse skin via phosphorylation-dependent degradation of IκBα [22]. The phosphorylation of IκBα is mediated by upstream kinases, such as IκB kinase (IKK) [40], ERK1/2 [22] or p38 MAP kinase [26]. In addition, pharmacological inhibition of TPA-induced activation of NF-κB has been shown to abrogate COX-2 expression [4], suggesting the role of NF-κB in regulating TPA-induced COX-2 expres-

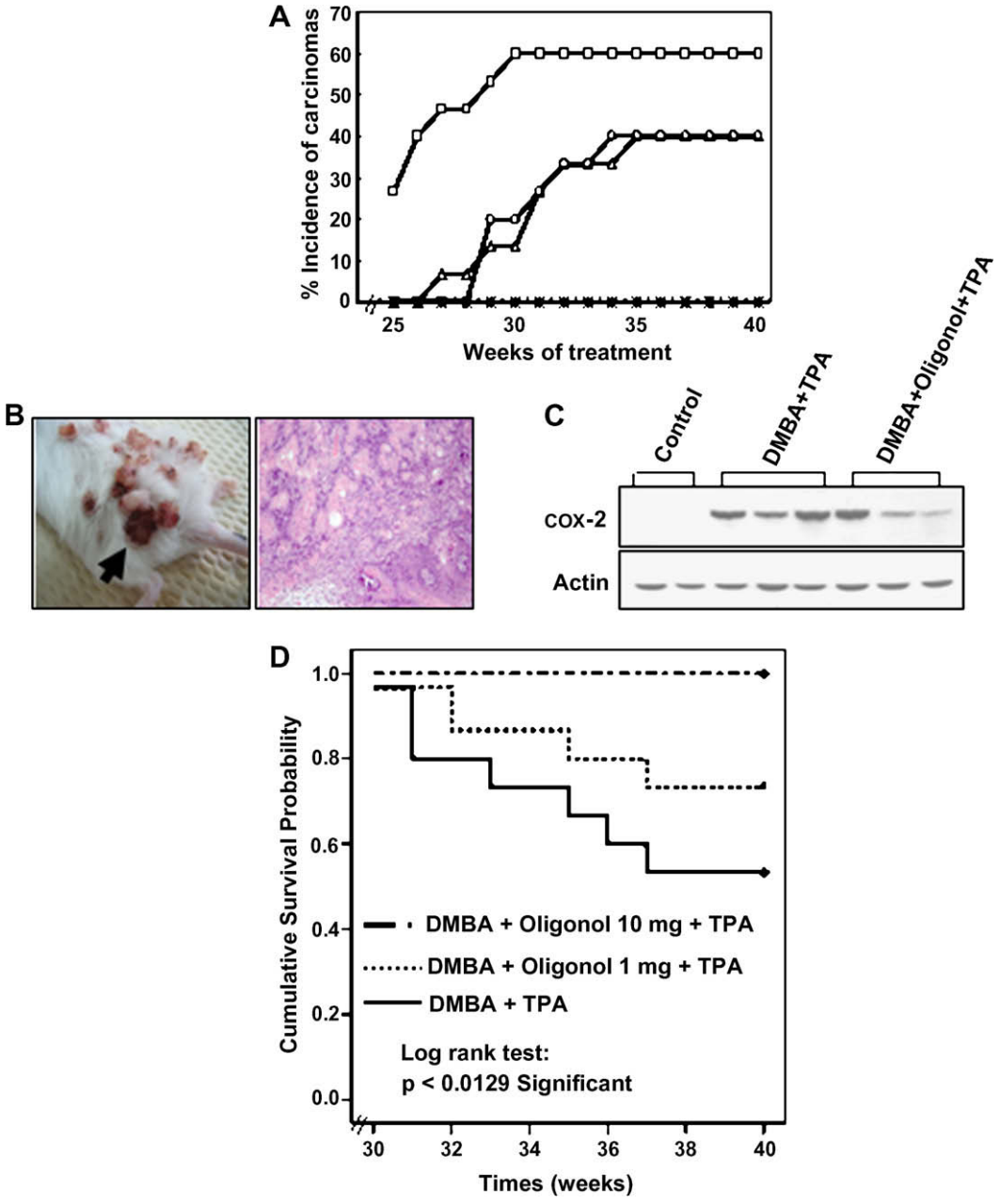

Fig. 6. Effects of oligonol on malignant conversion of papillomas to carcinomas and survival of tumor-bearing mice. Tumor-bearing mice from each group (n = 15 per group) were treated with TPA (10 nmol) twice a week in the presence or absence of oligonol (1 or 10 mg/mouse) till 40 weeks. Mice in group I and V were treated with acetone alone and acetone plus oligonol (10 mg/mouse), respectively. (A) The incidence of carcinoma formation in different treatment groups: (◇) Acetone only, (□) DMBA plus TPA, (△) DMBA plus 1 mg oligonol plus TPA, (○) DMBA plus 10 mg oligonol plus TPA, and (*) Acetone plus 10 mg oligonol. (B) The photographic image of typical mouse skin carcinomas from the DMBA plus TPA treatment group (left), and hematoxylin and eosin stained features of mouse skin carcinomas from the DMBA plus TPA treatment group (right). (C) Lysates from normal epidermis, and skin cancerous lesions from mice treated with DMBA plus TPA in presence or absence of oligonol (10 mg/mouse) for 40 weeks were subjected to Western blot analysis to determine the expression of COX-2. (D) Kaplan–Meyer analysis of survival among tumor-bearing mice.

sion in mouse skin *in vivo*. Although, the present study has revealed the inhibitory effects of oligonol on the nuclear translocation and the DNA binding of NF-κB in TPA-treated mouse skin, a previous study demonstrated that oligonol failed to abrogate NF-κB activation in UVB-stimulated mouse skin [21]. While oligonol has been reported to inhibit UVB-induced AP-1 DNA binding in HR-1 hairless mouse skin, it failed to affect AP-1 activation in TPA-treated ICR mouse skin (data not shown). Oligonol also modulates the activation of MAP kinases differentially in TPA-stimulated ICR and UVB-irradiated HR-1 hairless mouse skin. While oligonol attenuates the catalytic activity of ERK without affecting its phosphorylation in TPA-stimulated ICR mouse skin, topical application of this formulation inhibits the phosphorylation of ERK1/2 without affecting its catalytic activity in UVB-irradiated HR-1 hairless mouse skin [21]. These findings suggest that oligonol targets NF-κB and MAP kinases in a strain- or stimuli-specific manner.

Since pharmacological inhibitors of ERK and p38 MAP kinases have been shown to diminish the activation of NF-κB and the expression of COX-2 in TPA-treated mouse skin [22,26], the suppression of TPA-induced activation of ERK1/2 and p38 MAP kinase as well as NF-κB by oligonol suggests that this low-molecular weight polyphenol formulation targets ERK/p38 MAP kinase-NF-κB signaling in downregulating COX-2 expression in mouse skin. Besides inhibition of MAP kinases, possible inhibitory effects of oligonol on TPA-induced activation of other upstream kinases such as IKK, PI3K/Akt, or protein kinase C (PKC), which also contribute to TPA-induced NF-κB activation and COX-2 expression in mouse skin, merit further investigation.

The transcription factor C/EBP, which consists of three major isoforms C/EBPα, C/EBPβ, and C/EBPδ, plays a regulatory role in the induction of COX-2 by LPS or TPA [41]. All C/EBP isoforms have a common structure, with an N-terminal domain bearing the transactivation sequence and a basic DNA-binding domain, and a C-terminal domain containing a leucine zipper that allows the homo- or heterodimerization of these factors [42]. Since the expression of different C/EBP isoforms is quite cell type- and differentiation stage-specific, it is difficult to generalize the functions of C/EBPs in the regulation of COX-2 transcription. Even the same C/EBP isoform displays opposite effects depending on the cell type [43,44].

Unlike its differential effects on NF-κB and AP-1 activation, oligonol has been shown to attenuate the DNA binding of C/EBP in mouse skin stimulated with either UVB or TPA. We have previously reported that oligonol inhibits UVB-induced expression of C/EBPδ in UVB-irradiated HR-1 hairless mouse skin [21]. Since the expression of C/EBPδ, but not C/EBPα or C/EBPβ, has been shown to be elevated in mouse skin papillomas [23] and LPS-stimulated murine macrophage [28], it would be of further research interest to examine the effects of oligonol on the activation of specific C/EBP isoforms in TPA-treated mouse skin *in vivo*. The activation of C/EBP is regulated by upstream kinases ERK1/2 and/or p38 MAP kinase in cells exposed to LPS [27–29]. The present study provides the first evidence that p38 MAP kinase regulates C/EBP activation in TPA-stimulated mouse skin *in vivo*.

The inhibition of the COX-2 expression and/or activity is valuable for not only alleviating inflammation, but also for prevention of tumor promotion [4,45]. The COX-2 inhibitory effects of oligonol prompted us to examine the anti-tumor promoting potential of this oligomeric polyphenol formulation in mouse skin. The significant decrease in the incidence and the multiplicity of papillomas in DMBA-initiated and TPA-promoted mouse skin suggests oligonol as a potential cancer-chemopreventive agent. Tumor promotion involves clonal expansion of cells that have been transformed as a consequence of either genetic or epigenetic alterations [46].

Recently, Fujii et al. [47] demonstrated that the peak serum concentration of total phenolics attained at 2 h after an oral intake of oligonol (200 mg/day), and a gradual increase in serum polyphenol content was noted in healthy human subjects receiving oligonol (100 mg/day) for six months. The acute toxicity study in mice revealed that the LD_{50} of oligonol is 5.024 g/kg body weight, suggesting the safety of this formulation. Subacute and chronic toxicity studies in healthy volunteers revealed that the intake of oligonol at doses lower than 200 mg/day did not produce any remarkable signs of toxicity and appeared to be safe [47]. Our study also revealed that treatment with oligonol (10 mg/mouse) twice a week for 40 weeks did not produce any noticeable signs of adverse skin reactions, suggesting the safety of oligonol for topical application.

In conclusion, oligonol suppressed TPA-induced expression of COX-2 by inactivating NF-κB and C/EBP by blocking the activation of upstream ERK1/2 and p38 MAP kinases, which provides a mechanistic basis of its anti-inflammatory and anti-tumor promoting activity in mouse skin *in vivo*.

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