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Proanthocyanidins inhibit mitogenic and survival-signaling *in vitro* and tumor growth *in vivo*

Syed Musthapa Meeran[1], Santosh Kumar Katiyar[2]

[1]Department of Dermatology, University of Alabama at Birmingham, Birmingham, AL 35294, [2]Department of Dermatology, University of Alabama at Birmingham, Birmingham, AL 35294, and Birmingham VA Medical Center, Birmingham, AL 35294

TABLE OF CONTENTS

1. ABSTRACT

We have previously shown that treatment of human epidermoid carcinoma A431 cells with grape seed proanthocyanidins (GSPs) induces apoptosis of A431 cells. Here, we report that treatment of A431 cells with GSPs inhibits constitutive as well as EGF-induced higher levels of phosphorylated proteins of MAPK family in a dose-dependent manner. This effect is associated with the reactivation of MAP kinase phosphatases. Western blot analysis reveals that GSPs decrease: (i) the levels of phosphatidylinositol 3-kinase (PI3K) and the phosphorylation of Akt at ser^{473}, and (ii) the constitutive activation of NF-kappaB/p65. As NF-kappaB-targeted genes play crucial roles in tumor cell proliferation and differentiation, we assessed the effect of GSPs on proteins encoded by these genes. Treatment with GSPs results in inhibition of the expression of COX-2, iNOS, PCNA, cyclin D1 and MMP-9 in A431 cells compared with non-GSPs-treated controls. Treatment of athymic nude mice with GSPs by oral gavage (50 or 100 mg/kg body weight/mouse) reduces the growth of A431-xenografts in mice, which is associated with the inhibition of tumor cell proliferation in xenografts as indicated by the inhibition of mRNA expression of PCNA and cyclin D1, and of NF-kappaB activity. Together, the data suggest that GSPs might be effective in the treatment of skin cancers.

2. INTRODUCTION

Skin cancer represents a major, and growing, public health problem. The incidence of skin cancer is equivalent to the incidence of malignancies in all other organs combined (1). The constant increase in life expectancy, the depletion in ozone layer which allows more solar ultraviolet radiation to reach at the surface of the Earth, and changing dietary habits and lifestyle appear to be contributing towards the increase in the risk of skin cancer. According to current projections, one in five Americans will develop at least one nonmelanoma skin cancer during their life-time. The estimated cost of treating non-melanoma and melanoma skin cancers in the United States is US dollars 2.9 billion annually (www.cancer.org/statistics). The use of sunscreens does not adequately protect the skin from the risk of cutaneous malignancies (2). Therefore, it is important to develop effective chemopreventive or chemotherapeutic agents and strategies to address this issue.

Grape seeds are byproducts of grapes (*Vitis vinifera*) and are obtained during the industrial production of grape juice and wine. Seeds contain a larger fraction of proanthocyanidins which are primarily composed of dimers, trimers and oligomers of monomeric catechins (3,4). Grape seed proanthocyanidins (GSPs) have been shown to have antioxidant (5,6), anti-mutagenic, anti-

inflammatory and anti-carcinogenic (5, 7-9) properties. GSPs induce cytotoxic effects in various cancerous cell lines (5, 8-10) with no adverse biological effects on normal cells, i.e., human epidermal keratinocytes (11). The anti-skin carcinogenic effects of GSPs have been shown against chemical carcinogenesis (12) as well as in photocarcinogenesis animal models (6). Supplementation of GSPs with experimental diet inhibits UV radiation induced skin carcinogenesis in SKH-1 hairless mice in terms of tumor incidence, tumor multiplicity and tumor growth. Long-term feeding of GSPs supplemented with control diet did not show any apparent signs of toxicity in mice (6). These findings are important because the use of botanicals is constantly increasing by American people for the protection of skin disorders, including skin cancers. In elucidating the molecular mechanism of anticarcinogenic effects of GSPs, we observed that treatment of human epidermoid carcinoma A431 cells with GSPs inhibits the proliferation and induces apoptosis in these cells; however, the exact chemotherapeutic mechanism of GSPs and associated molecular targets in cancer cells are not clearly defined. To identify the molecular targets of GSPs on cancer cells and a clear understanding of the chemotherapeutic mechanism(s) associated with the skin cancer chemotherapeutic effects of GSPs may be useful in developing novel strategies and approaches for its prevention and treatment.

The present study was designed to assess the involvement of phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK)-signaling pathways with GSPs which are considered to play important roles in cellular proliferation, cell cycle progression and apoptosis. The defects in these signaling pathways result in the development of cancer. PI3K catalyses the formation of the 3′-phosphoinositides, phosphatidylinositol 3,4-diphosphate, and phosphatidylinositol 3,4,5-triphosphate. Increase in 3′-phosphoinositides leads to membrane translocation of down-stream effectors such as the serine/threonine protein kinase Akt resulting in increased cellular proliferation and inhibition of apoptosis (13). Similarly, the proteins of MAPK family are well recognized to play an important role in orchestrating intracellular events essential for cell proliferation, growth and apoptosis (14). Primarily three structurally related but functionally distinct MAPK signal transduction pathway have been identified in mammalian cells, which includes extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and the p38 (15,16). These proteins of MAPK family are mediators of signal transduction from the cell surface to the nucleus and play a major role in triggering and coordinating gene expression (17). The controlled activities of MAPK proteins are essential for normal cell growth and differentiation; however, the disruption in MAPK signaling pathways leads to oncogenesis and abnormal apoptosis (17,18). Nuclear factor-kappa B (NF-kappaB) is a well known downstream target of the MAPK signal transduction pathway, and constitutive activation of NF-kappaB has a crucial role in inflammatory diseases, including cancers. Therefore, the signaling pathways leading to the regulation of NF-kappaB activity have become the molecular targets for the chemopreventive or chemotherapeutic agents.

To better understand the chemotherapeutic effect and molecular mechanism along with the possible targets of GSPs against skin cancer, human epidermoid carcinoma A431 cells were selected as an *in vitro* model for the present investigation. We also tested the chemotherapeutic effect of GSPs on the growth of A431 cells *in vivo* using athymic nude mice. Herein we report that treatment of A431 cells with GSPs result in significant inhibition of: (i) constitutive activation of MAPK proteins, PI3K and phosphorylation of Akt, (ii) NF-kappaB pathway and NF-kappaB-targeted genes, and that may lead to the inhibition of cellular proliferation and induction of apoptosis in A431 cells. We also found that administration of GSPs by gavage inhibits the growth of A431-tumor xenograft in athymic nude mice without any apparent sign of toxicity. These results suggest a new chemotherapeutic strategy of skin cancer through the use of a novel dietary botanical agent, i.e. GSPs.

3. MATERIALS AND METHODS

3.1. Antibodies, PCR primers and chemicals

Antibodies for phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}), JNK (Thr^{183}/Tyr^{185}), p38 (Thr^{180}/Tyr^{182}), non-phosphorylated forms of MAPK and the anti-beta-actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies for NF-kappaB, I kappaBalpha, IKKalpha, iNOS, COX-2, cyclin D1, cleaved caspase-3, MMP-9, PCNA, PI3K (p85 and p110), Akt, p-AKT (ser^{473}), antibodies specific to MAP kinase phosphatases (MKP-1, V-15), and the anti-mouse IgG HRP-linked and anti-rabbit IgG HRP-linked secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The protein assay kit was purchased from Bio-Rad (Hercules, CA) and the enhanced chemiluminescence western blotting detection reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The manufacturer-supplied standardized real-time PCR primer pairs for the PCNA (cat. no. PPH00216A) and cyclin D1 (PPH00128A) were obtained from the SuperArray Bioscience Corporation (Frederick, MD). Grape seed proanthocyanidins (GSPs) fraction of grape seed extract was obtained from the Kikkoman Corporation (Noda city, Japan). All other chemicals employed in this study were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO).

3.2. Cell culture condition and treatment procedure

Human epidermoid carcinoma A431 cells were purchased from the American Type Culture Collection (Manassas, VA). The A431 cells were cultured as monolayers in DMEM supplemented with 10% heat inactivated fetal bovine serum, 100 microg/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA), and maintained in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For *in vitro* treatment purpose, GSPs were dissolved in a small amount of dimethyl sulfoxide (DMSO) and thereafter dissolved in PBS (pH 7.4) or cell culture medium. At about 70% confluency, cells were

starved overnight using serum-free medium and then treated with various concentrations of GSPs for desired time period. The final concentration of DMSO in culture medium was not more than 0.2% when cells were treated with GSPs. In GSPs and epidermal growth factor (EGF) combination studies, serum-starved cells were either untreated, or treated with GSPs for 3 h or with optimized dose of EGF (10ng/ml) for 1 h or treated with GSPs for 3 h followed by stimulation with EGF (10 ng/ml) for 1 h. An identical concentration of DMSO (0.2%, v/v) was added to non-GSPs-treated cells which were used as a control. After desired treatments, medium was aspirated, cells were washed with PBS and total cell lysates or nuclear fraction of cells prepared as described previously (10).

3.3. Western blot analysis

Cell lysates (25-50 micro g protein/sample) were resolved over 10-12% Tris-glycine gels and transferred onto a nitrocellulose membrane for western blot analysis, as detailed previously (10,19). The non-specific binding sites on the membranes were blocked with blocking buffer (5% non-fat dry milk, 1% Tween 20 in 20 mM TBS, pH 7.6) for 1 h at room temperature. The membranes were then incubated overnight with specific primary antibodies followed by incubation with the peroxidase-conjugated secondary antibody. After a wash, the protein expression was detected by chemiluminescence using an ECL detection system (Amersham Life Science, Inc.). The density of each band in an immunoblot was analyzed using the Scion Image Program (NIH, Bethesda, MD) and are expressed as the relative numerical values to control after normalization with beta-actin. The values for the non-GSPs-treated control were assigned the value "1" (arbitrary unit) and comparison was then made with densitometry values of other treatment groups. To ensure equal protein loading, each membrane was stripped and reprobed with anti-beta-actin antibodies. Each experiment was repeated at least two times.

3.4. *In vivo* tumor xenograft experiment

Female athymic nude mice of 5-6 weeks old were obtained from the National Cancer Institute (Bethesda, MD) and housed in Animal Resource Facility at University of Alabama at Birmingham in accordance with the Institutional Animal Care and Use Committee guidelines, and provided with sterilized AIN76A diet and water *ad libitum*. To determine the *in vivo* efficacy of GSPs as a chemotherapeutic agent, exponentially growing A431 cells in PBS were mixed in a 1:1 ratio with Matrigel (Becton Dickinson, Bedford, MA), and a 100 µl suspension containing $2x10^6$ cells was injected s.c. on the right flank of each mouse. After 24 h of tumor cell inoculation, animals were randomly divided into three groups and five mice per group. The treatment groups of mice (1^{st} and 2^{nd}) received 50 or 100 mg GSPs/kg body weight of mice in 100 ml of PBS through gavage 5 days a week (Monday through Friday) till the end of the experiment. The third group of mice received 100 µl of PBS by gavage and served as a control. The body weight and the consumption of diet per mouse were recorded on weekly basis throughout the study. Animals were also monitored if they become ill or suffering (lack normal grooming and avoidance behaviors) during the period of entire experiment. Tumor size on each mouse was measured regularly on 3 days interval. At the termination of the study, mice were sacrificed, tumors from control and GSPs-treated groups excised and weighed to record wet weight of each tumor in each group. A part of the tumor was used to prepare tumor lysate or isolate RNA for checking the expression level of proteins of interest using western blot analysis or real-time PCR.

3.5. RNA extraction and quantitative real-time PCR

The total RNA was extracted from the A431-tumor xenograft samples using TRIzol reagent (Invitrogen, CA) following the protocol recommended by the manufacturer. Briefly, tumor samples were homogenized with TRIzol reagent using Teflon homogenizer (Fisher Scientific, PA). The homogenate was centrifuged at 12,000 g for 10 min at 4^oC and the resulting supernatant was mixed with chloroform, and aqueous phase was separated. The RNA was precipitated by mixing the equal amounts of the aqueous phase and the isopropanol. The precipitate was washed with 75% ethanol in DEPC-treated RNAse-free water and stored at -70^oC. The concentration of total RNA was determined by measuring the optical density at 260 nm using Beckman/Coulter DU 530 spectrophotometer. The mRNA expression level of PCNA and cyclin D1 in tumor samples was determined using real-time PCR. For the mRNA quantification, complementary DNA (cDNA) was synthesized using 3 µg RNA through a reverse transcription reaction (iScript^{TM} cDNA Synthesis Kit, BIO-RAD, CA). Using SYBR Green/Fluorescein PCR Master Mix (SuperArray Bioscience Corporation, MD), cDNA was amplified using real-time PCR with a BioRad MyiQ thermocycler and SYBR green detection system (BioRad, CA). Samples were run in triplicate to ensure amplification integrity. Manufacturer-supplied (SuperArray, Bioscience Corporation, MD) specific primer pairs were used to measure the following: PCNA (cat. no. PPH00216A), cyclin D1 (cat. no. PPH00128A), and β-actin (cat. no. PPM02945A). The standard PCR conditions were: 95^oC, 15 min, then 40 cycles at 95^oC, 30 sec; 55^oC, 30 sec; and 72^oC, 30 sec, as recommended by the primer's manufacturer. The expression levels of genes were normalized to the expression level of the β-actin mRNA in each sample. The threshold for positivity of real-time PCR was determined based on negative controls. The calculations for the mRNA expression were made using the cycle threshold (Ct) method. The mean Ct values from duplicate measurements were used to calculate the expression of the target gene with normalization to a housekeeping gene as an internal control (beta-actin), and using the $2^{-\Delta Ct}$ formula.

3.6. NF-kappaB/p65 activity in tumor samples by ELISA

For quantitative analysis of NF-kappaB/p65 activity, Trans^{AM} ELISA kit (Active Motif, Carlsbad, CA) was used following the manufacturer's protocol. Absorbance was recorded at 450 nm with reference taken at 650 nm. The assay was performed in duplicate and the results are expressed as mean ± SD, n=5.

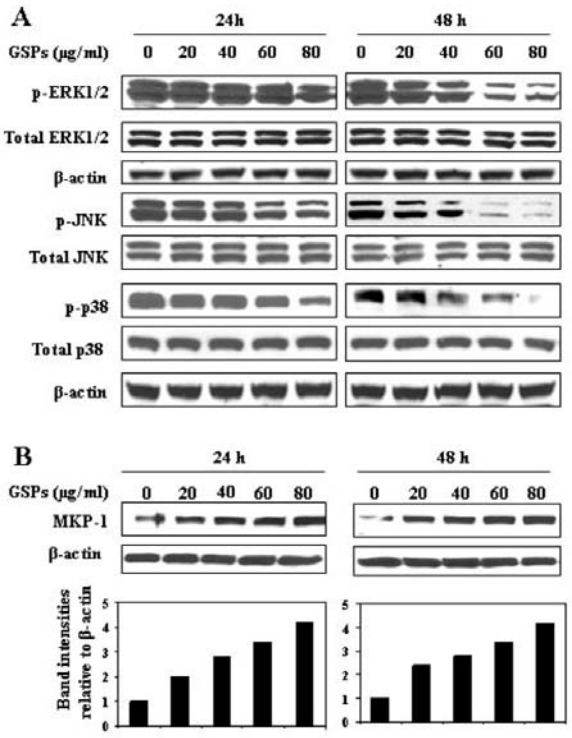

Figure 1. GSPs inhibit the constitutive expression of phosphorylated MAPK proteins, while increase the levels of MAP kinase phosphatases. Panel A, Treatment of human epidermoid carcinoma A431 cells with GSPs inhibits constitutive expression of phosphorylated forms of ERK1/2, JNK and p38 proteins of MAPK family. Marked inhibition was observed at 24 and 48 h after treatment of A431 cells with various concentrations of GSPs. Cells were treated with or without GSPs (0, 20, 40, 60 and 80 micro g/ml) for 24 and 48 h, then harvested and cell lysates prepared to determine the phosphorylated and total protein levels of ERK1/2, JNK and p38 using western blot analysis, as described in the Materials and methods. Panel B, Treatment of A431 cells with GSPs activates MAP kinase phosphatases (MKP-1) in a dose-dependent manner. The A431 cells were treated identically as detailed in Panel A. In each case, a representative blot is shown out of three independent experiments, where identical results were observed.

3.7. Statistical analysis

The results from the *in vitro* studies are representative of at least two or three independent experiments. In tumor xenograft study, the statistical significance of difference between control and GSPs-treated groups was determined by ANOVA and in each case $p<0.05$ was considered statistically significant.

4. RESULTS

4.1. GSPs inhibit constitutive expression of MAPK proteins in A431 cells

Human epidermoid carcinoma A431 cells like many other tumor cells express higher levels of phosphorylation of MAPK proteins compared to normal cells. In order to assess the chemotherapeutic effect of GSPs, we determined whether treatment of A431 cells with GSPs decreases the levels of phosphorylation of these proteins using western blot analysis. As shown in Figure 1A, treatment of A431 cells with GSPs for 24 h resulted in a dose-dependent (20, 40, 60 and 80 micro g/ml) reduction in the levels of phospho-ERK1/2, JNK and p38 proteins of MAPK family. Similar pattern but higher reduction in the levels of phosphorylation of MAPK proteins was observed with GSPs when A431 cells were treated for 48 h. In particular, the levels of phosphorylation of JNK and p38 proteins after the treatment of cells with GSPs at the dose of 80 micro g/ml for 48 h were markedly reduced. Moreover, the total amount of ERK1/2, JNK and p38 proteins remain unchanged at each dose of GSPs used and at each time point studied.

4.2. GSPs reactivate the levels of MAP kinase phosphatases in A431 cells

As MAP kinase phosphatases play a role in regulation of the phosphorylation of MAPK proteins (20), we determined the effect of GSPs on the levels of ERK1/2, JNK and p38-specific MAP kinase phosphatases in A431 cells. The cells were treated with various doses of GSPs for 24 and 48 h, and cell lysates were subjected to western blot analysis. The antibodies against MAP kinase phosphatases (MKP-1) have dual-specificity directed to phosphothreonine and phosphotyrosine residues within MAP kinases. Western blotting and subsequent analysis of the density of bands relative to beta-actin revealed that treatment of A431 cells with GSPs (20, 40, 60 and 80 micro g/ml) for 24 h resulted in upregulation of the levels of MAP kinase phosphatases specific to MAPK proteins (*e.g.*, ERK, JNK and p38) dose-dependently compared to non-GSPs-treated control cells (Figure 1B). Similar pattern was also noted when cells were treated with various concentrations of GSPs for 48 h.

4.3. GSPs inhibit EGF-induced phosphorylation of MAPK proteins in A431 cells

It has been shown that EGF-induces the activation of EGFR which leads to the phosphorylation of MAPK proteins. The translocation of phosphorylated proteins (*e.g.*, ERK1/2) to the nucleus activates transcription factors for cell growth and proliferation (21,22). Based on the inhibitory effects of GSPs on the phosphorylation of MAPK proteins in A431 cells, we further examined the effect of GSPs on EGF-induced activation of MAPK proteins in the present system. For this purpose A431 cells were either stimulated with EGF for 1 h, or treated with GSPs (60 and 80 micro g/ml) for 3 h followed by stimulation with EGF for 1 h. Cell lysates were prepared for the detection of the levels of phosphorylation of MAPK proteins. Western blot analysis revealed that treatment of A431 cells with EGF markedly enhanced the levels of phosphorylation of ERK1/2, JNK and p38 proteins compared to non-EGF-treated cells. (Figure 2A). The treatment of A431 cells with GSPs (60 and 80 micro g/ml) markedly inhibited EGF-induced phosphorylation of ERK1/2, JNK and p38 proteins compared with the cells which were only treated with EGF and not with GSPs (positive control).

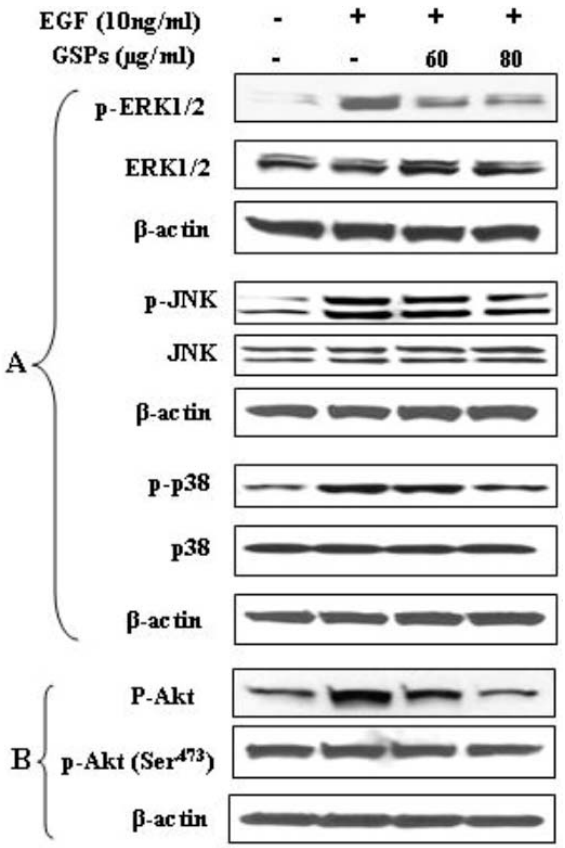

Figure 2. Effect of GSPs on EGF-induced activation of MAPK and Akt in A431 cells. Cells were pretreated with GSPs (60 micro g/ml) for 3 h followed by treatment with EGF (10 ng/ml) for 1 h or left untreated with GSPs, EGF alone or untreated with GSPs and EGF. Cells were harvested 1 h after EGF treatment and lysates were prepared to determine the levels of phosphorylated and total protein levels of MAPK (Panel A) and Akt (Panel B) proteins using western blot analysis, as described in Materials and methods. The representative blots are shown from three repeated experiments.

Further in this system, we checked the stimulatory effect of EGF on Akt protein of cell survival pathway, and determined whether the treatment of A431 cells with GSPs inhibits EGF-induced effects on phospho Akt. As shown in Figure 2B, treatment of A431 cells with EGF stimulated the phosphorylation of Akt compared to non-EGF-treated control cells. Moreover, the treatment of A431 cells with GSPs (60 and 80 micro g/ml) markedly inhibited EGF-induced phosphorylation of Akt protein compared with the cells which were only treated with EGF. The observed changes in phospho-specific MAPK and Akt proteins were not because of changes in total ERK1/2, JNK, p-38 or Akt proteins as the levels of individual total proteins remain constant (panels just below the phospho-specific proteins). And again the equal loading of samples was confirmed using beta-actin.

4.4. GSPs inhibit PI3K/ Akt phosphorylation in A431 cells

Because of the critical role of PI3K/Akt pathways in the development of cancers, we examined the effect of GSPs on this cell survival pathway in A431 cells. A key downstream effector of PI3K is the serine-threonine kinase Akt, which in response to PI3K activation phosphorylates and regulates the activity of a number of targets including kinases, transcription factors and other regulatory molecules. Activation of Akt modulates many downstream effectors for survival as well as antiapoptotic responses. To investigate the effect of GSPs on this pathway in A431 cells, cells were treated with various concentrations of GSPs (0, 20, 40, 60 and 80 micro g/ml) for 24 and 48 h, and the levels of PI3K (p85 and p110) and Akt at Ser^{473} were determined in cell lysates using western blot analysis. Western blot analysis revealed that treatment of A431 cells with GSPs for 24 h decreases the levels of phosphorylation of both regulatory (p85) and catalytic (p110) subunits of PI3K and also the phosphorylation of Akt at Ser^{473} in a concentration-dependent manner, as shown in Figure 3. Similar but relatively greater effect of GSPs on the phosphorylation of PI3K and Akt was observed when cells were treated for longer period of time, *i.e.*, 48 h, and at higher concentrations of GSPs (60 and 80 micro g/ml) used. Notably, the levels of total Akt remain unchanged at each dose of GSPs-treated and non-GSPs-treated control (Figures 3 and 2B) cells at 24 h of treatment; however, the levels of Akt was slightly decreased after 48 h of GSPs treatment. The results of beta-actin bands confirm the equal loading of samples.

4.5. GSPs inhibit constitutive expression of NF-kappaB/p65 in A431 cells via inhibitory effect on IKK alpha and decrease in degradation of I kappaB alpha protein

The NF-kappaB/p65 is a downstream target of the MAPK signal transduction pathways. It has been documented that one of the critical events in NF-kappaB activation is its dissociation with subsequent degradation of inhibitory protein I kappaB alpha via phosphorylation and ubiquitination (23). Therefore, we determined whether treatment of A431 cells with GSPs inhibits the constitutive expression or basal level of NF-kappaB/p65 in these cells. Western blot analysis and subsequent measurement of the intensity of bands relative to beta-actin indicated that treatment of A431 cells with various doses of GSPs (0, 20, 40, 60 and 80 micro g/ml) for 24 h resulted in dose-dependent inhibition of activation and translocation of NF-kappaB/p65 to the nucleus compared to non-GSPs-treated control A431 cells (Figure 4). Similar observations were also observed when cells were treated with GSPs for 48 h. The higher levels of IKKalpha have been shown to be essential for the greater activation of NF-kappaB and degradation of I kappaB alpha. The results from the western blot analysis indicated that the constitutive activation level of IKKalpha was higher in the A431 cells compared with the cells which were treated with GSPs for 24 and 48 h. In other words, treatment of A431 cells with various concentrations of GSPs results in a gradual decrease in the levels of IKKalpha in a concentration-dependent manner compared with non-GSPs-treated

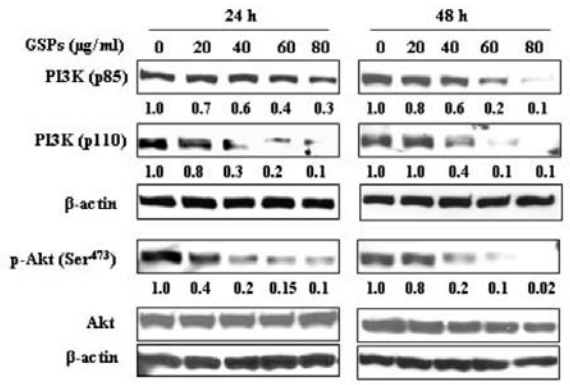

Figure 3. Treatment of A431 cells with GSPs decreases the expression level of PI3Kp85 (regulatory) and PI3Kp110 (catalytic), and phosphorylation of AKT (Ser^{473}) in a dose-dependent manner. As detailed in Materials and methods, the cells were treated with either vehicle (0.2% DMSO in medium) or GSPs (20, 40, 60 and 80 micro g/ml) for 24 h and 48 h and then harvested. Whole cell lysates were prepared and subjected to western blot analysis using specific antibodies. The presented immunoblot is a representative of three independent experiments with similar results. The relative intensity of each band is shown under each immunoblot after normalization for the levels of beta-actin.

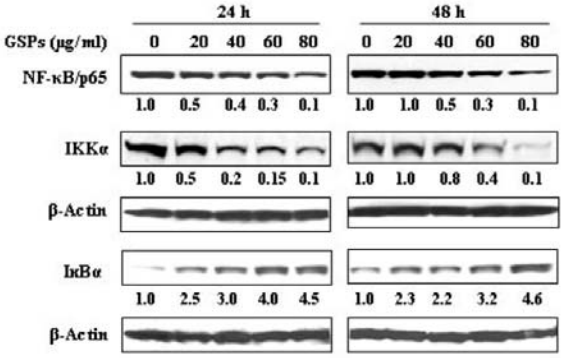

Figure 4. Treatment of A431 cells with GSPs inhibits constitutive activation of NF-kappaB and IKKalpha and degradation of I kappaB alpha in a dose-dependent manner. Cells were treated with GSPs (0, 20, 40, 60 and 80 micro g/ml) for 24 h and 48 h, then harvested and samples were prepared to determine the levels of NF-kappaB, IKKalpha or I kappaB alpha using western blot analysis, as described under Materials and methods. A representative blot is shown from three independent experiments with similar results. The relative intensity of each band after normalization for the levels of beta-actin is shown under each blot.

control cells. In contrast, the degradation of I kappaB alpha was strongly inhibited after the treatment of A431 cells with GSPs in a concentration-dependent manner and that was resulted in enhanced levels of I kappaB alpha after GSPs treatment compared to non-GSPs-treated cells. Similar but comparatively greater effect of GSPs was observed after their treatment for 48 h, as shown in Figure 4. The relative concentration-dependent decrease in the expression of the proteins of NF-kappaB and IKKalpha, and increase in I kappaB alpha after GSPs treatment is indicated by the intensity of bands shown under each immunoblot in terms of fold-change compared to non-GSPs-treated controls.

4.6. GSPs inhibit the expression of NF-kappaB/p65-targeted proteins in A431 cells

We next examined whether GSPs have the ability to influence the constitutive expression of NF-kappaB-responsive proteins, such as the expressions of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), proliferating cell nuclear antigen (PCNA), cyclin D1 and MMP-9 in human epidermoid carcinoma A431 cells. Western blot analysis revealed that treatment of A431 cells with varying concentrations of GSPs (0, 20, 40, 60 and 80 micro g/ml) for 24 h resulted in marked inhibition of constitutive expression of COX-2, iNOS, cyclin D1, PCNA and MMP-9 proteins compared to non-GSPs-treated control cells, as shown in Figure 5. Similar inhibitory effects of GSPs on the constitutive expression of these NF-kappaB-targeted proteins were also observed when cells were treated with GSPs for extended period of time, *i.e.* 48 h. The relative dose-dependent reduction in the expression of these NF-kappaB-targeted proteins after GSPs treatment is indicated by the intensity of bands shown under each immunoblot in terms of fold-change compared to non-GSPs-treated controls.

4.7. Feeding of GSPs by oral gavage inhibits growth of A431-xenografts without any apparent sign of toxicity in athymic nude mice

As we have found that treatment of A431 cells with GSPs decreases the constitutive expression of phosphorylated forms of MAPK proteins, the activation of NF-kappaB and NF-kappaB-targeted proteins, we further extended our study to determine the *in vivo* chemotherapeutic efficacy of GSPs using tumor xenograft mouse model. We determined the effect of GSPs administration by oral gavage on A431-xenograft growth in athymic nude mice. Mice were given 50 mg or 100 mg GSPs/kg body weight of mice by oral gavage which is equivalent to 1 mg or 2 mg of GSPs/20 g body weight of the mouse. The purpose of this study was also to check whether *in vivo* treatment of GSPs as a chemotherapeutic agent has any toxic effect to the animals. For this purpose the changes in body weights and consumption of diet and water per animal in each treatment group was recorded periodically. As can be seen in Figure 6A, the average body weight of the control (non-GSPs-treated) and GSPs-treated mice was almost identical and did not differ significantly throughout the experimental protocol. The daily an average consumption of diet (Figure 6B) or water (data not shown) by the animals in each group was also identical. Moreover, the feeding of GSPs by oral gavage did not exhibit impaired movement and posture or any sign of apparent physical toxicity in mice throughout the experiment for 5 weeks.

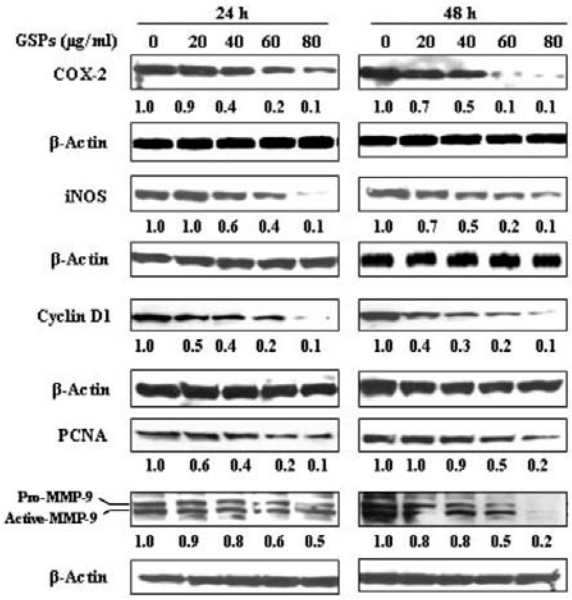

Figure 5. Treatment of A431 cells with GSPs decreases the expression of NF-kappaB-targeted genes, such as, COX-2, iNOS, Cyclin D1, PCNA and MMP-9 in a dose-dependent manner. Cells were treated as described in Figure 4, and the cell lysates were subjected to western blot analysis. A representative blot is shown from two independent experiments with almost identical pattern. The relative intensity of bands after normalization for the levels of beta-actin is shown under each blot.

As shown in Figure 6C, the average tumor growth in terms of total tumor volume in GSPs-treated mice was significantly lower throughout the experimental protocol, particularly with the dose of 100 mg GSPs/kg body weight of the mouse ($p<0.005$); however significant reduction in tumor growth was also noticed with the dose of 50 mg GSPs/kg body weight of the mouse at the most period of the experiment ($p<0.05$ to $p<0.01$). At the termination of the experiment at 5th week, the average tumor volume in GSPs-treated group was 1200±160 mm^3 and 607±85 mm^3 respectively for the feeding of 50 and 100 mg of GSPs/kg body weight/per animal compared to 1775±295 mm^3 in non-GSPs-fed control animals (Figure 6C). The experiment was stopped at this stage because of larger size of the skin tumor, and guidelines of Institutional Animal Care and Use Committee were followed. At the termination of the experiment, the animals were sacrificed and tumors were excised, and the wet weight of the tumor/animal in each treatment group was measured. The wet weight of tumor in 50 and 100 mg GSPs/kg body weight/animal-treated group was lower, respectively by 29% ($p<0.05$) and 63% ($p<0.005$), compared to the mice that were not given GSPs by gavage (Figure 6D). These results suggest an in vivo chemotherapeutic potential of GSPs without apparent toxicity in A431-skin tumor xenograft model.

4.8. GSPs inhibit the proliferation of tumor cells, inhibit NF-kappaB activity and induce apoptosis in A431-tumor xenograft

To further confirm that the growth of A431-tumor xenograft in GSPs-fed mice is inhibited because of inhibition of tumor cell proliferation, we analyzed the mRNA expression of PCNA and cyclin D1 as markers of proliferation using real-time PCR in the tumors of GSPs-treated mice and the data were compared with the tumors from the non-GSPs-treated controls. As shown in Figure 7, the mRNA expression of PCNA (Panel A) and cyclin D1 (Panel B) was significantly reduced, respectively by 37-54% and 32-57% ($p<0.05$ to $p<0.005$), in the tumors from the mice which were treated with GSPs compared to non-GSPs-treated control mice. NF-kappaB activity was also decreased in tumor samples after GSPs treatment (Panel C), as was also observed in *in vitro* A431 cells (Figure 4). Additionally, we also checked whether dietary GSPs induce apoptosis in tumor cells and that may result in inhibition of the growth of tumor xenograft. For this purpose we checked the level of cleaved caspase-3 as a marker of apoptotic cell death in tumor xenograft samples obtained from different treatment groups. As shown in Figure 7D, we found that the levels of cleaved caspase-3 in tumor xenograft samples obtained from GSPs treatment (0.2 and 0.5%, w/w) groups were higher compared to those tumor xenograft samples which were obtained from non-GSPs-treated group. These data indicated that treatment of GSPs inhibits the growth of tumor xenograft in mice through the inhibition of tumor cell proliferation and induction of apoptotic cells death of tumor cells.

5. DISCUSSION

The use of botanical agents, specifically dietary, is gaining constant attention for the health benefits of skin (2). Those botanicals which are specifically possessing anti-inflammatory and anti-oxidant properties are most suited for their health benefits. GSPs are among one of them, and in addition to anti-inflammatory and antioxidant properties, GSPs have been shown to have anti-carcinogenic properties in some *in vitro* and *in vivo* tumor models (2,5,8). We have shown previously that treatment of human epidermoid carcinoma A431 cells with GSPs induces cell growth inhibition and apoptosis of A431 cells but this effect was not seen in normal human epidermal keratinocytes, which indicates the cytotoxic effects of GSPs to cancer cells but not for normal skin cells. In the present study, we have further assessed the chemotherapeutic effect of GSPs on A431 cells *in vitro* and *in vivo* tumor-xenograft model with defined mechanism of action on the MAPK and NF-kappaB pathways. We observed that treatment of A431 cells with GSPs inhibits constitutive expression of phosphorylation of MAPK proteins. The higher levels of phosphorylation of MAPK proteins play major role in cell growth, differentiation and proliferation, and that may lead to clonal expansion of tumor cells into tumors (24). MAPK signaling pathway is an important upstream regulator of transcription factor activities and their signaling affects a wide variety of extracellular stimuli into intracellular events and thus control the activities of downstream transcription factors implicated in carcinogenesis (25). Our data demonstrate that constitutive expression of phosphorylated proteins of MAPK family, such as, ERK1/2, JNK and p38, in A431 cells, were significantly

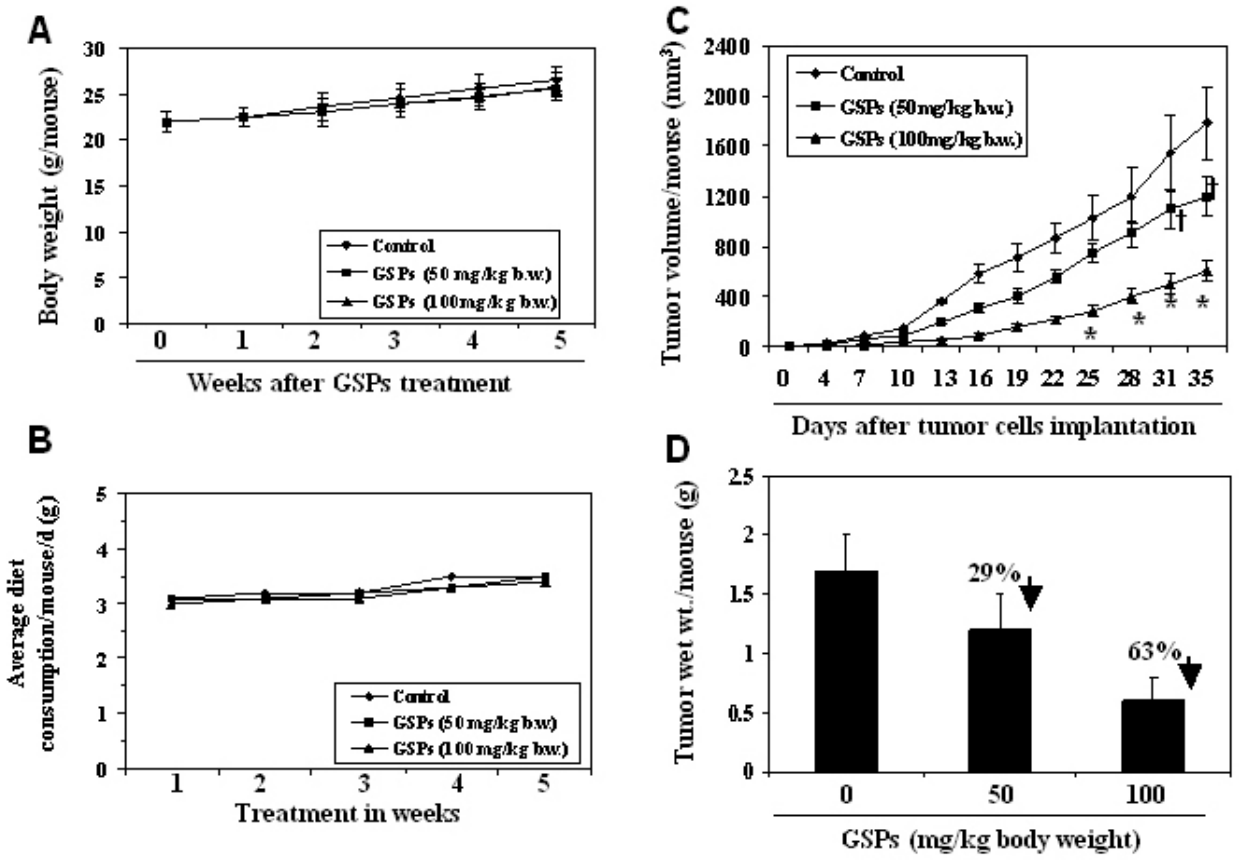

Figure 6. Treatment of GSPs by gavage inhibits the growth of tumor xenograft in athymic nude mice. Each mouse was s.c. implanted with $2x10^6$ A431 cells mixed with Matrigel on the right flank. Twenty-four h later, mice were given either PBS (100 micro l) or GSPs (50 mg or 100 mg/kg body weight/mouse in 100 micro l PBS) by gavage 5 days a week. A, Body weight of each mouse was recorded on weekly basis. The average body weights of the control and GSPs-fed mice did not differ significantly throughout the experiment period. B, Average diet consumption/ mouse/day (g); C, Average tumor volume ± SD/mouse (mm^3) in each group shown as a function of weeks of GSPs treatment. D, Tumors were harvested at the termination of the experiment and the wet weight of each tumor was measured and is reported in g as mean ± SD/group. Statistical significance of difference between control and GSPs-fed groups was calculated by one-way ANOVA followed by Bonferroni t test. n=5-7. Statistical significance *vs* control, $^{\dagger}p<0.01$; $^{*}p<0.005$.

lowered by GSPs treatment. The phosphorylation of ERK1/2 has been shown to play a critical role in transmitting signals from extracellular to intracellular molecules. Phosphorylation of JNK regulates activator protein-1 transcription in response to extracellular stimuli, such as oxidative stress, growth factors and inflammatory cytokines, which in turn may influence the activation of inflammation, invasion, metastasis and angiogenesis, and also in the promotion and progression of various types of cancers (15,16, 25). Therefore, inhibition of JNK activation may be a relevant molecular target for potential chemotherapeutic effects of GSPs. The decrease in the constitutive expression of phosphorylation of MAPK proteins by GSPs may, in turn, inhibit the downstream events, such as activation of activator protein-1 and NF-kappaB that can contribute to the development of skin cancers. We suggest that significant reduction in the constitutive expression of phosphorylated MAPKs by GSPs might be responsible for their inhibitory effects on the activation of transcription factor NF-kappaB, which we have found in this study. Therefore, the MAPK and NF-kappaB signaling pathways appear to be the potential targets of GSPs.

Our study also highlights that treatment of A431 cells with GSPs decreases EGF-induced phosphorylation of MAPK proteins. These data have significance as EGF-induced activation of EGFR leads to the activation of MAPK proteins, particularly ERK1/2, which translocate to the nucleus and activate transcription factors for cell growth and proliferation (15). Further, the decrease in constitutive levels of phosphorylated MAPK proteins in the A431 cells by GSPs treatment was associated with the dose-dependent increase or reactivation of MAP kinase phosphatases. The enhanced levels of phosphatases by GSPs in A431 cells can dephosphorylate both phosphorylated threonine and phosphorylated tyrosine (dual specificity) residues and might be able to inactivate or

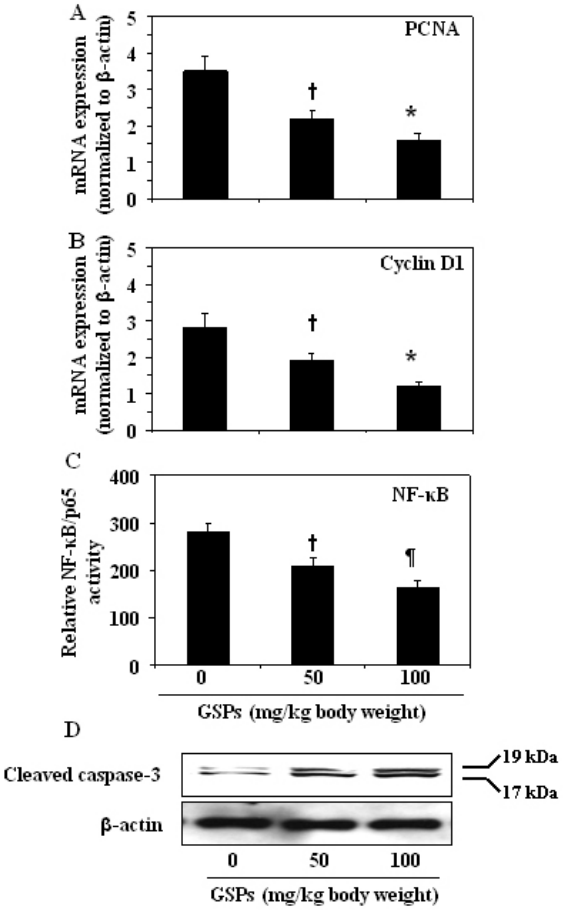

Figure 7. The treatment of GSPs by gavage inhibits the proliferation of tumor cells in A431-tumor xenograft. At the termination of the experiment, the tumors were collected, total RNA was isolated and mRNA expression of PCNA (Panel A) and cyclin D1 (Panel B) was analyzed using real-time PCR SYBR-Green System, as described in Materials and methods. The results are presented as the expression of the individual mRNA with normalization to beta-actin using the Ct method. Panel C: The activity of NF-kappaB/p65 was measured using ELISA. The results (mean ± SD) represent values from five tumor samples from five different mice. Statistical significance versus non-GSPs-treated controls; $^{\dagger}p<0.05$; $^{\P}p<0.01$; $^{*}p<0.005$. Panel D: Treatment of GSPs increases the levels of cleaved caspase-3 in tumor xenograft samples compared to non-GSPs-treated mice as analyzed by western blotting. Tumor lysates were prepared from the tumors collected at the termination of the experiment and subjected to western blot analysis. The levels of cleaved caspase-3 were determined as a marker of tumor cell apoptosis. Representative blot is shown from three independent experiments from six tumors with identical results. Equal protein loading was confirmed by probing stripped blots for beta-actin as shown.

suppress MAPK signaling (20). MKP-1 (a family of dual-specificity protein phosphatases) has been shown to inactivate all three major MAPK proteins (ERK, JNK and p38) (26). Therefore, it is possible that reduction in constitutive levels of MAPK phosphorylation in A431 cells is inhibited by the treatment of GSPs through activation of MAP kinase phosphatases.

Studies have shown that activation of PI3K plays an important role in carcinogenesis (27-29). The biological effects of PI3K are mediated through the activation or phosphorylation of down-stream target Akt. Akt, also known as protein kinase B, is a serine/threonine kinase, which has been identified as an important component of prosurvival signaling pathway (30). Our study demonstrates that treatment of A431 cells with GSPs inhibits the constitutive expression of PI3K and phosphorylation of Akt, as well as EGF-induced phosphorylation of Akt in our system. Reports indicate that well-known markers of tumor promotion and tumor cell proliferation, e.g., COX-2, iNOS, PCNA and cyclin D1, are regulated by NF-kappaB transcriptional factor (31). In addition to I kappaB alpha, NF-kappaB is also regulated by PI3K/Akt signaling pathway (32). Therefore, both NF-kappaB and PI3K/Akt signaling pathways have emerged as promising molecular targets in the prevention of cancer. Our study clearly demonstrates that treatment of A431 cells with GSPs decreases the expression of both regulatory (p85) and catalytic (p110) subunits of PI3K and phosphorylation of Akt at Ser^{473} compared to non-GSPs-treated control A431 cells. The activation of PI3K/Akt pathway promotes cell survival by activating NF-kappaB signaling pathway (33). Thus it can be suggested that inhibition of cellular proliferation of A431 cancer cells by GSPs is mediated, at least in part, through the down-regulation of PI3K/Akt pathway.

The signaling pathways controlling the regulation of NF-kappaB activity have been targeted in the development of chemotherapeutic agents. The activation of NF-kappaB has been implicated in inflammation, cell proliferation and oncogenic processes (34,35), and the activation of NF-kappaB depends on the phosphorylation and subsequent degradation of IkappaB proteins. Our studies show that treatment of A431 cells with GSPs markedly down-regulates the constitutive expression of NF-kappaB and simultaneously the expression of IKKalpha while prevents the degradation of I kappaB alpha. The IKK (IKKalpha and/or IKKbeta) phosphorylates IkappaB proteins and members of the NF-kappaB family. Therefore, the inhibitors of IKK have been considered as specific regulators of NF-kappaB. As GSPs block I kappaB alpha degradation in A431 cells, our study suggests that the down-regulatory effect of GSPs on NF-kappaB/p65 activation may be mediated through the inhibition of proteolysis of the I kappaB alpha protein.

The activation of NF-kappaB has been shown to up-regulate the expression of proinflammatory cytokines and inflammatory and oxidative gene products, such as COX-2 and iNOS (23,36), as key regulators of cell proliferation through its effects on cell cycle progression including its effects on cyclin D1, a cyclin that is expressed early in the cell cycle and is important for DNA synthesis (37). Majority of these genes have been shown to be up-regulated in human cancers, suggesting that down-

regulation of NF-kappaB and subsequently down-regulation of NF-kappaB-targeted genes may adversely affect the development of cancers. In this study, we observed that treatment of A431 cells with GSPs markedly inhibited the constitutive levels of NF-kappaB-responsive proteins, such as COX-2, iNOS, cyclin D1, PCNA and MMP-9. The down-regulation of these NF-kappaB-targeted proteins in human epidermoid carcinoma A431 cells may explain the anti-proliferative and/or anti-carcinogenic effects of GSPs. It also may be the possibility that the down-regulation of the constitutive expression of COX-2, iNOS, PCNA, cyclin D1 and MMP-9 in A431 cells by GSPs are independent to the inhibition of NF-kappaB, and therefore requires further studies. As it is well known that NF-kappaB pathway has a role in cell survival and proliferation, our findings suggest that down-regulation of NF-kappaB represents a key molecular target of GSPs.

In vitro cell culture model is useful to obtain mechanistic insights; however, the observations made in *in vitro* system need to be verified *in vivo* animal models to establish the relevance of the cellular findings. Therefore, *in vivo* experiments were conducted using athymic nude mice to confirm the chemotherapeutic potential of GSPs against A431 cells. The treatment of athymic nude mice with GSPs by oral gavage for 5 weeks do not exhibit weight loss, loss of appetite or any abnormal changes in physical behavior. As preclinical *in vivo* testing of potential chemotherapeutic agents in appropriate animal model is a prerequisite for their clinical development, we further determined the efficacy of GSPs by ectopic implantation of A431-xenograft in athymic nude mice. Our *in vivo* study provides evidence that administration of GSPs by gavage inhibits the growth of A431 tumor-xenograft without any apparent sign of toxicity in mice. The kinetics of tumor growth in non-GSPs-treated mice was rapid throughout the experiment compared to GSPs-treated mice. Moreover, the mechanism of inhibition of the growth of tumors by GSPs was further confirmed by the analysis of mRNA levels of PCNA and cyclin D1, as markers of tumor cell proliferation. The decrease in the mRNA expression of cyclin D1 and PCNA was observed in the tumor-xenograft samples of GSPs-treated mice compared to the tumor-xenograft samples of control mice which were not given GSPs by gavage. Further, the inhibition of the growth of tumor xenograft in athymic nude mice by GSPs was also associated with the induction of apoptotic cell death of tumor cells. These results suggest the inhibition of growth of A431-tumor xenograft via decreased proliferation and increased apoptotic cell death of tumor cells in GSPs-treated mice. Together, our studies show the chemotherapeutic potential of GSPs in controlling the growth of human epidermoid carcinoma cells including the possible mechanism of action of GSPs in *in vitro* and *in vivo* models.

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Abbreviations: GSPs: grape seed proanthocyanidins; EGF: epidermal growth factor; MAPK: mitogen-activated protein kinases; NF-kappaB: nuclear factor-kappa B; PI3K: phosphatidyl inositol-3-kinase; ERK: extracellular signal-regulated kinase; JNK: cJun N-terminal kinase; COX-2: cyclooxygenase-2; PCNA: proliferating cell nuclear antigen; iNOS: inducible nitric oxide synthase; MMP-9: matrix metalloproteinase-9

Key words: Grape Seed Proanthocyanidins; Mitogen-Activated Protein Kinases; Nuclear Factor-Kappab; Tumor Xenograft; Phosphatidylinositol 3-Kinase

Send correspondence to: Santosh K. Katiyar, Ph.D., Department of Dermatology, University of Alabama at Birmingham, 1670 University Boulevard, Volker Hall 557, P.O. Box 202, Birmingham, AL 35294, USA, Tel: 205-975-2608, Fax: 205-934-5745, E-mail: skatiyar@uab.edu

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