Grape Seed Proanthocyanidins Inhibit the Growth of Human Non-Small Cell Lung Cancer Xenografts by Targeting Insulin-Like Growth Factor Binding Protein-3, Tumor Cell Proliferation, and Angiogenic Factors

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Abstract

Purpose: Lung cancer is a leading cause of cancer-related deaths worldwide. Here, we assessed the chemotherapeutic effect of grape seed proanthocyanidins (GSPs) on human non-small cell lung cancer (NSCLC) cells in vitro and in vivo using a tumor xenograft model.

Experimental Design: The effects of GSPs on human NSCLC cell lines in terms of cellular proliferation were determined. The chemotherapeutic effects of a GSP-supplemented AIN76A control diet fed to nude mice bearing tumor xenografts (A549 and H1299) were evaluated in terms of biomarkers of cell proliferation and angiogenesis and on insulin-like growth factor binding protein-3 using immunohistochemical detection, ELISA, and Western blotting.

Results: In vitro treatment of NSCLC cells with GSPs resulted in inhibition of cellular proliferation. Administration of GSPs (0.1%, 0.2%, and 0.5%, w/w) as a supplement of an AIN76A control diet resulted in a dose-dependent inhibition of the growth of NSCLC (A549 and H1299) tumor xenografts in athymic nude mice (25–76%; P < 0.05–0.001). The growth-inhibitory effect of GSPs on the NSCLC xenograft tumors was associated with the enhancement of the levels of insulin-like growth factor binding protein-3 in the tumor microenvironment and plasma and antiproliferative, antiangiogenic, and proapoptotic effects.

Conclusions: This preclinical study reveals for the first time that dietary GSPs have the ability to inhibit the growth of human NSCLC tumor xenografts grown in vivo in athymic nude mice. More studies are needed to develop GSPs as a pharmacologically safe agent for the prevention of lung cancer in humans.

Lung cancer is the leading cause of cancer-related deaths in both men and women in the United States and worldwide (1). It is responsible for more deaths in the United States each year than breast, colon, and prostate cancers combined (2). One of every three cancer-related deaths is attributable to lung cancer with an overall 5-year survival of <15% (3, 4). Two types of lung cancer, small cell lung cancer and non-small cell lung cancer (NSCLC), account for 90% of all lung cancers. NSCLC represents ~80% of all types of lung cancer and includes squamous cell carcinomas, adenocarcinomas, and large cell carcinomas (5, 6). The current treatment strategies for advanced lung cancer include surgical resection, radiation, cytotoxic chemotherapy, and photodynamic therapy (7). In almost two-thirds of cases, the cancer has already spread beyond localized disease at the time of diagnosis, limiting therapeutic options (5, 6). Therefore, the exploration and development of more effective chemopreventive/chemotherapeutic agents and therapies that can target the molecules associated with tumor proliferation, angiogenesis, and apoptosis resistance will lead to improved outcomes in patients with lung cancer.

Botanical agents, particularly those that can be administered as dietary supplements, offer promising new options for the development of more effective chemopreventive and chemotherapeutic strategies. Grape seed proanthocyanidins (GSPs) are promising agents that have antioxidant properties (8–10) and appear to exhibit minimal toxicity (9, 10). GSPs are a mixture of polyphenols/flavanols and mainly contain proanthocyanidins (89%), which constitute dimers, trimers, tetramers, and oligomers/polymers of monomeric catechins and/or (-)-epicatechins, as described previously (10, 11). GSPs are readily available as an extract of grape seeds and this extract, rather than the individual constituents, is used in determining the chemotherapeutic effects of GSPs, as it represents a feasible and affordable botanical agent.

We reported recently that in vitro treatment of NSCLC cells with GSPs inhibited the migration of lung cancer cells (12), which was associated with the inhibition of the levels of nitric oxide production and inducible nitric oxide synthase, guanylate cyclase, and extracellular signal-regulated kinase 1/2. The migration of cancer cells is considered to be a major event in
the metastatic cascade of cancer cells. In the current study, we first assessed the effect of GSPs on the proliferation potential of 10 different NSCLC cell lines. These cell lines are genetically different, such as p53 wild-type (A549), p53-mutated (H1299), epidermal growth factor receptor (EGFR)-positive (A549, H1299, H226, H460, and H157), EGFR-mutated (H1975, H1650, HCC827, and H3255), and one lung cancer cell line from mouse (Lewis lung carcinoma cells). Thereafter, we determined the in vitro effect of dietary GSPs on the growth and progression of H1299 and A549 tumor xenografts implanted in immunocompromised athymic nude mice and coupled this study with the analysis of the tumors for the prognostic biomarkers commonly used in preclinical cancer models (tumor proliferation, tumor angiogenesis, and tumor cell apoptosis). It is well recognized that angiogenesis plays a crucial role in the growth and progression of solid tumors beyond the size of ~2 mm in diameter because these rapidly growing tumors require extra nutrient supply (13). Vascular endothelial growth factor (VEGF) is an important angiogenic factor and its overexpression has been linked to tumor-associated angiogenesis and a more aggressive tumor phenotype (14, 15). We therefore evaluated the antiangiogenic potential of the GSPs by determining the expression levels of VEGF and CD31 (a marker of neovascularization). Several studies have shown that higher circulating insulin-like growth factor-I (IGF-I) and lower levels of insulin-like growth factor binding protein-3 (IGFBP-3) are associated with an increased risk of malignancy (16, 17) and might be excellent determinants of lung cancer. We assessed the tumor-secreted levels of IGFBP-3 in the tumor microenvironment as well as in plasma to determine whether IGFBP-3 play a role in the growth-inhibitory effects of GSPs on the growth of lung tumors. We found that dietary GSPs resulted in significant inhibition of the growth of the lung tumor xenografts in nude mice, which was associated with the (a) enhancement in the levels of IGFBP-3 in both tumor microenvironment and plasma, (b) inhibition of tumor cell proliferation, (c) inhibition of angiogenic factors, and (d) induction of apoptosis of the tumor cells.

**Materials and Methods**

**Cell culture and cell lines.** The human NSCLC cell lines (A549, H1299, H226, H460, H157, H1975, H1650, H3255, and HCC827) and mouse Lewis lung carcinoma cells were purchased from the American Type Culture Collection. Human bronchial epithelial cells (BEAS-2B) from the American Type Culture Collection were used as a normal control. The lung cancer cell lines were cultured as monolayers in Ham’s F-12 medium or RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 100 μg/mL penicillin, and 100 μg/mL streptomycin and maintained in an incubator with a humidified atmosphere of 95% air and 5% CO2 at 37°C. The GSPs were dissolved in a small amount of DMSO, which was added to the complete cell culture medium [maximum concentration of DMSO, 0.1% (v/v) in medium] before addition to subconfluent cells (60-70% confluent). Cells treated only with DMSO served as a vehicle control.

**Dietary administration of GSPs.** We routinely receive GSPs for our research from Kikkoman. Quality control of GSPs is maintained by the company. GSPs contain ~89% proanthocyanidins, with dimers (6.6%), trimers (5.0%), tetramers (2.9%), and oligomers (74.8%), as described described earlier (10, 11). GSPs are stable for at least 2 years when refrigerated at 4°C. Experimental diets containing GSPs (0.1%, 0.2%, and 0.5%, w/w) are prepared in pellet form by TestDiet for our research and using the GSPs that we provide for this purpose.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for cell proliferation/survival.** The effect of GSPs on the proliferative capacity of the cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (18). The effect of GSPs on proliferation is reported as the percent cell viability of the treated cells compared with the vehicle-treated control cells, which were arbitrarily assigned a value of 100% viability.

**IGFBP-3 small interfering RNA transfection of A549 and H1299 cells.** Human-specific IGFBP-3 small interfering RNA (siRNA) was transfected into A549 and H1299 cells using siRNA Transfection Reagent Kit (sc-29528; Santa Cruz Biotechnology) according to the manufacturer’s protocol. Briefly, 2 × 10^5 cells per well were seeded in a 6-well plate and allowed to grow until 70% confluency. The IGFBP-3 siRNA mix with transfection reagents were overlaid on cells for ~6 h at 37°C and transferred into 2× growth medium for ~18 to 20 h. At 24 h post-transfection, fresh medium was added to the cells, and at 48 h post-transfection, cells were treated with GSPs (0, 40, and 60 μg/mL) for an additional 48 h; thereafter, cells were harvested and analyzed for viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Knockdown of IGFBP-3 after transfection was confirmed using Western blot analysis.

**Preparation of cell lysates and Western blot analysis.** Following treatment of NSCLC cells with or without GSPs, the cells were harvested, washed with cold PBS, and lysed with ice-cold lysis buffer supplemented with protease inhibitors as detailed previously (18). For immunoblotting, proteins (20-35 μg) were resolved on 10% Tris-glycine gels and transferred onto a nitrocellulose membrane. After blocking the nonspecific binding sites, the membrane was incubated with the primary antibody at 4°C overnight. The membrane was then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody and the immunoreactive bands were visualized using the enhanced chemiluminescence reagents (Amersham Biosciences). Each membrane was then stripped and reprobed with anti-β-actin antibody to verify equal protein loading.

**Animals and tumor xenograft assay.** Female athymic nude mice (6-7 weeks old) were used and purchased from the National Cancer Institute and provided the sterilized AIN76A diet and water ad libitum. All the mice were housed in the Animal Resource Facility of the University of Alabama at Birmingham and were maintained under the following conditions: 12-h dark/12-h light cycle, 24 ± 2°C temperature, and 50 ± 10% humidity. Mice in the experimental groups were given the AIN76A diet supplemented with GSPs (0.1%, 0.2%, and
subcutaneous implantation of tumor cells and (a tumor model in which the GSP-containing AIN76A diet was given to the mice at least 10 days before the subcutaneous implantation of tumor cells and a tumor model in which mice were switched to the GSP (0.5%)-containing AIN76A diet 29 days after the subcutaneous implantation of tumor cells). At this time, cells started growing into tumor. Mice were randomly divided into the treatment groups (n = 10). The experiment was terminated at 58 days after tumor cell inoculation following the guidelines of the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. Tumor growth was regularly monitored. Body weight per mouse per week and diet consumption per mouse per day also were recorded throughout the experiment. As per the guidelines of the Institutional Animal Care and Use Committee, the animals were regularly monitored for signs of illness or suffering (lack of normal grooming and avoidance behaviors). At the termination of the experiment, the entire tumor mass was recovered and weighed.

**Immunohistochemical detection of IGFBP-3-positive cells.** Immunohistochemical detection of IGFBP-3-negative or IGFBP-3-positive cells was done using specific primary antibodies. Briefly, tumor xenograft sections (5 μm thick) were incubated overnight with human reactive goat anti-IGFBP-3 primary antibody at 4°C (Santa Cruz Biotechnology) followed by incubation with the appropriate biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin. Antigen-antibody complexes were detected by peroxidase reaction using diaminobenzidine substrate and the cells were counterstained with hematoxylin.

**Quantitative assay for human IGFBP-3 using ELISA.** At the termination of the experiment, blood samples were collected in heparinized tubes and plasma was separated for the analysis of IGFBP-3. Sand standard curve generated using recombinant human IGFBP-3. The concentration of IGFBP-3 was extrapolated from the phase ELISA. The assay was based on the quantitative sandwich enzyme immunoassay employing monoclonal antibody specific for human IGFBP-3 precoated onto a microplate for solid-phase ELISA. The concentration of IGFBP-3 was extrapolated from the standard curve generated using recombinant human IGFBP-3.

**Immunohistochemical detection of proliferating cell nuclear antigen-positive and VEGF-positive cells.** Tumor sections (5 μm thick) were deparaffinized and rehydrated in a graded series of alcohols. Following rehydration, an antigen retrieval process was done by placing the slides in 10 mmol/L sodium citrate buffer (pH 6.0) at 95°C for 20 min followed by 20-min cooling. The sections were washed in PBS and nonspecific binding sites were blocked with 1% bovine serum albumin with 2% goat serum in PBS before incubation with either anti-proliferating cell nuclear antigen (PCNA) or anti-VEGF antibodies for 2 h at room temperature. After washing, the sections were incubated with biotinylated secondary antibody for 45 min followed by horseradish peroxidase-conjugated streptavidin, washing in PBS, incubation with diaminobenzidine substrate, and counterstaining with hematoxylin. Photographs of representative pictures were taken and the numbers of PCNA-positive or terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells were detected and counted using a light microscope. The results are presented as the number of positive cells x 100 divided by the total number of cells.

**Immunohistochemical detection of CD31+ cells.** The vascular density in the tumors was determined by immunostaining using an anti-CD31 antibody specific for vascular endothelial cell antigen (also known as PECAM-1). Immunostaining of CD31 was done using frozen tumor sections as described previously (19). Briefly, sections (5 μm thick) were fixed in cold acetone for 10 min. The nonspecific binding sites were blocked by incubating the sections in Tris-HCl (pH 7.6) containing goat serum (5%, v/v) and bovine serum albumin (0.5%, w/v). After blocking, the sections were incubated with primary antibodies for CD31 in blocking buffer for 1 h. Thereafter, antibody binding was detected by subsequent incubation of sections with streptavidin-phycocyanin-Cy5 secondary antibody for 1 h. After washing, the sections were counterstained with Hoechst 33342 dye.

**Immunohistochemical and statistical analysis.** Microscopic immunohistochemical analysis of tissue sections was done using an Olympus BX41 microscope fitted with a Q-color 5 Olympus camera. To compare the numbers of immunostaining-positive cells (PCNA and TUNEL) in different treatment groups, at least 6 different fields from each section were selected, and cells were counted. The numbers of cells are presented in terms of percent of total cells as necessary. Microscopic images were finalized using Microsoft PowerPoint software. The statistical significance of differences between the values of GSP-treated and non-GSP-treated controls was determined by one-way ANOVA followed by Tukey's test for multiple comparisons. In each case, P < 0.05 was considered statistically significant.

Results

**GSPs inhibit the proliferation of NSCLC cells in vitro.** We first determined the effect of GSPs on the proliferation of NSCLC cells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. NSCLC cell lines with different genetic characteristics, including p53 wild-type (A549), p53-mutated (H1299), EGFR-positive (A549, H1299, H226, and H460) and EGFR-mutated (H1975, H11650, HCC827, and H3255), were used for this purpose. Treatment of EGFR-positive human NSCLC cells with varying concentrations of GSPs (0, 20, 40, 60, and 80 μg/mL) for 24 h resulted in inhibition of proliferation of the cells (5-17%); however, significant greater inhibitory effect was observed at 48 h after GSP treatment (10-68%; P < 0.05-0.001) as assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 1A, left). The levels of inhibition also were time dependent (60 μg/mL GSPs; 20-75%; P < 0.05-0.001; Fig. 1A, right). The sensitivity of EGFR-mutated NSCLC cell lines also was determined after the treatment of cells with GSPs. As shown in Fig. 1B, treatment of EGFR-mutated cells with GSPs for 48 h also resulted in significant inhibition of proliferation rate (10-59%; P < 0.05-0.001); however, these cells are comparatively resistant to GSPs than EGFR-positive cells. Mouse Lewis lung carcinoma cells were highly sensitive to the cytotoxic effects of GSPs than the human NSCLC cell lines (Fig. 1B). Also, the p53-positive (A549) NSCLC cells were more sensitive to GSPs than p53-mutated cells (H1299). It is evident from the data that different NSCLC cells have different IC50 values and sensitivities to GSPs.

**GSPs inhibit proliferation of human lung cancer cells but not of normal human bronchial epithelial cells.** We also examined the effects of GSPs on normal (nonneoplastic) human bronchial epithelial cells (BEAS-2B) under identical conditions. We did not find significant inhibition of cell proliferation of normal human bronchial epithelial cells after GSP treatment at the concentrations of 20, 40, 60, or 80 μg/mL for 24, 48, or 72 h (Fig. 1C). Although an inhibitory effect of GSPs on normal bronchial epithelial cells was noted at the 96-h time point, the inhibition was significantly lower than the effect of equivalent concentrations of GSPs on the NSCLC cells at the same time point and equivalent concentrations of GSPs (P < 0.001).
GSP-induced inhibition of proliferation is blocked by knockdown of IGFBP-3 in A549 and H1299 cells. It has been shown that IGFBP-3 inhibits proliferation of cancer cells through IGF-I/IGFBP-3/IGF-I receptor axis (20–22); therefore, we examined whether the inhibition of proliferation of NSCLC cells by GSPs is mediated through IGFBP-3. For this purpose, IGFBP-3 was knocked down from the cells (A549 and H1299) using siRNA transfection. The transfection of IGFBP-3 siRNA resulted in marked reduction of IGFBP-3 (>80%) in both A549 and H1299 cells (Fig. 1D). The treatment of A549 cells with GSPs at the dose of 40 and 60 μg/mL along with the control siRNA for 48 h resulted in 38% and 62% inhibition of proliferation, respectively, whereas GSP-induced inhibition of proliferation of A549 cells in IGFBP-3 siRNA-transfected cells
was significantly blocked \( (P < 0.001; \text{Fig. 1D}) \). Similar results were also obtained with H1299 cells (p53-mutated) under identical experimental conditions; however, H1299 cells were comparatively resistant to GSPs than A549 cells. These data suggest that GSP-induced inhibition of NSCLC cells proliferation may be mediated through IGFBP-3.

Dietary GSPs inhibit the growth of lung tumor xenografts in athymic nude mice when the GSPs are provided in the diet before implantation of the lung tumor cells. As we have found that treatment of NSCLC cells with GSPs inhibited the growth or proliferation of these cells in vitro, we sought to determine whether dietary administration of GSPs inhibits the growth of NSCLC tumor cells in vivo. Two NSCLC cell lines, A549 (adenocarcinoma) and H1299 (large cell carcinoma), were used in these experiments. In this experiment, the cells were grown as xenografts in nude mice that had been fed a diet supplemented with GSPs for at least 10 days before injection of the tumor cells. The mice were provided the AIN76A control diet.

**Fig. 2.** Dietary GSPs inhibit the growth of A549 and H1299 NSCLC cells grown as xenografts in athymic nude mice. A, body weights of the mice were monitored on a weekly basis. The body weights of the GSP-treated and non-GSP-treated mice did not differ significantly throughout the duration of the experiment. B, average ± SD tumor volume per mouse (mm³) in each treatment group. C, tumor xenografts tissues were harvested at the termination of the experiment, and the wet weight of the tumor per mouse in each group is reported as mean ± SD (g). Statistical significance of difference between control and GSP-treated groups was analyzed by one-way ANOVA followed by the Bonferroni t test \( (n = 10) \). *, \( P < 0.05; \), \( P < 0.01; \), \( P < 0.001 \), versus non-GSP-treated controls.
diet alone or the same diet supplemented with three different doses of GSPs (0.1%, 0.2%, or 0.5%, w/w). The average body weights of the GSP-treated and control mice were comparable throughout the duration of the experiment (Fig. 2A). Similarly, there were no significant differences in the daily consumption of diet and drinking water by the mice among the different groups (data not shown) and the mice that were given GSPs in their diet did not exhibit any physical sign of toxicity. These data provide an indication that administration of GSPs in the diet at the concentrations used in these studies is not associated with gross toxicity.

Periodic measurement of the tumor volume indicated that the average tumor growth in terms of total tumor volume per mouse was higher in the non-GSP-treated mice than the GSP-treated groups. As can be seen in Fig. 2B, in mice that received GSPs at the doses of 0.1%, 0.2%, and 0.5% in their diet, the tumor volume at the termination of the experiment was 25%, 56% (P < 0.01), and 76% (P < 0.001) less, respectively, for A549 tumor xenografts (Fig. 2B, left). A similar inhibitory effect of dietary GSPs on the growth of H1299 tumor xenograft was also observed and this inhibition was dose-dependent (30-70%; P < 0.01-0.001; Fig. 2B, right).

The experiment was terminated on the 58th day after tumor cell implantation when the mice were sacrificed, the tumors were harvested, and the wet weight of the tumor per mouse in each treatment group was recorded. As shown in Fig. 2C (left), the wet weight of the A549 xenograft tumor per mouse was significantly lower in the mice administered GSPs than in the mice that did not receive GSPs in diet. The wet weight of the A549 tumors was 37% lower (P < 0.01) in mice administered 0.2% GSPs in the diet and 60% lower (P < 0.001) in mice administered 0.5% GSPs in the diet. Administration of GSPs at the same concentrations also resulted in reduction in the wet weight of the H1299 tumor xenografts. The wet weight of the H1299 tumors was 10% lower (not significant), 33% lower (P < 0.05), and 52% lower (P < 0.01) in mice administered 0.1%, 0.2%, and 0.5% GSPs, respectively, in the diet than in the mice fed the control diet. Thus, administration of GSPs in the diet, particularly at the doses of 0.2% and 0.5%, resulted in reduced growth of both A549 and H1299 lung cancer xenografts.

Therapeutic effect of dietary GSPs on the growth of lung tumor xenograft. The second *in vivo* tumor xenograft protocol was designed to evaluate the therapeutic effect of dietary GSPs on established tumors. The dietary treatment of GSPs (0.5%, w/w) was started when tumor size was ~200 to 250 mm³, on the 29th day after tumor cell implantation (Fig. 3). Although we did not observe any regression in the size of the established tumors...
tumors after GSPs were provided in the diet, the growth rate of the tumors was slower in the GSP-fed mice compared with the growth rate of the tumors in those mice that were not given GSPs in the diet. At the termination of the experiment on the 58th day after tumor cell implantation, the growth-inhibitory effect of dietary GSPs on tumor xenograft volume was 62\% (\( P < 0.005 \)) in A549 tumors compared with non-GSP-treated mice when the size of the tumors on the 29th and 58th days of the experiment were compared (Fig. 3A). Similar results were obtained for the H1299 xenografts (Fig. 3A, right). A reduction in the tumor wet weight at the termination of the experiment was also found with therapeutic treatment of mice with GSPs resulting in a 43\% (\( P < 0.01 \)) reduction in wet weight of A549 xenograft tumors compared with non-GSP-treated mice, whereas a 38\% (\( P < 0.01 \)) reduction was noted in the case of the H1299 xenograft tumors (Fig. 3B).

**Dietary GSPs enhance IGFBP-3 protein levels in plasma and lung tumors.** It has been shown that IGFBP-3 inhibits IGF action by competing with IGF receptors for IGF peptides and that increased IGF-I signaling stimulates proliferation and promotes metastasis of cancer cells (20–22). IGFBP-3 therefore represents a promising target for treatment as well as prevention of cancer. We therefore determined whether dietary GSPs affect the levels of IGFBP-3 in NSCLC tumor xenograft tissues or in the plasma. For this purpose, tumor samples were collected and used from the first tumor xenograft experiment (Fig. 2). Also, because 0.5\% of dietary GSPs induced significantly higher protective effects, the tumor samples from this treatment modality were used for further studies. To assess the effect of GSPs, paraffin-embedded sections of the tumor samples from each group were subjected to immunostaining using an antibody specific for human IGFBP-3. As shown in Fig. 4A, the levels of IGFBP-3 were higher, as indicated by the intensity of the staining, in samples of tumors from GSP (0.5\%)-treated mice compared with the tumors of non-GSP-treated control group of mice. This effect of GSPs on the levels of IGFBP-3 was observed in both A549 and H1299 xenografts. As IGFBP-3 secreted by tumor cells could enter the circulation, we also analyzed its levels in blood plasma using a human IGFBP-3-specific ELISA. The levels of human IGFBP-3 in the

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**Fig. 4.** Dietary GSPs increase the expression levels of IGFBP-3 in NSCLC tumor xenograft tissues and in plasma. A, frozen sections (5 \( \mu m \)) of the tumors were subjected to immunoperoxidase staining to detect IGFBP-3-positive cells as detailed in Materials and Methods. Tumor tissues were used from the tumors detailed in Fig. 2. IGFBP-positive cells are dark brown (arrows). B, levels of IGFBP-3 protein in plasma samples were determined using ELISA. Mean ± SD (\( n = 5 \)). †, \( P < 0.001 \), versus control.
plasma of the mice were 2- to 3-fold higher in the GSP-treated xenograft-bearing mice compared with non-GSP-treated control xenograft-bearing mice (Fig. 4B). These observations suggest that up-regulation of IGFBP-3 by dietary GSPs could be a potential in vivo mechanism by which the GSPs inhibit the growth of the NSCLC cells in this xenograft model.

Dietary GSPs inhibit tumor cell proliferation and enhance apoptotic cell death in lung tumor xenografts. Uncontrolled cell proliferation and resistance to apoptosis are the characteristic features of almost every type of cancer, including NSCLC tumors. We therefore analyzed the lung tumor xenografts for the potential antiproliferative and apoptotic effects of GSPs that may have played a role in their overall anticarcinogenic efficacy. For this purpose, tumor samples were collected and used from the experiment of Fig. 2 and subjected to immunohistochemical evaluation and Western blot analysis. Immunohistochemical detection of PCNA-positive cells in tumor xenograft tissues indicated that the percentage of proliferating cells was significantly lower in both A549 (38%; \( P < 0.005 \)) and H1299 (32%; \( P < 0.005 \)) tumor xenografts from GSP (0.5%)-treated mice than the xenograft tumors from the control mice (Fig. 5A). The intensity of immunostaining of PCNA-positive cells was higher in the non-GSP-fed control xenograft samples than the xenograft samples of GSP-treated mice,
indicating the higher expression of PCNA protein. This observation was further confirmed by Western blot analysis. As shown in Fig. 5B, the treatment of mice with dietary GSPs (0.5%, w/w) resulted in inhibition of the expression level of PCNA protein in both A549 and H1299 tumor xenograft samples compared with non-GSP-treated mice.

To determine whether the GSPs, in addition to inducing a loss of the proliferation capacity of the NSCLC cells, also induced apoptosis of the tumor cells, we evaluated the effect of GSPs on the apoptotic index of tumor cells in xenograft samples using the TUNEL assay. Immunohistochemical analysis indicated higher numbers of TUNEL-positive cells in the tumor samples of GSP-fed groups of mice compared with the numbers in the tumor samples from the non-GSP-fed groups of mice (Fig. 5C). The numbers of TUNEL-positive cells in the GSP-treated tumors were ~2-fold higher in both A549 and H1299 tumor xenograft samples than the non-GSP-treated tumors as shown and summarized in Fig. 5C. To further verify that dietary GSPs induce apoptosis of the lung tumor cells, tumor lysates from each treatment group were subjected to Western blot analysis and the levels of activated caspase-3 were determined. As shown in Fig. 5D, Western blot analysis indicated that the levels of activated caspase-3 (a marker of apoptosis) were higher in both A549 and H1299 xenografts grown in GSP-fed mice than the tumors grown in mice that were not fed GSPs. These data suggest that antitumor efficacy of GSPs against in vivo NSCLC tumor growth involved both inhibition of tumor cell proliferation and induction of apoptosis.

**Dietary GSPs inhibit VEGF expression in NSCLC tumors.** The evidence supporting a central role for VEGF in tumor-induced angiogenesis, tumor growth, and metastasis identifies VEGF as a promising target for antitumor therapy (23, 24). As we have observed that dietary GSPs inhibited tumor growth of NSCLC cells in vivo, we sought to determine whether GSPs modulate VEGF expression in tumors. To determine the in vivo effect of dietary GSPs on VEGF protein expression in lung tumor xenografts, the tumor sections described above were subjected to immunohistochemical detection of VEGF. Microscopic evaluation of the VEGF staining pattern indicated that treatment of mice with GSPs resulted in a reduction in the intensity of VEGF-positive staining as well as a decrease in the number of VEGF-positive cells in the A549 and H1299 xenograft tissue samples compared with the non-GSP-treated samples.

![Fig. 6.](image)

**Fig. 6.** Dietary GSPs reduce the expression levels of VEGF and CD31 in A549 and H1299 NSCLC tumor xenograft tissues obtained from the experiment of Fig. 2. A, frozen sections (5 μm thick) were subjected to immunohistochemical staining to detect the expression of VEGF. B, expression levels of VEGF in tumor lysate samples were determined by Western blot analysis. C, immunohistochemical detection of CD31 + cells was done using a phycoerythrin-conjugated CD31 antibody and counterstaining with Hoechst 33342 as detailed in Materials and Methods. Dietary GSPs reduce the number of CD31 + (red) cells as well as a reduction in the clustering of CD31 + cells in tumors compared with non-GSP-treated tumors. Representative micrographs are shown from tumor xenografts samples from 5 to 6 mice.
control A549 and H1299 xenograft tissue samples (Fig. 6A). This observation was further confirmed by Western blot analysis. As shown in Fig. 6B, Western blot analysis indicated that the treatment of mice with dietary GSPs (0.5%, w/w) resulted in a reduction in the levels of VEGF protein in the A549 and H1299 xenografts compared with the xenografts from non-GSP-treated mice.

**Dietary GSPs inhibit the development of neovascularization in the lung tumor xenografts.** Tumor angiogenesis is considered as a prominent target for the control of the growth of solid tumors and also as a prognostic biomarker for cancer management or treatment. Therefore, we sought to assess the effect of GSPs on the extent of neovascularization of the lung tumor xenografts by examining the expression of CD31, which is known to contribute to the formation of new vasculature and is used as a biomarker of angiogenesis (25). Microscopic examination indicated that the numbers of CD31+ cells and the intensity of the CD31 immunofluorescent staining (red) in the A549 and H1299 tumor xenografts from mice that were not fed GSPs was higher than that of the equivalent xenografts from GSP-treated mice (Fig. 6C). These results further suggest that GSPs exert an antiangiogenic effect in the NSCLC tumor xenografts.

### Discussion

GSPs represent one such dietary botanical agent that has been shown to have anticarcinogenic activity (10, 26, 27). We have shown previously that long-term dietary feeding of animals with GSPs does not induce apparent signs of toxicity (10). Similar effects were also noted in the current lung tumor xenograft experiment. We therefore undertook a comprehensive analysis of the effects of GSPs on lung cancer cells using both in vitro and in vivo models. In this study, we found that treatment of human NSCLC cells with different genetic characteristics, p53 wild-type, p53-mutated, EGFR-positive, EGFR-mutated, and mouse Lewis lung carcinoma cells with GSPs, resulted in inhibition of cell proliferation, whereas these effects of GSPs were not observed in normal human bronchial epithelial cells. These data indicate that GSPs affect the proliferation of NSCLC cells and further support the concept that inhibition of tumor cell proliferation may be the fundamental mechanisms by which GSPs exert their anticancer effects. Our data also suggest that the inhibitory effect of GSPs on the proliferation of NSCLC cells is mediated through IGFBP-3 expression.

The most significant findings in the present study are those that show the effects of dietary GSPs on the growth and development of lung tumor xenografts in vivo. In these studies, two strategies were employed for dietary administration of the GSPs. In one strategy, the mice were given GSPs in diet at least 10 days before implantation of the tumor cells. In the second strategy, the mice were given GSPs in the diet after the establishment of the xenograft tumors. Our study provides evidence that dietary administration of GSPs inhibits the growth of NSCLC (A549 and H1299) tumor xenograft development under both strategies employed without any apparent sign of toxicity in athymic nude mice. We observed that the tumor growth-inhibitory effect of dietary GSPs (70-76% inhibition) was greater in first strategy than the second therapeutic approach (61-62% inhibition). It is suggested that the mice may be better adapted to dietary GSPs and better prepared to deal with the carcinogenic effect of lung tumor cells compared with the second therapeutic approach (Fig. 3) wherein mice received GSPs when the tumor growth was already started and therefore were not well adapted.

The analysis of biomarkers is an important consideration for the evaluation of cancer chemopreventive or therapeutic efficacy of any test agent. IGF-I and its major binding protein IGFBP-3 have been implicated in lung cancer and other malignancies (28–30). The antitumor efficacy of some phytochemicals, such as green tea polyphenols, curcumin and genistein (28), and β-carotene and α-tocopherol (31), has been associated with the up-regulation of IGFBP-3 levels in animal tumor models. Consistent with these reports, we observed that in vivo inhibition of lung tumor xenograft growth by dietary GSPs is associated with enhanced levels of IGFBP-3 in tumor xenograft tissues as well as its increased secretion in plasma, suggesting that the enhancement of IGFBP-3 level may be one of the prominent mechanisms through which dietary GSPs inhibit the growth of lung tumor xenograft in nude mice. This in vivo observation is supported by our in vitro data (Fig. 1D) where we have found that knockdown of IGF-BP-3 from human lung cancer cells blocked the inhibitory effect of GSPs on cellular proliferation. Some reports suggest no association among serum IGF-I, IGFBP-3, and lung cancer risk (32); thus, further studies are needed to support and define the effect of GSPs on the IGF-I/IGF-I receptor/IGFBP-3/phosphatidylinositol 3-kinase signaling cascade in lung cancer.

Biomarkers of proliferation and angiogenesis include PCNA (as a cofactor for DNA polymerase), apoptosis (DNA fragmentation as determined by TUNEL analysis), levels of CD31 (a marker of neovascularization), and VEGF (another marker of angiogenesis). Dietary GSPs reduced the rate of tumor cell proliferation (PCNA) and the extent of angiogenesis (CD31) as well as enhanced tumor cell death (TUNEL-positive cells) in the NSCLC tumor xenografts. Several types of solid tumors of epithelial origin have been shown to express and secrete VEGF, which has been implicated in uncontrolled tumor growth. Antiangiogenic interventions using monoclonal antibodies of VEGF or by blocking the receptors of VEGF have been shown to inhibit the growth of tumors (33–35). The reduction in the levels of VEGF in the NSCLC tumor xenografts of mice fed GSPs might indicate that this represents one of the important mechanisms by which GSPs exert their inhibition of the growth and progression of the NSCLC tumors may be by controlling the angiogenic process. This observation suggests that antiangiogenic potential of GSPs could be explored as one of the strategies for lowering the risk of NSCLC in human patients.

In summary, the results from the current study show for the first time the chemotherapeutic efficacy of GSPs in controlling the growth of human NSCLC cells in vitro and tumor xenograft growth in vivo. The in vitro studies show that inhibition of the growth of lung tumor xenografts in nude mice by dietary GSPs is associated with the inhibition of tumor cell proliferation, angiogenesis, and up-regulation of IGFBP-3. Further studies are required to address if these changes are mere markers of the effects of GSPs or are essential for their function in NSCLC. The present findings therefore provide preclinical information,
suggesting that GSPs have the potential to be developed as a pharmacologically safe agent for the prevention of human NSCLC risk.

References

Proanthocyanidins Inhibit Lung Tumor Growth

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
Grape Seed Proanthocyanidins Inhibit the Growth of Human Non-Small Cell Lung Cancer Xenografts by Targeting Insulin-Like Growth Factor Binding Protein-3, Tumor Cell Proliferation, and Angiogenic Factors

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