

Grape Seed Procyanidin Extract Mediates Antineoplastic Effects against Lung Cancer via Modulations of Prostacyclin and 15-HETE Eicosanoid Pathways

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Abstract

Grape seed procyanidin extract (GSE) has been reported to exert antineoplastic properties via the inhibition of cyclooxygenase-2 (COX-2)/prostaglandin E₂ (PGE₂) eicosanoid pathways. In addition, ample data link carcinogenesis to inflammatory events involving other major eicosanoid metabolic pathways, including prostacyclin (PGI₂) and 15-hydroxyeicosatetraenoic acid (15-HETE). We therefore evaluated the effects of GSE on prostacyclin synthase (PTGIS)/PGI₂ and 15-lipoxygenase-2 (15-LOX-2)/15-HETE productions by human lung premalignant and malignant cells and correlated the findings with antiproliferative or proapoptotic effects of GSE. The effects of GSE on PGI₂ and 15-HETE productions by human bronchoalveolar lavage (BAL) cells *ex vivo* were also determined. We further evaluated the bioactivity of oral administration of leucoselect phytosome (a standardized GSE) in the lungs of subjects participating in a lung cancer chemoprevention trial,

by comparing the antiproliferative effects of coculturing matched pre- versus posttreatment BAL fluids with lung premalignant and malignant cells. GSE significantly increased PGI₂ (as measured by 6-keto PGF1 α) and 15-HETE productions by these cells. Transfections of *PTGIS* or *15-LOX-2*-specific siRNA partially abrogated the antiproliferative or proapoptotic effects of GSE in lung premalignant and malignant cells, respectively. GSE also increased PTGIS and inhibition of caspase-3, and transfection of *15-LOX-2* siRNA abrogated the GSE-induced apoptosis in A549 cells. In addition, culture supernatants from *ex vivo* GSE-treated baseline BAL cells, as well as BAL fluids from subjects treated with leucoselect phytosome, significantly decreased proliferations of lung premalignant and malignant cells. Our findings support the continued investigation of GSE as an anti-neoplastic and chemopreventive agent against lung cancer. *Cancer Prev Res*; 9(12); 925–32. ©2016 AACR.

Introduction

Derived from seeds of grapes (*Vitis vinifera*), grape seed procyanidin extract (GSE) has been shown to have antioxidant capabilities 20 and 50 times that of vitamin C and E, respectively (1). Widely available as a health food supplement, GSE is used to improve cardiovascular health, such as hypertension, hyperlipidemia, atherosclerosis, and chronic venous insufficiency. Small randomized trials have found beneficial effects of GSE for diabetic retinopathy and for vascular fragility (2). Recently, preclinical studies have shown that GSE inhibited human non-small cell lung cancer (NSCLC) xenografts growth in mice, in part, through the inhibition and downregulation of cyclooxygenase-2 (COX-2) and prostaglandin (PG) E₂ eicosanoid pathways (3).

Ample data link carcinogenesis to inflammatory events involving various eicosanoid metabolic pathways. Arachidonic acid

(AA), released from membrane phospholipids upon cell stimulation, is converted to prostanoids by cyclooxygenases (COX), to leukotrienes by 5-lipoxygenase (5-LOX), and to 15(S)-hydroxyeicosatetraenoic acid (15-HETE) by 15-lipoxygenases-2 (15-LOX-2; ref. 4). Overexpression of COX-2 and subsequent overproduction of prostaglandin E₂ (PGE₂) are associated with a variety of well-established lung cancer risk factors (5–9). In two independent phase IIb lung cancer chemoprevention trials involving heavy former and/or active smokers, COX-2 inhibition with celecoxib significantly reduced bronchial Ki67 expressions, a marker of cell proliferation and the primary endpoint for the trials (10, 11). Unfortunately, the increase in cardiovascular risks associated with selective, pharmaceutical COX-2 inhibitors prevented further evaluation of celecoxib in phase III trials. Such an increase in cardiovascular risk by COX-2 selective inhibitors is thought to be, in part, due to the reduction of PGI₂, leading to an imbalance with thromboxane and is prothrombotic.

PGI₂ is an eicosanoid derived from PGH₂ by prostacyclin synthase (PTGIS); it is a potent vasodilator and inhibitor of platelet activation and aggregation (12). A majority of NSCLCs have decreased PTGIS expression (13). The potential of oral iloprost, a synthetic analogue of PGI₂, as a chemopreventive agent for lung cancer has been evaluated in a multicenter phase IIb study. In that study, iloprost has been shown to improve endobronchial dysplasia in former smokers (14). However, the short half-life, side effects, and costs of the agent have dampened enthusiasm for moving forward into phase III trials.

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Whereas 15-HETE is primarily derived from AA by 15-LOX-2, 15-HETE has been shown to bind to PPAR- γ nuclear receptors and induce apoptosis in A549 cells (15, 16), and reductions in 15-HETE have been associated with lung carcinogenesis (17). We therefore evaluated the effects of GSE on PTGIS/PGI₂ and 15-LOX-2/15-HETE pathways in lung neoplastic cells and found that GSE also concomitantly and favorably modulated these important eicosanoids signaling pathways known to be involved in lung tumorigenesis.

As part of a pilot study to determine the feasibility of leucoselect phytosome, a standardized GSE preparation complexed with soy phospholipid to improve bioavailability, for lung cancer chemoprevention, heavy current and former smokers are being recruited and treated with a 3-month course of leucoselect phytosome. To determine the effects of leucoselect phytosome on altering surrogate endpoint biomarkers (SEBM) of cancerization in the lung, serial bronchoscopies with pre- and posttreatment BAL were performed. *Ex vivo* conditioning of baseline BAL cells with GSE significantly increased the production of PGI₂ and 15-HETE, and coculture with these GSE-treated BAL cell culture supernatants decreased lung premalignant and malignant cell proliferations. Moreover, proliferation of lung neoplastic cells was significantly reduced by coculturing with post-leucoselect phytosome treatment BAL fluid in comparison with matched, pretreatment BAL fluid from the same subjects.

Materials and Methods

Leucoselect phytosome clinical study design

A single arm, dose-escalation phase I lung cancer chemoprevention study of 3 months of oral leucoselect phytosome, composed of standardized oligomeric procyanidins complexed with soy phospholipid (1:2.6 w/w; Indena), is being conducted in 20 high-risk heavy active or ex-smokers 21 years of age or older with a smoking history of at least 30 pack-years. Written informed consent is obtained in accordance with the New Mexico VA Health Care System (Albuquerque, NM) Institutional Review Board. Participants are screened with chest X-ray and fluorescence bronchoscopy (Onco-Life, Novadaq Technologies, Inc.) to rule out the presence of lung cancer. Qualified participants meeting all entry criteria (Table 1) were enrolled

and treated with 1 capsule per day for week one, 2 capsules per day for week 2, 3 capsules per day for week 3, and then 4 capsules per day for the rest of the treatment duration as tolerated. Repeat fluorescence bronchoscopy is performed at the end of treatment with BAL, bronchial brushings, bronchial biopsies to collect samples for biomarker analysis.

Bronchoalveolar lavage and *ex vivo* conditioning of alveolar macrophages

Bronchoscopies were performed as described previously (18). Briefly, subjects were prepped with a combination of topical anesthesia (4% lidocaine atomized to posterior pharynx plus 1%–2% aliquots of topical lidocaine along the trachea-laryngo-bronchial tree as needed) and moderate sedation using incremental doses of midazolam and fentanyl according to institutional guidelines. A fiberoptic video bronchoscope (BF 20D, Olympus America) was advanced into the airway and wedged into a subsegment of the right middle lobe. Four 60-mL aliquots of room temperature saline were serially lavaged and recovered by manual syringe suction. Recovered fluid was passed through a 100- μ m sterile nylon filter (Becton Dickinson) to remove mucus and particulates, pooled, and centrifuged at 300 \times g for 8 minutes at 4°C. The BAL cells and fluid were then harvested, processed, aliquoted, and stored at –80°C until analysis.

BAL cell pellets were first washed in Hank's balanced salt solution (Sigma-Aldrich) and then resuspended in X-VIVO 15 serum-free medium (BioWhittaker) to a concentration of 0.5 \times 10⁶ cells/mL. After 2-hour incubation/adherence, cells were treated with GSE overnight at 37°C for 18 to 24 hours. Culture supernatants were harvested for eicosanoids determination.

Cell cultures

As models to evaluate the antineoplastic effect of GSE against lung cancer, the human NSCLC cell lines A549 and H23, the small-cell lung cancer (SCLC) cell line DMS114 (ATCC), and the bronchial premalignant cell line 1198 (derived from tracheal transplant of BEAS-2B cells treated with cigarette smoke condensate, generously provided by Dr. Klein-Santos, Fox Chase Cancer Center, Philadelphia, PA; ref. 19) were studied *in vitro*. Experiments involving all commercial cell lines were initiated within 6 months of purchase. Cell lines were not further

Table 1. Entry criteria for the phase I lung cancer chemoprevention trial with leucoselect phytosome

Inclusion criteria:

- 1 Age over 21.
- 2 Smoking history >30 pack years.

Exclusion criteria:

- 1 Inability to provide informed consent (e.g., cognitive impairment, severe psychiatric disorders).
- 2 Hypersensitivity to grapes and related products.
- 3 Liver dysfunction (abnormal liver function tests).
- 4 Renal dysfunction (abnormal serum creatinine).
- 5 End-stage respiratory disease (FEV1 < 0.8 liters, resting or exertional hypoxemia, to select patients with adequate reserve to undergo bronchoscopy and complete the study).
- 6 Unstable angina.
- 7 Malignancy within 5 years, excluding nonmelanoma type skin cancer or stage I NSCLC postcurative resection without evidence of recurrence.
- 8 Pregnancy.
- 9 Systemic corticoid steroid therapy.
- 10 Coagulopathy.
- 11 Concurrent use of grapes or grape-related products.
- 12 Unwilling to refrain from drinking more than 1 glass of wine a day.
- 13 Patients with concurrent medical conditions that may interfere with completion of tests, therapy, or the follow-up schedule.

authenticated. ATCC uses short tandem repeat profiling for cell line authentication. Cells were maintained as monolayers in an atmosphere of 5% CO₂ in air at 37°C in 25-cm² tissue culture flasks containing 5.0 mL of RPMI1640 medium supplemented with 10% FBS, 100 units/mL of penicillin, 0.1 mg/mL of streptomycin, and 2 mmol/L of glutamine (JRH Biosciences) for A549; RPMI1640 medium with 2 mmol/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, and 1.0 mmol/L sodium pyruvate, 10% FBS, for H23 cells; and Waymouth's MB 752/1 medium with L-glutamine and 10% FBS for DMS114.

Aliquots of 0.1×10^6 cells were incubated at 37°C for 2 hours. Varying doses of GSE (6, 12, 15, 18, 30, 45, or 60 µg/mL) were added, and the cells were incubated at 37°C for 18 to 44 hours. The dose range of GSE was chosen based on considerations from prior published studies (20, 21). Primary normal human bronchial epithelial (NHBE) cells (Lonza) and BEAS-2B cells (nontumorigenic immortalized bronchial epithelial cells, ATCC) were used as controls and maintained according to the manufacturer's instructions. NHBE and BEAS-2B cells were plated in wells at 0.5×10^6 cells/mL and treated with varying doses of GSE for 18 to 24 hours.

To ascertain the involvement of caspase-3 in our model systems, the caspase-3 inhibitor Z-DEVD-FMK (100 µmol/L, R&D Systems) was added one hour prior to the addition of GSE. Experiments with dose titrations of the inhibitor were first carried out to determine the optimal inhibitory dose without compromising cell viability. Standardized GSE (90% procyanidins) was purchased from commercially available sources (Organic Herb Inc.). Stock solutions of GSE were made by dissolving the extract with deionized water. Aliquots of the stock were stored at -80°C and used only once for each set of experiments.

For MTT assays, cells were plated at concentration of 8–15 × 10³ cells per well in 96-well plates, treated and cultured, then subjected to the MTT assay.

All culture supernatants, cell lysates for total protein, and total RNA harvested from cell lysates were stored at -80°C until analysis, when applicable. Samples of mRNA were collected using the miRNeasy Mini Kit (Qiagen Inc.), per the manufacturer's instructions.

MTT assay

To quantify cellular proliferation in conditioned cells, the MTT Cell Proliferation Assay (ATCC) was used to measure the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells according to the manufacturer's instructions.

Cell death ELISA

For quantification of apoptosis, the Cell Death Detection ELISA Kit (Roche) was used. To quantify apoptosis in the conditioned cells, specific measurements of mono- and oligonucleosomes by immunochemical determination of histone-complexed DNA fragments in the cytoplasmic fraction of cell culture lysates were performed using the Cell Death ELISA Kit according to the manufacturer's instructions.

Quantigene assay

Following overnight treatment with GSE, cells were washed twice with PBS, counted, followed by addition of lysis buffer to

400 cells/µL. The gene expression level was determined using the QuantiGene Plex 2.0 Assay Kit (Affymetrix) per the manufacturer's instructions. The assay is based on DIRECT quantification of the RNA targets using xMAP Luminex beads and branched DNA signal amplification technology. For each RNA molecule of interest, an oligonucleotide probe set, containing three types of synthetic probes, capture extenders, label extenders, and blockers that hybridize and span contiguous sequences of the target RNA, is used.

siRNA transfection

Transfections of *PTGIS*- and *15-LOX-2*-specific siRNA into lung neoplastic cells were achieved using specific siRNA and transfecting reagents from Qiagen Inc. The cell numbers and the amount of siRNA mimics and transfection reagent were first optimized according to manufacturer's instructions. Briefly, $6-8 \times 10^3$ of

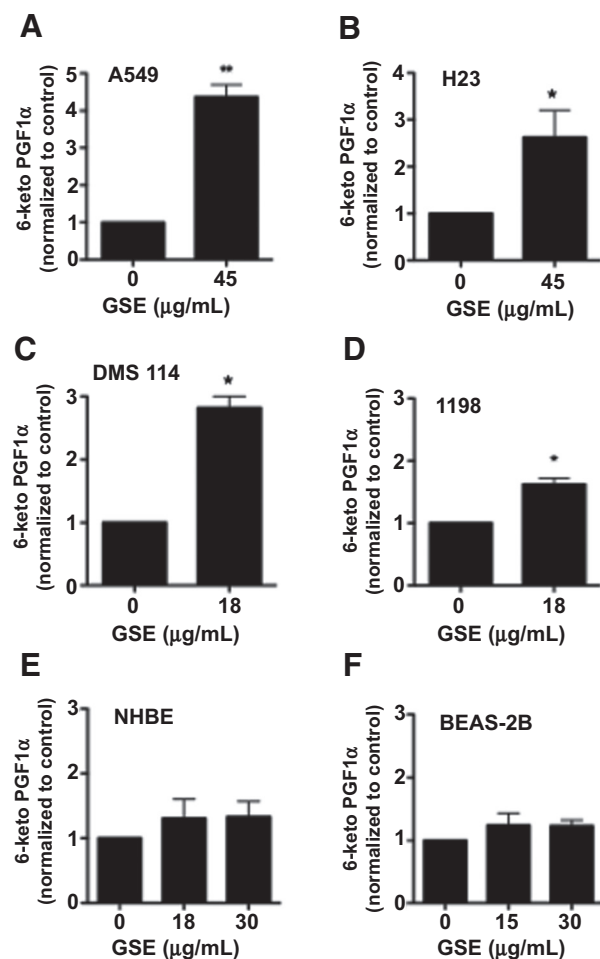


Figure 1.

A-F, overnight treatment with GSE increased production of 6-keto PGF1 α , a stable metabolite of PGI₂, by NSCLC cell lines A549 (**A**) and H23 (**B**), SCLC cell line DMS114 (**C**), and the bronchial pre-malignant cell line 1198 (**D**), but not by NHBE (**E**) or BEAS-2B (**F**) cells (cell proliferation as measured by MTT also did not change significantly, data not shown). The levels of 6-keto PGF1 α were measured in culture supernatants as a surrogate for PGI₂ production in response to GSE treatment. The range of control PGI₂ levels for various malignant and pre-malignant cell lines is 45 to 219 pg/mL. Columns, mean; bars, SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$.

cells in 150 μ L medium were aliquoted into 96-well plates. After 1 hour of adherence, 50 μ L of specific miRNA transfection complexes were gently added to the cells with gentle swirling. The cells were incubated for 42 hours and then treated overnight with GSE for 24 hours, followed by the MTT assay. Cell conditions were scaled up 10-fold in 12-well plates for total RNA harvest.

Quantification of 15-HETE

Measurements of 15-HETE concentrations in conditioned culture supernatants were performed using enzyme-specific immunoassay (EIA) according to the manufacturer's EIA Kit protocol (Cayman Chemical).

Quantification of 6-keto PGF1 α

Measurements of 6-keto PGF1 α concentrations in conditioned culture supernatants were performed using EIA according to the manufacturer's EIA Kit protocol (Cayman Chemical).

PTGIS ELISA

Levels of PTGIS protein were measured in GSE-treated A549 cell lysate using a PTGIS-specific ELISA per the manufacturer's instruction (Cloud-Clone Corp.).

Quantification of caspase-3 activity

For quantification of caspase-3 activity, the Caspase-3/CPP32 Colorimetric Assay Kits (BioVision) were used. The assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA. The pNA light emission was quantified using a microtiter plate reader at 405 nm. Freshly harvested cell extracts from conditioned cultures were assayed per the manufacturer's instructions.

Statistical analysis

Data are expressed as the mean \pm SD in all circumstances where mean values are compared. Data were analyzed by paired Student *t* test and/or ANOVA. Batch analyses were performed for each

comparison group to eliminate interassay variability. Differences are considered significant when $P < 0.05$.

Results

GSE increased 6-keto PGF1 α production by lung premalignant and malignant cells

To explore the effects of GSE on PGI₂ production by lung neoplastic cells, NSCLC cell lines A549 and H23, the SCLC cell line DMS114, and the bronchial preneoplastic cell line 1198 were treated with GSE overnight. Because of the short half-life of PGI₂, levels of 6-keto PGF1 α were measured in culture supernatants as a surrogate for PGI₂, production in response to GSE treatment. GSE significantly increased the production of 6-keto PGF1 α in A549 (Fig. 1A) and H23 cells (Fig. 1B), DMS114 (Fig. 1C) and 1198 (Fig. 1D) cells, but not in NHBE (Fig. 1E) or BEAS-2B (Fig. 1F) cells.

GSE increased *PTGIS* mRNA expression and protein production, and *PTGIS*-siRNA transfection abrogated the antiproliferative effects of GSE in lung premalignant and malignant cells

To further characterize the molecular mechanisms involved in GSE-mediated increase in PGI₂ production, the effects of GSE treatment on *PTGIS* mRNA expression in A549 cells were measured following overnight treatment. GSE dose dependently increased *PTGIS* mRNA expression (Fig. 2A). GSE also dose dependently increased PTGIS protein production in A549 cells (Fig. 2B). Moreover, transfection of *PTGIS*-siRNA partially reversed the GSE-induced antiproliferative effects on both A549 (Fig. 2C) and 1198 cells (Fig. 2D).

GSE increased 15-HETE production and 15-LOX-siRNA transfection abrogated the apoptotic effects of GSE by lung premalignant and malignant cells

15-HETE is one of the most abundant eicosanoid in the bronchi, has been shown to induce apoptosis in A549 cells, and is believed to be anticarcinogenic (15–17). To determine whether 15-HETE plays a role in GSE-induced apoptosis, the effects of GSE

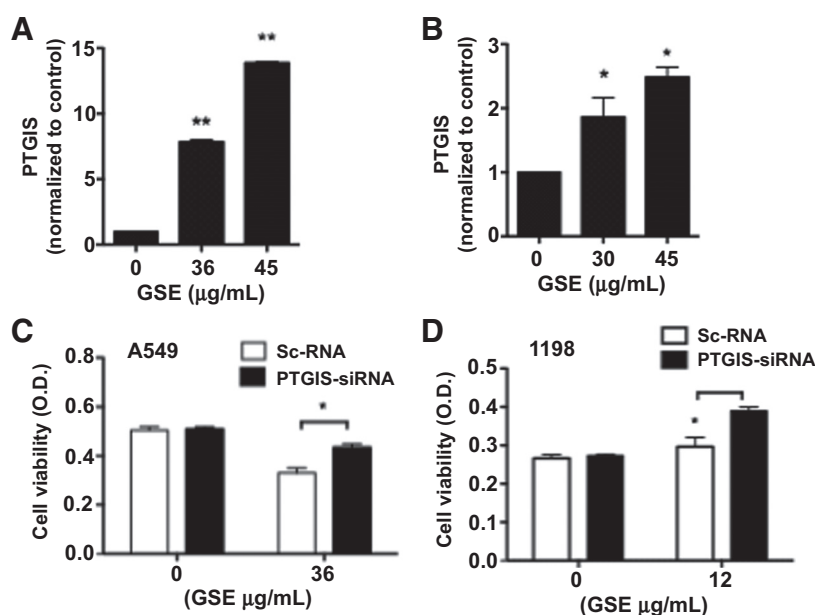


Figure 2.

A–C, GSE dose dependently increased *PTGIS* mRNA expression (**A**) and protein production (**B**) in A549 cells. **C and D,** moreover, transfection of *PTGIS*-siRNA partially reversed the GSE-induced antiproliferative effects on both A549 (**C**) and 1198 cells (**D**). O.D., optical density. Control, cells transfected with scrambled RNA (Sc-RNA). Columns, mean; bars, SD ($n = 3$). *, $P < 0.05$; ** $P < 0.01$.

treatment on 15-HETE production by A549 (Fig. 3A), 1198 (Fig. 3B), and DMS114 (Fig. 3C) cells were evaluated. GSE significantly increased 15-HETE production by these cells. Furthermore, transfection with 15-LOX-siRNA abrogated the apoptotic effects of GSE in A549 (Fig. 3D) and 1198 (Fig. 3E) cells.

GSE increased caspase-3 activation and inhibition of caspase-3 abrogated the apoptotic effect of GSE in A549 cells

We previously reported the involvement of the 15-LOX/15-HETE pathway in caspase-3 activation (16). To determine whether the GSE-induced apoptosis is mediated by caspase-3, A549 cells were treated with GSE in the presence or absence of the caspase-3 inhibitor, Z-DEVD-FMK (DEVD). GSE significantly increased caspase-3 activation, whereas DEVD abrogated the GSE-induced apoptosis (Fig. 4). Similar findings were observed in 1198 cells (data not shown).

GSE dose dependently increased 6-keto PGF1 α and 15-HETE production by human BAL cells

BAL represents a minimally invasive bronchoscopic sampling procedure to obtain cells and molecules from the lung microenvironment. BAL cells are composed of predominantly alveolar macrophages (AM), the major immunoeffector cells

and source of eicosanoids in the lung microenvironment. Through modulations of eicosanoids, AMs likely play a central role in mediating immune surveillance and antitumor immunity in the lung microenvironment. As such, we evaluated the effects of GSE on 6-keto PGF1 α and 15-HETE productions by human BAL cells *ex vivo*. Treatment with GSE for 24 hours significantly increased both 15-HETE (Fig. 5A) and 6-keto PGF1 α (Fig. 5B) production by BAL cells. This finding suggests that GSE may favorably modulate anticarcinogenic forces in the lung microenvironment, in part, via increasing PGI₂ and 15-HETE productions from BAL cells.

Culture supernatants from GSE-treated BAL cells, as well as BAL fluid from human subjects treated with leucoselect phytosome, significantly inhibited growth of lung premalignant and malignant cells

To better simulate the lung microenvironment, culture supernatants from baseline BAL cells pretreated with GSE (6 μ g/mL) *ex vivo* were used in coculture with A549 and significantly inhibited its cell proliferation, at one tenth of the dose required to induce similar effects when A549 cells were treated with GSE alone (Fig. 5C). Furthermore, A549, DMS114, and 1198 cells were treated with matched BAL fluid obtained from

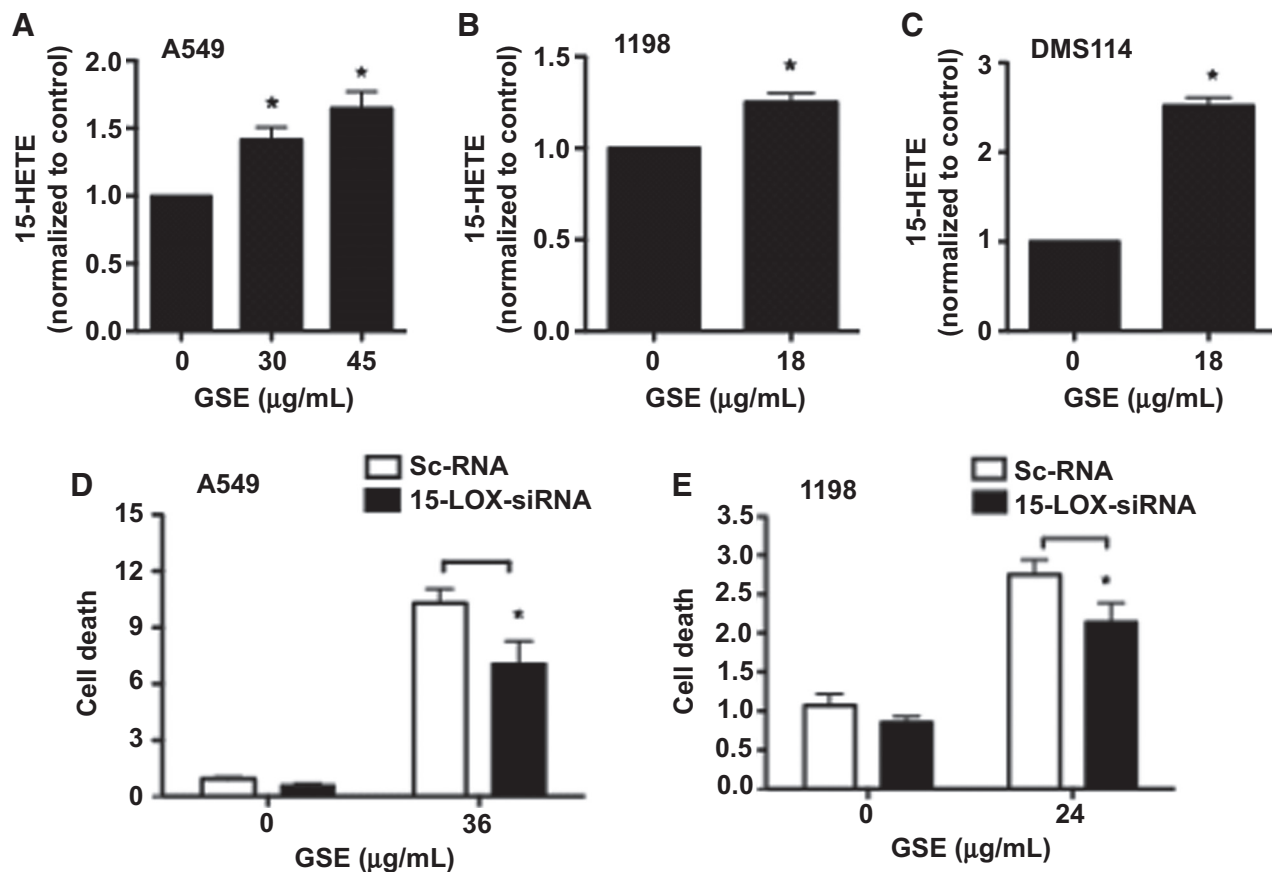


Figure 3.

A–C, GSE significantly increased 15-HETE production by both A549 (**A**) and 1198 (**B**) and DMS 114 (**C**) cells, correlating with GSE-induced apoptosis. The range of control 15-HETE levels is approximately 6 to 800, 2 to 400, and 2 to 3,000 pg/mL for A549, 1198, and DMS114 cells, respectively. **D** and **E,** transfection with 15-LOX-siRNA partially abrogated the apoptotic effects of GSE in A549 (**D**) and 1198 (**E**) cells. Control, cells transfected with scrambled RNA (Sc-RNA). Columns, mean; bars, SD ($n = 3$). *, $P < 0.05$.

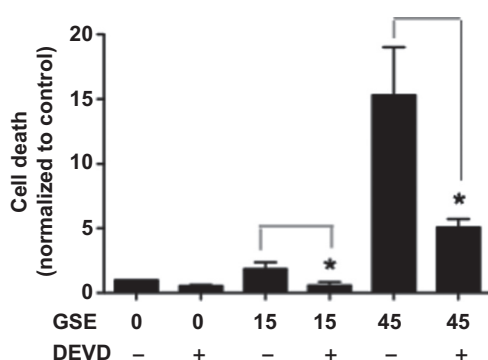


Figure 4.

A549 cells were conditioned with GSE in the presence or absence of the caspase-3 inhibitor, Z-DEVD-FMK (DEVD). GSE significantly increased caspase-3 activation, whereas DEVD abrogated the GSE-induced apoptosis. Similar findings were observed in 1198 cells (data not shown). Columns, mean; bars, SD ($n = 3$). *, $P < 0.05$.

subjects pre- and post 3 months of oral leucoselect phytosome treatment. Posttreatment BAL fluid, despite 1:4 dilution in culture medium, significantly reduced proliferation of A549 (Fig. 6A), 1198 (Fig. 6B), and DMS114 cells (Fig. 6C).

Discussion

In this study, we demonstrate, for the first time, the roles of PTGIS/PGI₂ and 15-LOX-2/15-HETE in mediating the antineoplastic and chemopreventive properties of GSE against lung cancer. GSE increases PGI₂ (as measured by 6-keto PGF1 α) productions in human lung premalignant, malignant, and BAL cells, suggesting that GSE may function both as a natural COX-2 inhibitor and an inducer of PGI₂. Furthermore, GSE significantly increased 15-HETE production by lung premalignant, malignant and BAL cells, and inhibition of caspase-3 abrogated the GSE-induced apoptosis in A549 cells. In addition, culture supernatants from GSE-treated BAL cells, as well as BAL fluid from human subjects treated with leucoselect phytosome, significantly inhibited growth of lung premalignant and malignant cells. The fact that GSE did not significantly alter PGI₂ production in NHBE and BEAS-2B cells suggests that GSE acts on later stages of carcinogenesis. Our findings reveal novel antineoplastic mechanisms involving major eicosanoid signaling pathways by GSE and support the continue investigation of GSE as an antineoplastic and chemopreventive agent for lung cancer.

Whereas the anticancer mechanism of GSE is still incompletely understood, preclinical studies have shown that the anticancer effects of GSE are mediated, at least in part, through the inhibition and downregulation of COX-2/PGE₂ pathways (3). The unexpected increase in cardiovascular risk associated with pharmaceutical COX-2 inhibitors has dampened enthusiasm for their use in chemoprevention. Exploring the potential utility of a natural COX-2 inhibitor with favorable side effect profiles that may also be cardio-protective, such as GSE, for treatment and prevention of lung cancer is clearly justified.

To translate the preclinical findings into a lung cancer chemoprevention trial, we first performed a literature search to identify and select a GSE agent. Because the absorption of GSE is affected by molecular weight (22), variable compositions of GSE polyphenol in different products on the market further contribute to

low and erratic bioavailability (22, 23). An inexpensive GSE standardized to smaller size oligomeric procyanidins and complexed with soy phospholipids into phytosomes to improve bioavailability (leucoselect) is available over the counter. Leucoselect had been shown to improve the total antioxidant capacity of plasma and reduce LDL susceptibility to oxidative stress in heavy smokers (24). As such, we evaluated its efficacy in our preclinical study, using an A549 lung cancer xenograft nude mouse model, and demonstrated significant efficacy in tumor growth inhibition (25). These findings set the stage for our lung cancer chemoprevention trial with leucoselect phytosome. Although leucoselect phytosome has been used as a health food supplement to promote cardiovascular health for decades, the mechanism by which leucoselect may exert benefits on cardiovascular health is poorly understood. Our findings suggest that the increase in PGI₂ production may contribute to its cardiovascular benefits.

In view of the fact that the ability of GSE to inhibit COX-2 may lead to shunting of AA toward 15-LOX pathways, we evaluated the effects of GSE on 15-HETE productions in lung premalignant and malignant cells and human BAL cells (*ex vivo*). GSE dose dependently increased 15-HETE productions by these cells, but GSE did not increase expression of 15-LOX (1 or 2) in A549 cells (data not shown), suggesting that the increase of 15-HETE by GSE is also a result of shunting, as was previously found in a phase IIb clinical trial with celecoxib (11). Taken together, these findings reflect the

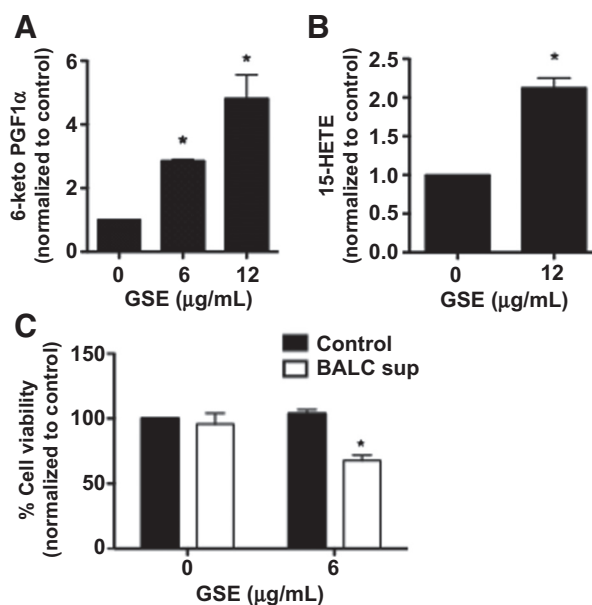


Figure 5.

A and **B**, GSE dose dependently increases 6-keto PGF1 α (**A**) and 15-HETE (**B**) productions by human BAL cells *ex vivo*. 6-keto PGF1 α and 15-HETE levels were measured in culture supernatants after overnight incubation with varying doses of GSE ($\mu\text{g/mL}$). The range of control 6-keto PGF1 α and 15-HETE levels are ~85 to 125 pg/mL and ~6 to 7,000 pg/mL, respectively. Much less GSE is required to significantly increase 6-keto PGF1 α and 15-HETE productions by BAL cells than A549 cells. Columns, mean; bars, SD ($n = 3$). *, $P < 0.05$. Proliferation of A549 (**C**) cells is significantly inhibited when the cells are treated with culture supernatants from GSE-pretreated BAL cells [6 $\mu\text{g/mL}$, 1:1 GSE treated BAL culture supernatant (BALC sup): RPMI], at a much lower dose (final concentration of GSE = 3 $\mu\text{g/mL}$) than the dose required (30 $\mu\text{g/mL}$) to induce a similar response when A549 cells are treated with GSE alone. Columns, mean; bars, SD ($n = 3$). *, $P < 0.05$.

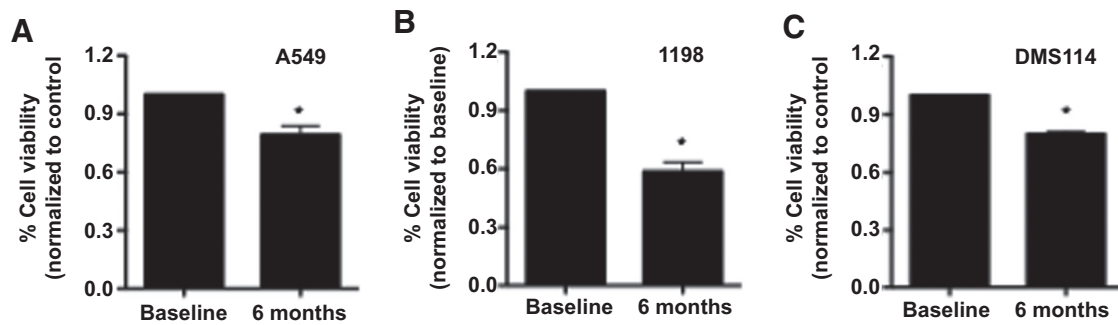


Figure 6.

To determine the bioactivity of oral administration of leucoselect phytosome in the lung, A549, 1198, and DMS114 cells were treated with matched BAL fluid (BAL: culture medium = 1:4) obtained from the first three subjects pre- and post 3 months of oral leucoselect phytosome treatment. **A-C**, posttreatment BAL fluid significantly reduced proliferation of A549 (**A**), 1198 cells (**B**), and DMS114 cells (**C**). Columns, mean; bars, SD ($n = 3$). *, $P < 0.05$.

additional influence of increased 15-HETE in the setting of COX-2 inhibition on dampening the driving force of cancerization in the lungs.

During the sequence of events associated with apoptosis, caspase-3, an intracellular cysteine protease that exists as a proenzyme, becomes activated. Caspase-3 activation represents an important mechanism of apoptosis induced by a wide variety of apoptotic signals (26). In our study, we find that GSE-induced apoptosis in NSCLC cells involves activation of caspase-3, and inhibition of caspase-3 significantly abrogates the GSE-induced apoptosis in A549 cells. Our group previously described the involvement of 15-LOX pathway in caspase-3 activation (16). Accordingly, the increase of 15-HETE production by GSE in lung premalignant and malignant cells likely plays a role in activation of caspase-3 and induction of apoptosis. GSE-mediated apoptotic cell death in NSCLC cell lines has been previously shown to involve the activation of caspase-9 (27), and 15-HETE has also been reported to activate caspase-9 (28). Caspase-9 is an initiator caspase that cleaves/activates procaspase-3. As such, GSE-induced 15-HETE production likely activates caspase-3 via caspase-9. Whether or not GSE activates caspase-9 through other mechanisms independent of 15-HETE is unclear and remains to be elucidated.

Another interesting observation from the current study is that the SCLC cell line DMS114 is far more sensitive to the antiproliferative effects of GSE with an *in vitro* IC₅₀ of approximately 3-fold less than that of the NSCLC cell lines (data not shown). Approximately 10% to 15% of lung cancer cases are SCLC (29). Although SCLC is very responsive to platinum-based chemotherapy and radiation (30), resistance to therapy quickly develops. The overall prognosis of SCLC is dismal (31, 32). People with advanced stage SCLC usually survive for less than 1 year. Even with limited-stage disease, the median survival time is less than 2 years. Furthermore, people with SCLC have the highest rate of developing a second primary cancer, usually in 5% to 10% of cases, but as high as 30%. Almost four decades since the introduction of the platinum-etoposide doublet chemotherapy, therapeutic options have remained virtually unchanged (33). To our knowledge, this is the first report on antineoplastic effects of GSE against SCLC cancer. Conceivably, GSE may also present important opportunities for SCLC chemoprevention and treatment.

Lung cancer is the leading cause of cancer death in the world (32). Despite advancements in anticancer treatments, the 5-year

survival for lung cancer remains dismal (32). The lack of effective therapy provides the impetus to search for alternative, safe, and efficacious agents for lung cancer chemoprevention, to impede the driving force of cancerization, and prevent lung cancer development in at-risk individuals (34). The encouraging findings from phase IIb lung cancer chemoprevention trials with celecoxib and iloprost have engendered discussions on developing a study using a combination of the two agents, so that iloprost may negate the increased cardiovascular risk associated with pharmaceutical COX-2 inhibitors, while exerting synergistic antineoplastic effects. In the end, the various issues associated with both agents have prevented further investigation, yet opening doors to explore the potential utility of natural COX-2 inhibitors that may simultaneously increase PGI₂ and promote cardiovascular health, such as GSE. In this study, we demonstrate novel GSE-mediated antineoplastic mechanisms; modulation of these mechanisms may also be useful as SEBM in clinical trials. Our findings provide important rationales to continue the clinical investigation of the potential of GSE for lung cancer chemoprevention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.T. Mao

Development of methodology: J.T. Mao, J. Smoake, B. Xue

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.T. Mao, J. Smoake, H.K. Park, Q.-Y. Lu, B. Xue

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.T. Mao, J. Smoake, B. Xue

Writing, review, and/or revision of the manuscript: J.T. Mao, Q.-Y. Lu

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.T. Mao, J. Smoake

Study supervision: J.T. Mao

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