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Research Article

Cancer Prevention Research

Synergistic Effects of Combined Phytochemicals and Skin Cancer Prevention in SENCAR Mice

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Abstract

The purpose of our study was to determine the inhibitory effect of combined phytochemicals on chemically induced murine skin tumorigenesis. Our hypothesis was that concurrent topical and dietary treatment with selected compounds would lead to more efficient prevention of skin cancer. We tested ellagic acid and calcium D-glucarate as components of diets, while resveratrol was applied topically; grape seed extract was applied topically or in the diet. The 4-week inflammatory-hyperplasia assay based on the 7,12-dimethylbenz[*a*]anthracene (DMBA)–induced skin carcinogenesis model in SENCAR mice was used. We have found that all the selected combinations caused a marked decrease of epidermal thickness compared with the DMBA-treated group and also with groups treated with a single compound and DMBA. All combinations of resveratrol with other compounds showed a synergistic effect on hyperplasia and Ha-*ras* mutations. Skin tissue of mice receiving the combinations showed decreased cell proliferation and Bcl2 expression; decreased p21, a regulator of cell cycle; and decreased marker of inflammation cyclooxygenase-2. All the selected combinations diminished the DMBA-induced mRNA expression of the CYP1B1 level, and also caused a marked decrease of proto-oncogenes c-jun and c-fos, components of transcription factor activator protein. In conclusion, all combinations showed either additive or synergistic effects and their joint actions allowed for decreasing the doses of the compounds. Especially, resveratrol combinations with ellagic acid, grape seed extract, and other phytochemicals are very potent inhibitors of skin tumorgenesis, based on the suppression of epidermal hyperplasia as well as on the modulation of intermediate biomarkers of cell proliferation, cell survival, inflammation, oncogene mutation, and apoptosis.

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Introduction

The induction of cancer is a multistage process and its stages have been defined experimentally as initiation, promotion, and progression. Carcinogenesis depends on inherited and acquired susceptibility factors, such oncogenes and tumor suppressor genes (1, 2), on exposure to initiation factors, i.e., exogenous and endogenous carcinogens, and on promotion and progression factors. The greatest understanding of the important cellular and molecular events involved in tumor initiation, promotion, and progression has been provided by studies in the mouse skin carcinogenesis model (3, 4). It is well known that a variety of chemical and physical agents can cause skin cancer in rodents and man. Repetitive treatment with known skin carcinogens will lead to skin damage followed by inflammation and regenerative hyperplasia, dysplasia, papillomas, basal, and/or squamous cell carcinomas (3–5).

The mouse skin cancer model has provided an important system not only for studying the mechanisms involved in the various stages of carcinogenesis and for the bioassay of tumor-promoting and carcinogenetic agents, but also for the study of the inhibitors of tumor formation and malignant conversion (5). The studies on the mechanism(s) of antitumor-initiating and antitumor-promoting properties of a variety of naturally occurring phytochemicals suggest that they are very important for the prevention of skin cancer as well as for the prevention of other epithelial cancers in humans (6). The mouse skin cancer model relates very well to other models where squamous cell carcinomas are induced. It is however important to choose for such studies the compounds that act through one or more different mechanisms. The inhibitors may modify the carcinogen activation; enhance phase II enzyme detoxification; modify antioxidant enzymes; prevent oxidative damage to DNA bases and mutations; decrease inflammation, cell proliferation, and hyperplasia; modulate the immune response; and induce apoptosis.

The phytochemicals selected for this study occur in many medicinal herbs and plants. Grape seed extract

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(GSE) is a rich source of one of the most beneficial groups of plant flavonoids, proanthocyanidin oligomers. They exert many health-promoting effects (7), including antioxidant activity (8) or the inhibition of the growth of cancer cells in culture (9). Ellagic acid (ELA) is a phenolic compound presenting a variety of biological activities including potent antioxidant (10), anticancer (11), and antimutagen (12) properties. Resveratrol, a naturally occurring phytoalexin, associated with many health benefits, most notably the mitigation of age-related diseases, including neurodegeneration, carcinogenesis, and atherosclerosis (13, 14). Calcium D-glucarate (CG) is the salt and the commercial form of D-glucaric acid, which occurs naturally in a variety of foods, including broccoli, oranges, and apples. Following oral administration, D-glucarate is converted to D-glucaro-1,4-lactone, which inhibits β-glucuronidase and enhances phase II detoxification (15). Some *in vitro* and animal data suggest that the inhibition of β-glucuronidase may suppress carcinogenesis (16), as well as inhibit the initiation and promotion/progression stages of tumorigenesis (6, 17, 18).

The overall goal of the present study was to determine the effects of synergistic action of the selected natural source phytochemicals used in the combinations in the 7,12-dimethylbenz(*a*)anthracene (DMBA)–induced mouse skin inflammatory hyperplasia assay. Our hypothesis was that concurrent topical and dietary treatment with selected compounds would lead to more efficient prevention of chemically induced murine skin tumorigenesis. Epithelial thickness, bromodeoxyuridine (BrdUrd) index of proliferation, percentage of mice with mutation in 61 codon of Ha-*ras*, and expression of a panel of intermediate biomarkers that are reliable indicators of cell proliferation and survival, apoptosis, and inflammation were analyzed by reverse transcription-PCR (RT-PCR) and immunohistochemical analyses.

Materials and Methods

Natural source phytochemicals and chemicals

Powdered GSE was purchased from the Kalyx, Inc. It was standardized to yield 95 mg (95%) proanthocyanidins. The proanthocyanidins extract described by Llopiz et al. (19), which obtained from *Vitis vinifera* grape seeds, consisted of 21.3% monomers, 17.4% dimers, 16.3% trimers, 13.3% tetramers, and 31.7% higher polymers. The concentration of the proanthocyanidin extract was calculated by taking a mean molecular weight of 1,399 and was used in our studies to provide micromolar concentration of GSE. Resveratrol, ELA, and CG as well as other chemicals used in this study were obtained from Sigma-Aldrich and were of analytic grade or the highest grade available. DMBA, used as the complete carcinogen for appropriate control and experimental groups, was also obtained from Sigma-Aldrich; dosing solution was prepared using high-purity acetone and was subject to concentration analysis before use.

Animals

Five-week-old female SENCAR mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center, and were placed into quarantine for 1 wk before the beginning of the carcinogen treatment. Mice were housed in groups of five under conditions of constant temperature and humidity, and were maintained on a 12-h light/dark cycle with *ad libitum* access to food and water. All animal procedures were done in accordance with the NIH Guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Carcinogen and phytochemical administration

At 6 to 7 wk of age, the backs of the mice were shaved. The dose of 100 nmol DMBA dissolved in acetone (200 μL) was applied twice weekly and the selected inhibitors were applied in a total volume of 200 μL of acetone, either topically 20 min before DMBA or were given continually in the diet, beginning 2 wk before the first dose of DMBA. The inhibitors and DMBA were given twice weekly for 4 wk. Two days after the last treatment, mice were sacrificed and samples of skin were collected for immunostaining and DNA and RNA isolations. CG and ELA were incorporated in the AIN-93G diet (Dyets, Inc.) at the concentrations ranging from 1% up to 4% of the test compounds in diets. Resveratrol was applied topically (2.5 and 5 μmol per dose) to the dorsal surface of the mice. GSE (a major antioxidant being proanthocyanidin B-2-gallate) was applied topically (1 and 2.5 μmol per dose) or in the AIN-93G diet (2 and 4%). When diets and topical treatments were applied in combinations, we have used low doses of compounds, i.e., 2.5 μmol resveratrol with 2% CG, 1% ELA, or 2% GSE diet, and 1 μmol GSE (see above for the molecular weight calculation) with 2% CG or 1% ELA diet.

Tissue preparation

Mice were sacrificed by carbon dioxide suffocation and skin samples were collected from the dosed area of each animal. Sections specified for histologic evaluation were fixed in 10% neutral-buffered formalin overnight and were transferred to 70% ethanol the next day for paraffin embedment using standard procedures. Skin sections (5 μm) were cut on a microtome and mounted on polylysine–treated glass slides until processing for immunohistochemistry. Two thirds of the dorsal skin tissues were frozen using liquid nitrogen for RNA/DNA extraction.

Histologic evaluation

The tissues were prepared for histologic evaluation by using conventional paraffin sections and H&E staining. Approximately 1 cm^2 of each skin was preserved in formalin for immunohistochemistry. Epithelial thickness was determined, using an Olympus microscope B45 (Olympus Corp., Leeds Instruments, Inc.), from at least 20 randomly selected sites in formalin-fixed skin samples. For proliferative index analyses, mice were given an i.p. injection of

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BrdUrd (1.5 mg per mouse) 60 min before sacrifice. The tissue sections were immunostained with anti-BrdUrd antibody (Lab Vision Co.). Proliferative indexes were calculated as the mean percentage of basal layer keratinocytes having BrdUrd-incorporated nuclei. The number of BrdUrd-positive cells within the basal cells layer was determined from at least 20 randomly selected sites. For the evaluation of inflammation and transcription activity, we have used an anti–cyclooxygenase-2 (COX-2) antibody obtained also from Lab Vision Co. and anti–c-jun antibody from BD Transduction Laboratories.

Extraction of RNA

Total RNA from the mice skin was extracted using TRI Reagent (MRC, Inc.) according to the manufacturer's protocol. The RNA was resuspended in 100 μL of diethylpyrocarbonate-treated water and stored at −80°C until further use. The RNA concentration was determined from the absorbance at a wavelength of 260 nm (by using an OD_{260} unit equivalent to 40 μg/mL of RNA).

Reverse transcriptase reaction and real-time PCR

cDNA was synthesized using 1 μg RNA, previously treated with DNase I (Sigma-Aldrich), with the combination of specific reverse primers using the cMaster RT kit (Eppendorf North America) according to the manufacturer's protocol. The reaction mixture was incubated at 50°C for 60 min and was terminated by heating at 80°C for 10 min. The resultant cDNA was stored at −80°C until further use.

Primer sets of 5-CTCAGACACCAGAGTGC-3 and 5-GACAGTGAGCAGTTGCG-3 for p21, 5-ATCCTGCCAGCTCCACCG-3 and 5-TGGTCAAATCCTGTGCTCATACAT-3 for COX-2, 5-GATGTGGAGCAACTTGGAAT-3 and 5-AGCTCTCCACTTGCAGAAAA-3 for PCNA, 5-CTCGTCGCTACCGTCGTGACTTCG-3 and 5-CAGATGCCGGTTCAGGTACTCAGTC-3 for Bcl-2, 5-CAGATGCCGGTTCAGGTACTCAGTC-3 and 5-CTCCTTCTCCAGCATGGGC-3 for c-fos, 5-TTGACCCCATAGGAAACTGC-3 and 5-GCTGTCTCTTGGTAGGAGGA-3 for CYP1B1, and 5-CATCCTGGCCTCGCTGTC-3 and 5-CTCGTCGTACTCCTGCTTGGT-3 for β-actin gave the unique products. Standard quantitative RT-PCR was done in triplicate using SYBR Green RealMasterMix (Eppendorf North America) on the Realplex MasterCycler (Eppendorf). cDNA was diluted 1:10 before use in real-time PCR with HotMaster Taq DNA Polymerase. PCR was performed in an Eppendorf Mastercyler *ep*gradient realplex^2 in 96-well microplates using a final volume of 20 μL. Optimum reaction conditions were obtained with 9 μL of RealMasterMix, 250 nmol/L specific forward primer, 250 nmol/L specific reverse primer, and 10 μL template cDNA. Amplifications were done starting with a 2-min template denaturation step at 94°C, followed by 40 cycles of denaturation at 94°C for 20 s, annealing (depending on primers) for 20 s, and extension at 68°C for 20 s. Fluorescence increase of SYBR Green was automatically measured during PCR. RT-PCR cycle thresholds of candidate genes were normalized to control gene. The formula 2 cycle threshold (candidate)/2 cycle threshold (control) was used to calculate the normalized ratios.

Analysis of Ha-*ras* mutations in codon 61

RT-PCRs were carried out by using genomic DNA isolated from freshly frozen skin tissue by using DNAzol (MRC, Inc.) according to the instructions provided by the manufacturer. Detection of CAA→CTA transversion in codon 61 of Ha-*ras* gene was done by DNA amplification using two different sets of primers: the first was able to amplify the wild-type of Ha-*ras* only and the second, with mutated primer, was capable to amplify the mutated DNA only (20). Substitution at the 3′ end, for example in a mutated primer, enables it to anneal only to a sequence with mutation, but in the case of wild-type DNA, it is impossible for polymerase to start DNA synthesis. For RT-PCR, the primers used were as follows: forward primer for both wild-type and mutant Ha-*ras*, 5′-CTAAGCCTGTTGTTTTGCAGGAC-3′; reverse primer for wild-type Ha-*ras*, 5′-CATGGCACTATACTCTTCTT-3′; and reverse primer for mutant Ha-*ras*, 5′-CATGGCACTATACTCTTCTA-3′. PCR was done at 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 58°C for 20 s, and 78°C for 20 s.

Statistical analysis

The results were expressed as means ± SD. For comparison of the differences between the groups, a two-tailed, unpaired, Student's *t* test was used. A P value of <0.05 was considered statistically significant.

Results

Effects of compounds on sustained epidermal hyperplasia

The induction of epidermal hyperplasia after multiple treatments with each compound tested is shown in Fig. 1A. The results are the averages of 20 measurements at various locations along the epidermis of the skin specimen from each treatment group. We have found out that DMBA alone increased epidermal thickness five times from on average of 12.2 ± 0.9 μm in normal mouse skin to 61.1 ± 3.1 μm in DMBA-treated skin. All phytochemicals inhibited hyperplasia in a dose-dependent manner. The most potent inhibition was observed in the group of mice treated with ELA, which reduced the DMBA-induced epidermal thickness by 86% at 2% ELA in the diet. Resveratrol alone had the weakest effect on decreasing DMBA-induced epidermal thickness. Topical GSE showed a good dose-dependent effect, slightly better than the dietary GSE. However, almost all treatments showed statistically significant ($P < 0.05$) inhibitory effects on DMBA-induced hyperplasia.

The treatment of mice with two compounds used together inhibited DMBA-induced epidermal hyperplasia to a greater extent compared with single test compound treatments. A simple equation was used to check if the combined treatments show synergism (21). The data were analyzed by the isobole method for a combination of

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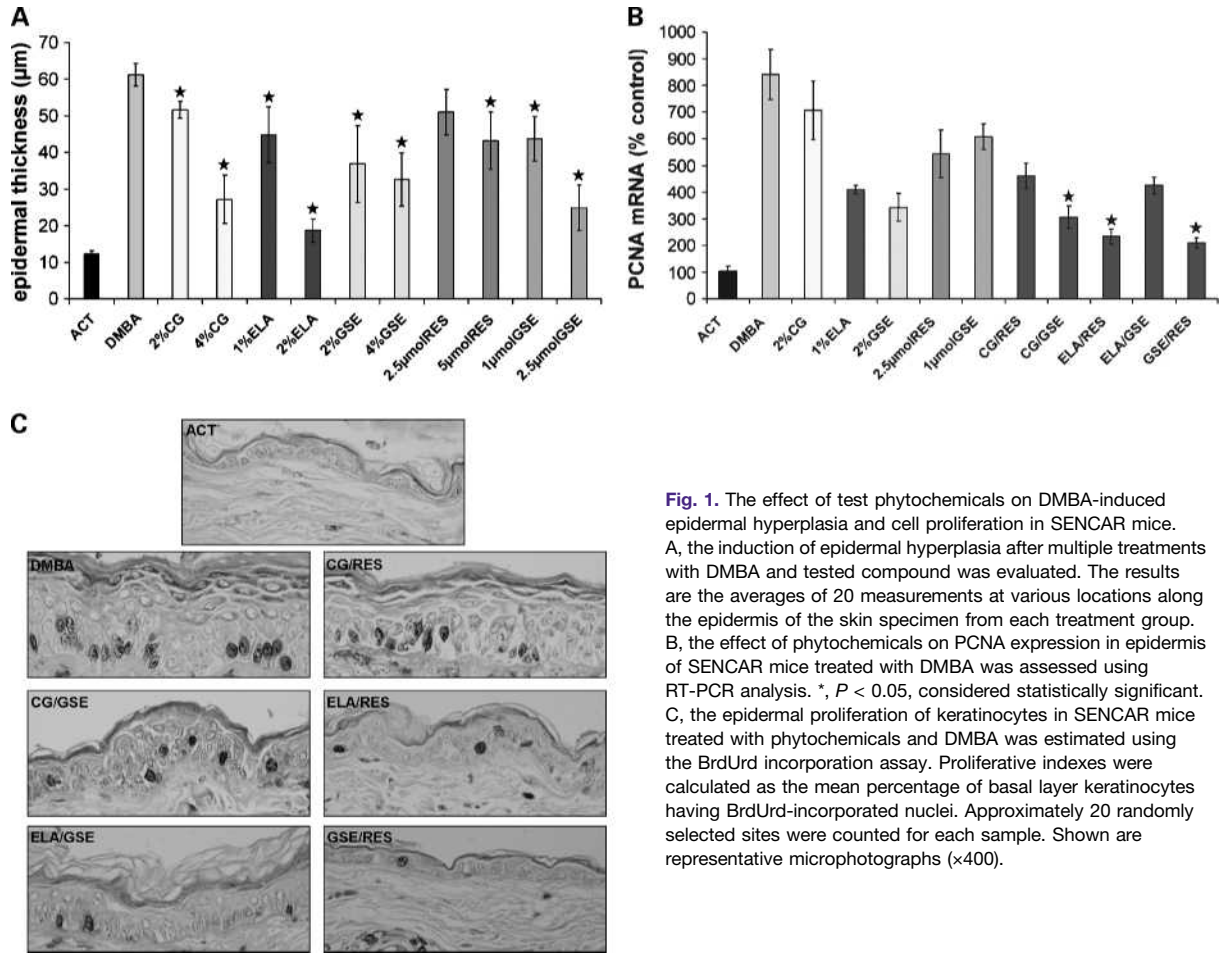

Fig. 1. The effect of test phytochemicals on DMBA-induced epidermal hyperplasia and cell proliferation in SENCAR mice. A, the induction of epidermal hyperplasia after multiple treatments with DMBA and tested compound was evaluated. The results are the averages of 20 measurements at various locations along the epidermis of the skin specimen from each treatment group. B, the effect of phytochemicals on PCNA expression in epidermis of SENCAR mice treated with DMBA was assessed using RT-PCR analysis. *, $P < 0.05$, considered statistically significant. C, the epidermal proliferation of keratinocytes in SENCAR mice treated with phytochemicals and DMBA was estimated using the BrdUrd incorporation assay. Proliferative indexes were calculated as the mean percentage of basal layer keratinocytes having BrdUrd-incorporated nuclei. Approximately 20 randomly selected sites were counted for each sample. Shown are representative microphotographs (×400).

drugs A and B, applying the equation $A_C/A_E + B_C/B_E = D$, where A_C and B_C correspond to concentrations of drugs used in the combination treatment, and A_E and B_E correspond to concentrations of drugs able to produce, by itself, the same magnitude effect. If the combination index D is smaller than 1, the effect of combination is synergistic, whereas if D equals 1 or D is larger than 1, the effect is additive or antagonistic, respectively.

All combinations of resveratrol with dietary compounds showed strong synergistic effects in decreasing DMBA-induced epidermal hyperplasia. For a combination of ELA with resveratrol, D was equal to 0.72, and for a combination of CG with resveratrol, D was equal to 0.75. Dietary GSE in combination with the topically administered resveratrol gave the strongest response, reaching the 93% inhibition of the epidermal hyperplasia ($D = 0.22$), i.e., a decrease in epidermal thickness from 61 μm (DMBA control) to 15 μm. Dietary CG in combination with the topical GSE also had a strong effect on epidermal hyperplasia ($D = 0.76$). The weakest effect was visible for ELA in combination with GSE ($D = 0.97$).

Effect of phytochemicals on cell proliferation in epidermis of SENCAR mice as determined by proliferating cell nuclear antigen expression and the incorporation of BrdUrd

Proliferating cell nuclear antigen (PCNA), an auxiliary protein of DNA polymerase δ that plays a pivotal role in the cell cycle, was reported to be overexpressed in highly proliferating tumors (22). The topical application of DMBA significantly increased the PCNA mRNA level compared with control (Fig. 1B). All treatments significantly reduced the PCNA mRNA and three combinations showed statistically better effects comparing to single treatments. The epidermal proliferation of keratinocytes in SENCAR mice was also estimated using the BrdUrd incorporation assay. Immunohistochemical assessment of tissue sections showed that essentially all of the BrdUrd-positive cells were located in the basal layer of control and treated epidermis (Fig. 1C). Some of the proliferating keratinocytes were also detected in the suprabasal layers. Dietary and topical combinations of two test compounds showed markedly lower levels of proliferative basal keratinocytes

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compared with the control and compounds applied alone. Dietary GSE combined with topically administered resveratrol showed the best results in the proliferation assay. The value of proliferation index was identical as in the negative control (6%).

Analysis of mutations in codon 61 of Ha-*ras* oncogene in skin of SENCAR mice

After the initiation with DMBA *in vivo*, the majority of the papillomas that appear have an A to T mutation in the second position of codon 61 of the Ha-*ras* oncogene (23). This transversion in *ras* oncogene is presumed as an initiating event, triggering multiple signaling cascades, distortion of the cellular balance between proliferation, and apoptosis, eventually leading to progression of *ras*-initiated cells to malignancy (24, 25). The mutation can be detected very early after the initiating treatment. Taking the cognizance of real-time PCR as a very sensitive method recognizing even one copy of substrate, we decided to apply this method for mutation screening. This very precise method allowed for faster searching in a large number of samples. Most of the tested compounds showed only a weak inhibitory effect on the number of mice with Ha-*ras* mutations when used alone (Fig. 2A). However, all combinations were markedly effective in inhibiting Ha-*ras* mutations. The best results were obtained for the following combinations: dietary CG with topical resveratrol (80% inhibition), dietary GSE with topically administered resveratrol, and ELA in diet with topical resveratrol where we observed the complete inhibition of Ha-*ras* mutations. Thus, combinations of the test compounds showed very strong anti-initiation effects simultaneously with the strong antitumor promotion effects, i.e., on DMBA-induced hyperplasia and proliferation.

Effect of phytochemicals on COX-2 expression in murine skin treated with combined inhibitors and DMBA

The hyperplasia in DMBA-treated skin was associated with increasingly higher levels of COX-2 expression, an enzyme associated with inflammation and tumorigenesis (26). As shown in Fig. 2C, epithelia treated with acetone were COX-2 negative. The microphotographs of representative skin specimens, immunostained with anti–COX-2 antibody, showed that all combinations markedly reduced COX-2 expression compared to positive control and they also worked better than single agents (data not shown). Quantitative analysis with RT-PCR showed that only GSE and resveratrol were able to decrease the COX-2 expression level alone, but all combinations reduced the COX-2 mRNA level, and combinations of resveratrol with ELA or GSE reached the negative control level (Fig. 2B).

Effects of phytochemicals on p21 mRNA level in skin treated with DMBA

Real-time PCR analysis of RNA samples isolated from skin tissues treated with DMBA showed the elevated level of cyclin-dependent kinase inhibitor 1A (p21) mRNA, which functions as a regulator of cell cycle progression at G_1 (27). This can lead to terminal differentiation, repair of a damaged DNA with subsequent progress into the S phase, or apoptosis (28).

The p21 level that was increased by repeated doses of DMBA was reduced by ELA, resveratrol, and dietary GSE, but not by topically administrated GSE (Fig. 3A). Among the combination, the treatment of mice skin with the combinations of CG with GSE, and ELA with resveratrol caused a significant decrease of the p21 mRNA level.

Effect of phytochemicals on Bcl-2 expression in murine skin treated with combined inhibitors and DMBA

Bcl-2 is an antiapoptotic protein, and the deregulation of *bcl-2* in oncogenesis leads to the synthesis of a large amount of the Bcl-2 protein, which can lead to uncontrolled cell proliferation (29). In normal skin, single cells of the basal layer were found to express Bcl-2 (30, 31). In neoplastic skin, Bcl-2 has been found in basal cell carcinomas, malignant melanomas, and more seldom in squamous cell carcinomas (31). Topical application of DMBA on murine skin significantly increased the Bcl-2 level, when compared with the negative control (Fig. 3B). Pretreatment with tested compounds caused the decrease in the Bcl-2 mRNA level, but only combinations of resveratrol with ELA or GSE showed a significantly important effect when compared with single treatments.

Effect of phytochemicals on c-jun and c-fos expression in murine skin treated with combined inhibitors and DMBA

Activator protein (AP-1)–dependent gene transcription is required for tumor promotion as was shown in mouse JB6 cells in culture or *in vivo* in mouse skin carcinogenesis (32). In 1993, Brown et al. (33) created a dominant-negative mutant form of c-jun (TAM67), which was then used to confirm this mechanism. TAM67 interacts with any of the three jun or four fos family proteins, inhibiting the transactivational activity of AP-1 and NF-κB. Mice expressing TAM67 are protected from tumorigenesis induced by DMBA and 12-*O*-tetradecanoylphorbol-13-acetate (32). AP-1 has also been shown to regulate the induction of COX-2 (26).

Our results show that twice weekly applications of DMBA for 4 weeks significantly increased the mRNA expression of c-fos in skin compared with that in vehicle-treated animals (Fig. 4A). In fact, the epidermis of DMBA-treated animals showed a significant increase in the protein levels of c-jun as analyzed by immunohistochemistry (Fig. 4B). When used alone, only ELA and resveratrol were able to diminish the DMBA-induced mRNA expression of proto-oncogene c-fos leading to decreased protein levels. On the other hand, all tested combinations significantly decreased the DMBA-induced c-jun and c-fos levels. The c-fos mRNA level after treatment with combinations of resveratrol with GSE and ELA decreased to the negative control level.

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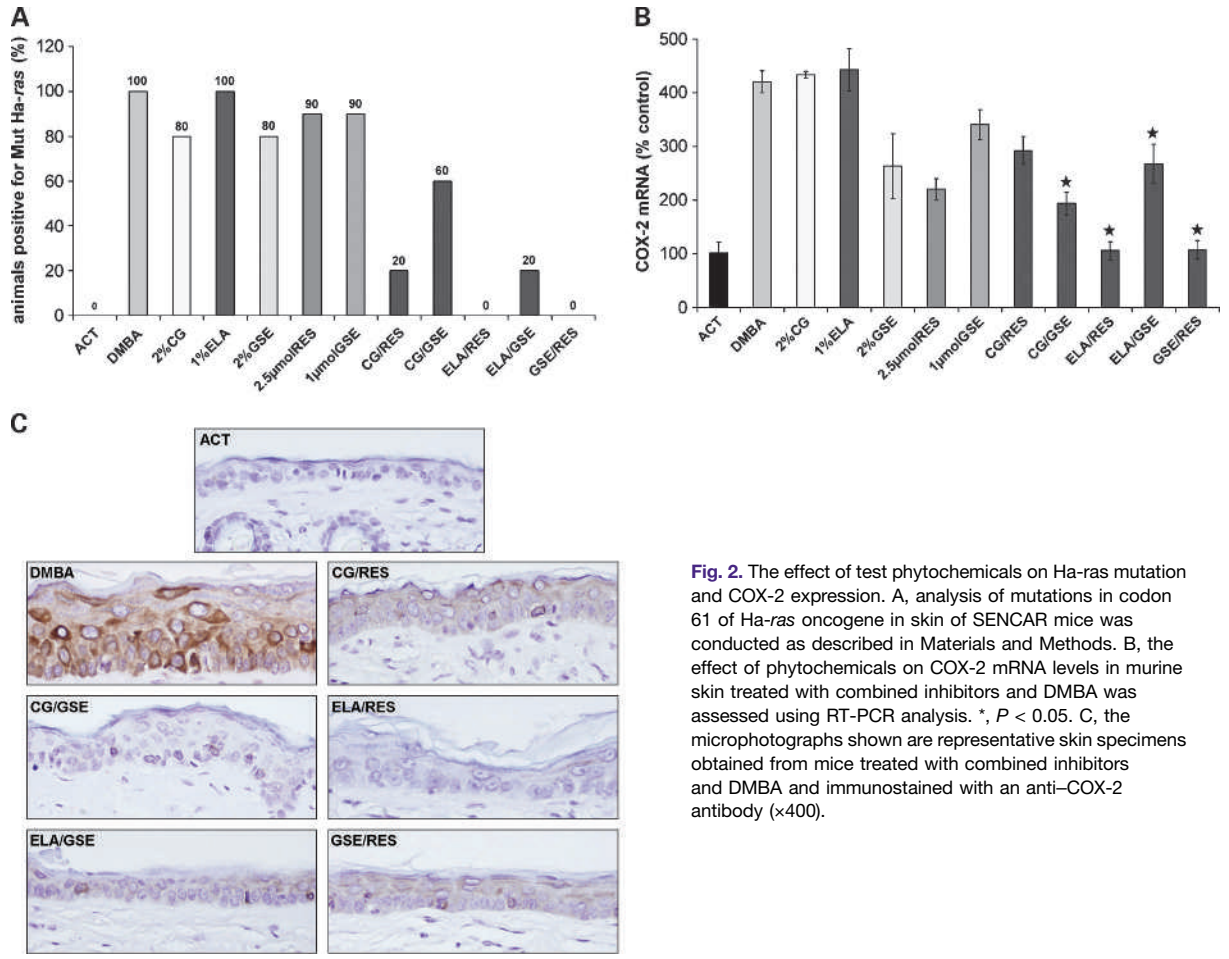

Fig. 2. The effect of test phytochemicals on Ha-ras mutation and COX-2 expression. A, analysis of mutations in codon 61 of Ha-*ras* oncogene in skin of SENCAR mice was conducted as described in Materials and Methods. B, the effect of phytochemicals on COX-2 mRNA levels in murine skin treated with combined inhibitors and DMBA was assessed using RT-PCR analysis. *, $P < 0.05$. C, the microphotographs shown are representative skin specimens obtained from mice treated with combined inhibitors and DMBA and immunostained with an anti–COX-2 antibody (×400).

Effects of phytochemicals on CYP1B1 level in murine skin treated with DMBA

One of the most important factors determining the sensitivity of a tissue to carcinogenesis is the activation of the carcinogenic agent by the cytochrome p450 system. DMBA is a procarcinogen and requires metabolic conversion to its ultimate carcinogenic metabolite by oxidation, which is conducted by CYP450 enzymes. Real-time PCR analysis of RNA samples from skin tissues treated with DMBA showed the 9-fold elevated level of CYP1B1 (Fig. 4C). Not all phytochemicals had a significant effect on this enzyme, but all their combinations were able to modulate the phase I drug metabolizing enzyme CYP1B1. DMBA treatment produces carcinogen-DNA adducts that may induce A → T transversion, and such point mutations in codon 61 are associated with the overexpression of the Ha-*ras* gene in hamster buccal pouch carcinogenesis (34). Combinations of resveratrol with ELA and GSE decreased the expression of CYP1B1 to the control level and caused that no mutations in Ha-*ras* gene were detected in DMBA-treated mice.

Discussion

The present study was designed to evaluate the combined effects of GSE, ELA, resveratrol, and CG on DMBA-induced skin carcinogenesis. Because no single marker can fully explain the mechanism underlying chemoprevention, we analyzed the expression of a panel of markers that are reliable indicators of cell proliferation and survival by RT-PCR and immunohistochemical analyses. In addition, Ha-*ras* mutations were analyzed by RT-PCR in genomic DNA isolated from the skin.

Topical application of DMBA for 4 weeks resulted in epidermal hyperplasia manifested as enhanced thickness due to an increase in cellularity up to 10 cell layers. The hyperplasia in the mouse skin was associated with increased PCNA expression and with BrdUrd labeling indicative of accelerated cell cycle progression and proliferation. We also observed the significant increase in the mRNA levels of p21, Bcl-2, and AP-1 components (c-jun and c-fos), with simultaneous increased expression of the inflammation

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marker COX-2 and the phase I CYP1B1 enzyme in the skin of DMBA-painted mice compared with untreated controls.

We have found that all tested compounds showed dose-dependent effects, and although dietary or topical administration, especially at higher doses, reduced hyperplasia and regulated the expression of some genes, combined administration of studied phytochemicals was more effective. Resveratrol showed the weakest effect when used alone, but unexpectedly, all combinations of resveratrol with other compounds showed a synergistic effect on DMBA-induced hyperplasia and Ha-*ras* mutations. The combination of topical resveratrol and dietary GSE gave the strongest response and decreased the epidermal thickness from 61 μm (DMBA control) to 15 μm (93% inhibition) with no detectable mutations in Ha-*ras*. Combinations of dietary CG or ELA with topical resveratrol reduced the epidermal thickness to 29 μm (65% inhibition) in the CG/resveratrol group and to 25 μm (74% inhibition) in the ELA/resveratrol group. After the CG/resveratrol treatment, one mouse of the group with five mice per group had mutations in the Ha-*ras* gene, but there were no detectable mutations after the ELA/resveratrol treatment.

Combinations of diets with topical treatments significantly decreased cell proliferation (PCNA and BrdUrd indexes) and Bcl-2 expression (Bcl-2 is an antiapoptotic protein and its overexpression can lead to uncontrolled cell proliferation). They also decreased p21, a regulator of the cell cycle. p21 displays a selectivity toward the inhibition of CDK/cyclin complexes that are involved in the transition from the G_1 to the S phase and acts as a tumor suppressor. There is a lot of data that p21 is expressed at a higher level in gliomas, ovarian, or lung cancers, and this high level of p21 protein is not associated with any inhibition of cellular proliferation (35–37). Overexpression of p21 was also associated with malignancy in DMBA-induced ovarian tumors (38).

According to Kim et al. (38), the increased expression of p21 may be the result of a feedback mechanism designed to halt proliferation. p21 is present in the cytoplasm and the nucleus of normal and cancer cells. p21 found in the nucleus could bind to CDK and inhibit kinase activity, whereas p21 found in the cytoplasm could correspond to a pool of inactive p21. In other words, the compartmentalization of p21 in cancer cells could remove its inhibitory effect. Not all our combinations showed inhibitory effect on the p21 mRNA level, but some of them downregulated the expression of the p21 gene. Because p21 can suppress tumors by promoting cell cycle arrest in response to various stimuli, and can also promote cellular proliferation and oncogenicity in many kind of cancers, we did not try to show our combinations as an inducer or inhibitor of p21, but rather as agents of proper control of the mammalian cell cycle.

All combinations also diminished the DMBA-induced mRNA expression of proto-oncogenes c-jun and c-fos, components of the transcription factor AP-1, which can regulate the induction of COX-2, an enzyme associated with inflammation and tumorigenesis. In fact, COX-2 expression was also reduced following treatment with combined inhibitors.

Combinations of the phytochemicals seemed to exert some effect on the modulation of a phase I drug-metabolizing enzyme. All the selected combinations caused a statistically significant decrease of the CYP1B1 level compared with the DMBA-treated group and also to groups treated with a single compound and DMBA. Cytochrome *P*450 enzymes are a superfamily of hemoprotein monooxygenases that catalyze the oxidation of a wide variety of both endogenous and xenobiotic compounds (39). They are involved in the metabolic activation of the polycyclic aromatic hydrocarbons, which are procarcinogens commonly found in our environment (40). We have studied the effect of these phytochemicals on CYP1B1 because the inhibition of CYP enzymes seems

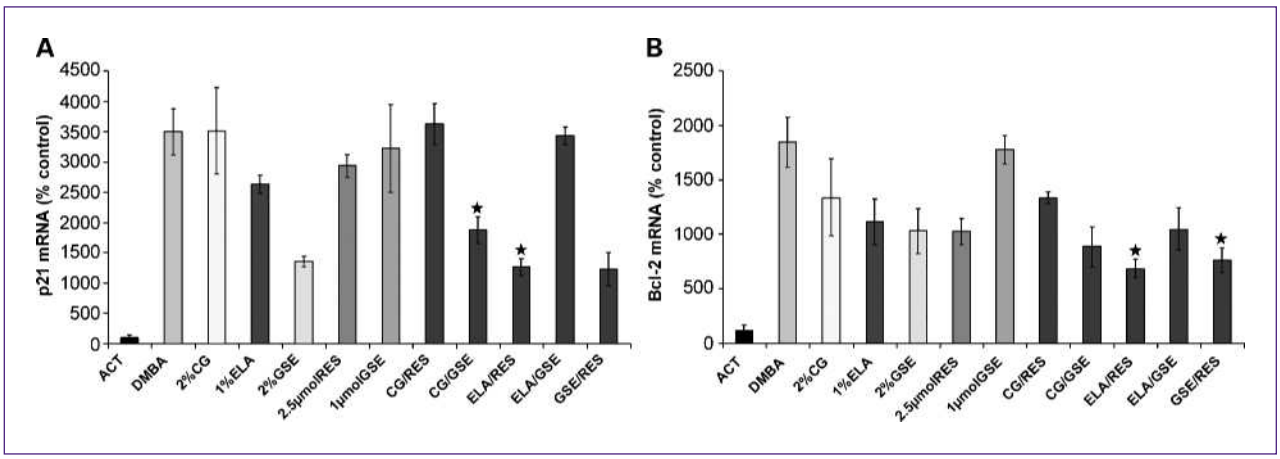

Fig. 3. The effects of test phytochemicals on p21 (A) and Bcl-2 (B) mRNA levels in murine skin treated with combined inhibitors and DMBA.

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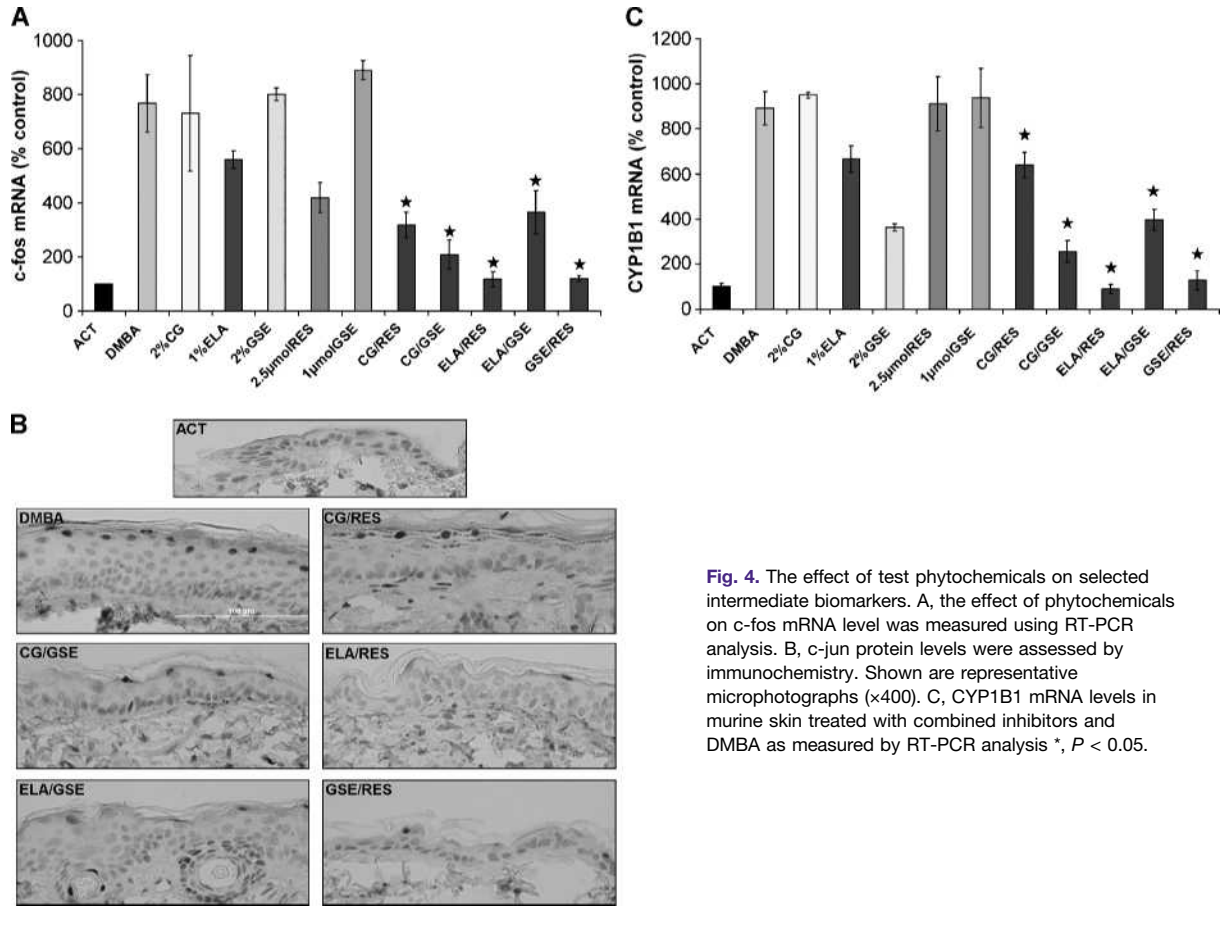

Fig. 4. The effect of test phytochemicals on selected intermediate biomarkers. A, the effect of phytochemicals on c-fos mRNA level was measured using RT-PCR analysis. B, c-jun protein levels were assessed by immunochemistry. Shown are representative microphotographs (×400). C, CYP1B1 mRNA levels in murine skin treated with combined inhibitors and DMBA as measured by RT-PCR analysis *, $P < 0.05$.

to be beneficial in the prevention of DMBA-DNA adduct formation *in vivo* and *in vitro* (41).

In conclusion, all combinations of test phytochemicals showed either additive or synergistic effects. Their joint actions allowed for decreasing the doses of the compounds while they were still showing some synergistic effects. Our results indicate that resveratrol combinations with ELA, GSE, and other phytochemicals are very potent inhibitors of skin tumorgenesis based on the suppression of epidermal hyperplasia as well as on the modulation of biomarkers of cell proliferation, cell survival, inflammation, oncogene mutation, and apoptosis. Combinations of resveratrol with ELA and GSE completely reversed DMBA effect on AP-1 components levels, resulting in a decrease of the p21 level, decreased expression of CYP1B1 (to the acetone control level), and simultaneously, these combinations seemed to prevent mutations in the Ha-*ras* gene.

In summary, our studies on the mechanism(s) of anti-initiating– and antitumor-promoting properties of a variety of phytochemicals suggest that their combinations may be very useful in the prevention of skin cancer and other epithelial cancers in humans, achieving a higher efficacy and potency with reduced toxicity. In particular, combination regimens that use resveratrol as one of the constituents are potentially very effective in chemoprevention. The obtained results suggest that anti-initiation and antitumor properties of topical resveratrol may be significantly augmented by dietary agents such as ELA, CG, or GSE, but the mechanism of synergistic action of these compounds needs further studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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