Dietary intake of a plant phospholipid/lipid conjugate reduces lung cancer growth and tumor angiogenesis

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It is well recognized that early detection and cancer prevention are significant armaments in the ‘war against cancer’. Changes in lifestyle and diet have significant impact on the global incidence of cancer. For over 30 years, many investigators have studied the concept of chemoprevention. More recently, with the demonstration that antiangiogenic activity reduces tumor growth, the concept of angioprevention has emerged as a novel strategy in the deterrence of cancer development (carcinogenesis). In this study, we utilized a fast growing, highly aggressive murine Lewis lung cancer model to examine the in vivo antitumor effects of a novel, dietary supplement, known as plant phospholipid/lipid conjugate (pPLC). Our goal was to determine if pPLC possessed direct antitumor activity with relatively little toxicity that could be developed as a chemoprevention therapy. We used pPLC directly in this in vivo model due to the lack of aqueous solubility of this novel formulation, which precludes in vitro experimentation. pPLC contains known antioxidants, ferulic acid and lipoic acid, as well as soy sterols, formulated in a unique aqueous-insoluble matrix. The pPLC dietary supplement was shown to suppress in vivo growth of this tumor model by 30%. We also demonstrated a significant decrease in tumor angiogenesis accompanied by increased apoptosis and present preliminary evidence of enhanced expression of the hypoxia-related genes pentraxin-3 and metallothionein-3, by 24.9-fold and 10.9-fold, respectively, compared with vehicle control. These findings lead us to propose using this plant phospholipid/lipid conjugate as a dietary supplement that may be useful in cancer prevention.

Introduction

The National Cancer Institute recognizes that prevention is an essential component to the strategic plan to eliminate the suffering and death due to cancer. It is estimated that 30–40% of cancers can be directly linked to dietary habits. People who eat a diet high in plant foods have a much lower risk of developing cancer. Despite a growing body of evidence supporting an association between diets rich in foods and at reduced risk of cancer. For over 30 years, many investigators have studied the concept of chemoprevention. More recently, with the concept of antiangiogenesis as a novel strategy in the deterrence of cancer development (carcinogenesis). In this study, we utilized a fast growing, highly aggressive murine Lewis lung cancer model to examine the in vivo antitumor effects of a novel, dietary supplement, known as plant phospholipid/lipid conjugate (pPLC). Our goal was to determine if pPLC possessed direct antitumor activity with relatively little toxicity that could be developed as a chemoprevention therapy. We used pPLC directly in this in vivo model due to the lack of aqueous solubility of this novel formulation, which precludes in vitro experimentation. pPLC contains known antioxidants, ferulic acid and lipoic acid, as well as soy sterols, formulated in a unique aqueous-insoluble matrix. The pPLC dietary supplement was shown to suppress in vivo growth of this tumor model by 30%. We also demonstrated a significant decrease in tumor angiogenesis accompanied by increased apoptosis and present preliminary evidence of enhanced expression of the hypoxia-related genes pentraxin-3 and metallothionein-3, by 24.9-fold and 10.9-fold, respectively, compared with vehicle control. These findings lead us to propose using this plant phospholipid/lipid conjugate as a dietary supplement that may be useful in cancer prevention.

Abbreviations: FGF2, fibroblast growth factor-2; IL, interleukin; LL2-LUC, luciferase-labeled Lewis lung carcinoma; MAP, mouse antibody production; pPLC, plant phospholipid/lipid conjugate; RT-PCR, real-time PCR.
chelation. The reduced form known as dihydrolipoic acid has potent antioxidant activity and inactivates reactive oxidative species (23). Given that food intake may reduce the absorption of lipoic acid, it is therefore recommended that lipoic acid be taken for therapeutic purposes from exogenous sources such as dietary supplements between meals. Dietary supplements containing up to 600 mg are available providing 1000 times greater dose than that available from diet alone (22). Lipoic acid supplements in dosages as high as 1800 mg/day are associated with significant weight loss, and doses of 2400 mg/day have been shown to be useful in clinical trials for the prevention of cardiovascular disease with little or no adverse effects (24–26). Lipoic acid acts as an important coenzyme for several mitochondrial enzymes and protects these organelles from oxidative stress. Lipoic acid also inhibits growth of a variety of cancer cells in vitro including human colon cancer (HT-29) cells, Jurkat (acute T-cell leukemia) and FaDu (pharyngeal squamous carcinoma) cells, as well as murine B16F10 melanoma cells. The principal mechanisms involved in these in vitro effects were antioxidant activity and induction of apoptosis (8,23,27,28).

We identified a commercially available dietary supplement plant phospholipid/lipid conjugate (pPLC) available from Conjugated Functional Foods (Hackensack, NJ), which combines soy sterols, ferulic acid and lipoic acid through a proprietary process with a variety of other ingredients that are crucial to the formulation and conjugation process. As discussed above, previous studies have shown that soy sterols, ferulic acid and lipoic acid each possesses low bioavailability but overlapping biological antioxidant and antiangiogenic activities. Given the long time course involved in true ‘chemoprevention and angioprevention’ studies, we instead selected a highly aggressive syngeneic mouse lung cancer model (Lewis lung carcinoma) in order to more rapidly (24–28 days) evaluate the in vivo antitumorigenic and antiangiogenic potential of pPLC. In addition, we utilized histologic staining and messenger RNA expression profiling to examine changes in gene expression possibly associated with antitumor mechanisms.

Materials and methods

Feed composition and preparation

The composition of pPLC and vehicle control conjugates are shown as weight percent in Table I. Purina mouse chow is the standard diet given to all mice housed in the Clinical Cancer Research Mouse Vivarium at National Institutes of Health. Accordingly, we used this feed to deliver both the pPLC-containing and control-conjugated formulations since they are insoluble in aqueous solution. In these experiments, pPLC or the vehicle control conjugates were physically milled into the Purina mouse chow, resulting in powdered chow containing different weight percent concentrations of the vehicle or pPLC conjugates. Powdered chow was fed ad libitum using Follower feeders. Preliminary experiments demonstrated that mice fed chow containing concentrations of pPLC greater than 5% failed to ingest sufficient feed to demonstrate the normal gain in body weight observed in control mice.

Mice and tumorigenicity studies

Five- to 8-week-old female C57BL/6j mice (Jackson Laboratories, Bar Harbor, ME) were housed 5 mice/cage, under a 12-h light/dark cycle with access to Purina Chow containing 5% vehicle control or 5% pPLC conjugate and water ad libitum in accordance with an approved National Institutes of Health Institutional Animal Care and Use Committee protocol (LJ-003). Luciferase-labeled Lewis lung carcinoma (LL2-LUC) cells (Caliper Life Sciences, Hopkinton, MA) were grown in Dulbecco’s Modified Eagle Medium/F-12 media 1:1 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma–Aldrich, St. Louis, MO) in a humidified incubator containing 5% CO2 at 37°C. Prior to subcutaneous injection, LL2-LUC cells were mouse antibody production tested (NCI/FCRF, Frederick, MD), grown to ~80% confluence and counted using the Z1 Counter Particle Counter (Beckman Coulter, Brea, CA). The viability of LL2-LUC cell cultures were determined by Trypan Blue staining (Invitrogen, Carlsbad, CA). Each mouse was injected subcutaneously in the right flank with 0.25 × 106 LL2-LUC cells resuspended in 100 µl phosphate-buffered saline. Five or 15 mice per experimental group were used in three replicate experiments. Mice were switched to chow containing 5% weight volume pPLC or vehicle control conjugate 3 days post tumor cell inoculation to allow time for tumor take. Feed was changed every 48 h. At the indicated number of days posttumor cell injection, mice were weighed, tumor growth was monitored by luminescence using the IVIS Spectrum Imaging System (Perkin Elmer) and the Living Image Software v3.1 (Caliper Life Sciences, Hopkinton, MA), and tumor volumes were calculated by manual caliper measurements using the following formula: Volume = π x (Width/2)² x Length.

Feed was changed every 48 h. At the indicated number of days posttumor cell injection, mice were weighed, tumor growth was monitored by luminescence using the IVIS Spectrum Imaging System (Perkin Elmer) and the Living Image Software v3.1 (Caliper Life Sciences, Hopkinton, MA), and tumor volumes were calculated by manual caliper measurements using the following formula: Volume = π x (Width/2)² x Length. For luminescence measurements, mice were injected intraperitoneally with 100 µl of 37.5 mg/ml D-luciferin diluted in sterile phosphate-buffered saline 15 min prior to imaging. The experiment was repeated at least 3 times using either 5 or 15 mice per group in each experiment.

Immunohistochemistry

Immunohistochemistry was performed to examine CD-31 expression in paraffin-embedded formalin-fixed tumor tissue using the avidin–biotin peroxidase method with the Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Prior to staining, slides were deparaffinized in a series of xylene and graded alcohols, followed by antigen retrieval in 0.01 M Citrate buffer (pH 6) and incubation in 3% hydrogen peroxide to quench endogenous peroxidase. Immunohistochemistry was performed to examine CD-31 expression in paraffin-embedded formalin-fixed tumor tissue using the avidin–biotin peroxidase method with the Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Prior to staining, slides were deparaffinized in a series of xylene and graded alcohols, followed by antigen retrieval in 0.01 M Citrate buffer (pH 6) and incubation in 3% hydrogen peroxide to quench endogenous peroxidase. Immunohistochemistry was performed to examine CD-31 expression in paraffin-embedded formalin-fixed tumor tissue using the avidin–biotin peroxidase method with the Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Prior to staining, slides were deparaffinized in a series of xylene and graded alcohols, followed by antigen retrieval in 0.01 M Citrate buffer (pH 6) and incubation in 3% hydrogen peroxide to quench endogenous peroxidase. Immunohistochemistry was performed to examine CD-31 expression in paraffin-embedded formalin-fixed tumor tissue using the avidin–biotin peroxidase method with the Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Prior to staining, slides were deparaffinized in a series of xylene and graded alcohols, followed by antigen retrieval in 0.01 M Citrate buffer (pH 6) and incubation in 3% hydrogen peroxide to quench endogenous peroxidase. Immunohistochemistry was performed to examine CD-31 expression in paraffin-embedded formalin-fixed tumor tissue using the avidin–biotin peroxidase method with the Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Prior to staining, slides were deparaffinized in a series of xylene and graded alcohols, followed by antigen retrieval in 0.01 M Citrate buffer (pH 6) and incubation in 3% hydrogen peroxide to quench endogenous peroxidase. Immunohistochemistry was performed to examine CD-31 expression in paraffin-embedded formalin-fixed tumor tissue using the avidin–biotin peroxidase method with the Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Prior to staining, slides were deparaffinized in a series of xylene and graded alcohols, followed by antigen retrieval in 0.01 M Citrate buffer (pH 6) and incubation in 3% hydrogen peroxide to quench endogenous peroxidase. Immunohistochemistry was performed to examine CD-31 expression in paraffin-embedded formalin-fixed tumor tissue using the avidin–biotin peroxidase method with the Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Prior to staining, slides were deparaffinized in a series of xylene and graded alcohols, followed by antigen retrieval in 0.01 M Citrate buffer (pH 6) and incubation in 3% hydrogen peroxide to quench endogenous peroxidase. Immunohistochemistry was performed to examine CD-31 expression in paraffin-embedded formalin-fixed tumor tissue using the avidin–biotin peroxidase method with the Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Prior to staining, slides were deparaffinized in a series of xylene and graded alcohols, followed by antigen retrieval in 0.01 M Citrate buffer (pH 6) and incubation in 3% hydrogen peroxide to quench endogenous peroxidase. Immunohistochemistry was performed to examine CD-31 expression in paraffin-embedded formalin-fixed tumor tissue using the avidin–biotin peroxidase method with the Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Prior to staining, slides were deparaffinized in a series of xylene and graded alcohols, followed by antigen retrieval in 0.01 M Citrate buffer (pH 6) and incubation in 3% hydrogen peroxide to quench endogenous peroxidase. Immunohistochemistry was performed to examine CD-31 expression in paraffin-embedded formalin-fixed tumor tissue using the avidin–biotin peroxidase method with the Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Prior to staining, slides were deparaffinized in a series of xylene and graded alcohols, followed by antigen retrieval in 0.01 M Citrate buffer (pH 6) and incubation in 3% hydrogen peroxide to quench endogenous peroxidase.
Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay

Apoptotic cell death was investigated using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay (ApopTag Plus Peroxidase In Situ Detection Kit, Chemicon, Temecula, CA) according to the vendor’s instructions. Quantification of staining was assessed by calculating the mean number of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling positive cell nuclei within 10 high-power (×200) fields per animal in each group (vehicle control n = 5 and pPLC n = 6) and comparisons between groups were performed as previously described.

SA Biosciences real-time PCR array on tumor tissue

At the termination of the experiment, tumors were resected and a portion was submerged in RNAlater® Solution (Ambion®, Life Technologies, Grand Island, NY) and stored at 4°C until RNA isolation. Total RNA was isolated from ~30 mg of tumor tissue using the RNeasy™ Kit (Qiagen, Valencia, CA). Tumor tissue was homogenized using the gentleMACS™ Dissociator (Miltenyi Biotec, Cambridge, MA) and RNA purity and concentration was determined using the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The complementary DNA was generated for each tumor from 2 μg RNA using the RT² First Strand™ Kit (Qiagen, 330401) according to the manufacturer’s protocol. All real-time (RT)-PCRs contained 2X SA Biosciences RT² SYBR Green/ROX Master Mix (Qiagen, 330521) and one reverse transcription reaction containing 2 μg RNA for every 96 wells of the Mouse Hypoxia Signaling Pathway RT² Profiler™ PCR Array (SA Biosciences, A Qiagen Company, PAMM-0324-4) or Mouse Inflammatory Cytokines and Receptors RT² Profiler™ PCR Array (SA Biosciences, A Qiagen Company, PAMM-011A). Each array contained five housekeeping genes, one genomic DNA control, three reverse transcription controls, and three positive PCR controls in order to control for template concentration and consistency between arrays, as well as to detect any sample contamination or impurities. RT-PCR was performed on 10 tumor samples (five per group) and normalized to glyceraldehyde 3-phosphate dehydrogenase by the comparative Ct method.

Pentraxin-3 (Ptx3) enzyme-linked immunosorbent assay and RT-PCR

Murine dermal fibroblasts isolated from C57BL/6 pups and Lewis lung carcinoma cells were plated at 1 × 10⁶ cells/well in a 12-well plate and grown until 80% confluent. Cells were serum starved for 4 h prior to treatment with 0.1 ng/ml interleukin (IL)-1β, 10 ng/ml IL-1α, or 1 ng/ml transforming growth factor-β. Supernatant and cells were collected 24 h later for mPtx3 enzyme-linked immunosorbent assay and RT-PCR analysis. Ptx3 levels were measured in tissue culture supernatants according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN—MPTX30). Sensitivity was 0.23 ng/ml. The complementary DNA was generated from 2 μg RNA using the RT² First Strand™ Kit (Qiagen, 330401) according to the manufacturer’s protocol. RT-PCR reactions were run using the Applied Biosystems 7900HT Fast Real-Time PCR System. SYBR Green master mix (Applied Biosystems) and the following primer sequences were added to each complementary DNA sample:

- CTATGGCTGCGAAGCAAATTT
- ΔΔ

Mean Ct values for Ptx3 were normalized to the Ct values of the endogenous control, glyceraldehyde 3-phosphate dehydrogenase, and the ΔΔCt method was used to analyze fold change in Ptx3 expression.

Statistical analyses

Tumor sizes and weights were tested for statistical significance by using a 1-tailed t-test with F-tests to evaluate variance (GraphPad Software) and P values ≤ 0.05 were considered statistically significant.

Results

Dietary intake of pPLC conjugate decreases Lewis lung carcinoma tumor growth

In order to test if milled chow containing the pPLC and vehicle control conjugates were consumed by non-tumor bearing C57BL/6 mice animal body weights were monitored on a daily basis. We first tested chow containing 25% of total weight as vehicle or pPLC milled with chow to determine if this high concentration was palatable and to deliver maximal concentrations of the pPLC constituents. Accordingly, at this concentration the mice lost an average of 14.4% total body weight compared with controls fed chow containing no additive, which gained an average of 4% total body weight over the 6-day test period (data not shown). We concluded that the 25% weight concentration of the vehicle or pPLC was too high and that the mice did not consume this mix sufficiently to increase body weight. Alternatively, the observed weight loss could be associated with the antiangiogenic effects of high-dose lipoic acid, as previously reported in rodents and human clinical trials (26,29).

To ensure milled chow consumption and avoid the weight loss associated with high-dose lipoic acid consumption, we tested chow with 5% of total weight concentrations of the pPLC or vehicle control conjugates. Using an in vivo, highly aggressive tumor model of short duration, the 5% weight composition was calculated to deliver adequate levels of bioavailable pPLC in the limited dosing period of the experiment. The groups of animals on this percent weight composition gained weight at exactly the same rate as the mice fed Purina chow containing no added conjugate. These results suggest that at 5% weight composition, the mice consumed the feed containing the conjugates at approximately the same rate as control feed to maintain a normal increase in body weight throughout the duration of the study and avoid the antiangiogenic (weight loss) associated with high-dose lipoic acid consumption (data not shown). The mean daily feed intake for C57BL/6 mice in this experiment was 4.0 g/mouse/day, very close to the overall average daily intake of 4.4 g/mouse determined in 26 different mouse strains (30). We used a mean daily feed intake of 4.9 g/mouse to calculate the daily intake of components in the 5% pPLC and vehicle control feed using the percent compositions shown in Table 1 (total converted intake of pPLC or vehicle 6.7 g/kg/day).

To study the in vivo impact of pPLC conjugate on primary tumor growth, we injected C57BL/6 mice subcutaneously with luciferase-labeled Lewis lung carcinoma cells. Three days after tumor cell inoculation to allow tumor take, mice were switched from conventional diet to a milled diet containing 5% pPLC or vehicle control. At the indicated number of days posttumor cell inoculation, primary tumor growth was measured by luminescence (Figure 1A and B). Luminescence could be detected as early as 7 days posttumor cell inoculation in all mice, but manual caliper measurements could not be accurately determined until 14 days posttumor cell inoculation (data not shown), but demonstrated a rough correlation with the increase in luminescence measurements from day 14–24. In contrast, luminescence values became unreliable after 24 days, although tumor volume could still be calculated from manual caliper measurements at day 27 (when the experiments were terminated according to the animal study protocol with tumor volumes in excess of 2000 mm³). These findings are consistent with the greater sensitivity of the luminescence imaging compared with conventional caliper measurement.

Luminescence values revealed a statistically significant (P ≤ 0.05) decrease in tumor cell growth in mice on the 5% pPLC diet compared with vehicle control diet on days 19 (42.23 ± 20.03 × 10⁶ photon flux versus 46.20 ± 5.77 × 10⁶ photon flux, respectively, P ≤ 0.01) and 24 (95.92 ± 20.03 × 10⁶ photon flux versus 153.67 ± 32.91 × 10⁶ photon flux, respectively, P ≤ 0.04) posttumor cell inoculation (Figure 1A, upper and lower panels). A statistically significant decrease in tumor volume was also recorded by manual caliper measurements 27 days posttumor cell inoculation in mice receiving 5% pPLC (2418.5 ± 312.0 mm³ versus 1654.1 ± 195.1 mm³, P ≤ 0.05; data not shown). These results suggest that dietary intake of pPLC conjugate suppresses tumor growth of a highly aggressive syngeneic mouse lung cancer model.

To confirm the tumor inhibitory effect of pPLC conjugate in vivo, we compared the average tumor weights between the two treatment groups at the conclusion of the experiment (27 days posttumor cell inoculation). Primary tumors isolated from C57BL/6 mice fed the pPLC conjugate diet weighed significantly less than tumors from mice fed the vehicle control diet (1.57 ± 0.20 g compared with 2.20 ± 0.21 g, respectively, P ≤ 0.05; Figure 1B, right panel).

Dietary pPLC decreases lung tumor vascularity and increases tumor cell apoptosis in vivo

The previously reported in vitro antiangiogenic and/or antiapoptotic effects of soy sterols, lipoic acid and ferulic acid led us to examine whether similar mechanisms contributed to decreased tumor growth in vivo. Formalin-fixed, paraffin-embedded tumor sections were analyzed for microvascular density (CD-31 staining, Figure 1C upper panels) and tumor cell apoptosis (terminal deoxynucleotidyl
Fig. 1. Lewis lung carcinoma tumor growth, tumor angiogenesis and apoptosis in vivo. (A, upper and lower panels) C57BL/6J mice were injected (subcutaneous) with luciferase-labeled Lewis lung carcinoma cells and at day three started on a diet with 5% weight percent pPLC (upper panel) or vehicle control (lower panel). At the indicated number of days posttumor cell inoculation, primary tumor growth was measured using an IVIS spectrum imaging system to determine photon flux within the areas outlined in red using the Living Image Software v3.1. (B, left panel) Tumor growth over time measured by luminescence is shown in units of photon flux (photons/s/cm²/sr). (B, right panel) Tumor weights were obtained at the termination of the experiment on day 27 posttumor cell inoculation. Experiment was repeated three times and each time the pPLC diet showed a 22–30% reduction in tumor volume by luminescence and manual caliper measurements (data not shown). Shown is a representative experiment with n = 15 mice per condition. * indicates P ≤ 0.05. (C, upper panel, red arrows) Tumors were harvested 27 days posttumor cell inoculation and stained for CD-31 expression to measure blood vessel formation. (D, upper panel) Expression was
transferase-mediated dUTP nick end-labeling staining, Figure 1C lower panels) from at least five mice per experimental group. Comparison of the CD-31 positive vascular structures revealed a significant decrease in microvascular density in tumor samples isolated from mice fed the pPLC conjugate diet compared with vehicle control (6.52±0.33 versus 3.93±0.30, respectively, *P ≤ 0.05; Figure 1D upper panel). A significant increase in the mean tumor cell apoptosis was observed in tumors from mice on the pPLC conjugate diet compared with vehicle controls (7.98±0.45 versus 4.08±0.36, respectively, *P ≤ 0.05, Figure 1D, lower panel). Taken together, these results suggest that decreased tumor angiogenesis and increased tumor cell apoptosis contributed to reduced growth of Lewis lung carcinoma in mice fed a diet containing pPLC conjugate. Moreover, these findings are the first report of a combined effect of soy sterols, ferulic acid and lipoic acid conjugate on tumor growth in vivo and suggest that combined therapy with several phytochemicals may be more effective than a single supplement, possibly due to combined effects and/or enhanced bioavailability.

**Dietary consumption of pPLC increases metallothionein-3 and pentraxin-3 gene expression within LL2 tumors**

The growth of primary tumors can occur more quickly than the infiltration of new blood vessels, thereby creating hypoxic microregions within the tumor mass. In areas of these hypoxic conditions, tumor cells generate excess production of reactive oxygen species. Since, soy sterols, ferulic and lipoic acid, the active ingredients in pPLC, are all known to have potent antioxidant activity, we analyzed differential gene expression in tumors from the two experimental groups using a murine hypoxic signaling pathway RNA microarray (RT*Profiler™*). Results of these analyses on tumors from mice receiving pPLC conjugate feed showed a significant upregulation of at least five genes and downregulation of four genes compared with mice fed vehicle control conjugate, Table II. The greatest increases were found in metallothionein-3 (Mt3) and pentraxin-3 (Ptx3) expression with a 10.9 and 24.1-fold increase, respectively. Further investigation of the cells expressing Mt3 were hampered by the lack of antibodies that worked well in immunohistochemical staining or western blot.

In contrast, pentraxin 3 (Ptx3) is a member of the long pentraxin superfamily that is rapidly produced and released by several cell types including mononuclear phagocytes, dendritic cells, fibroblasts and endothelial cells in response to primary inflammatory signals (including IL-1β) and has been reported to be a significant biomarker for human non–small lung cancer through its elevation in the inflammatory tumor microenvironment and adipose tissue (31–33). We tested the contribution of the LL2-LUC cells to observed elevated levels both in vivo and in vitro. Immunohistochemical analysis of LL2-LUC tumor-bearing mice treated with pPLC showed a significant increase in highly positive cell staining in non-tumor cells at both the periphery and the center of the tumors (Figure 2B and D–F). Whereas, in control tumors, only slight non-specific staining for Ptx3 was observed at the periphery of the tumor mass. These findings are consistent with the increased cell expression of Ptx3 not in the LL2-LUC tumor cells, but in other, probably inflammatory, cell types in a statistically significant fashion, both in the center and periphery of the tumors grown in vivo, (Figures 2E and F). These findings are highly consistent with the evolving concept of the tumor microenvironment in the regulation of tumor progression, and that cellular suppression of tumor growth by host cells may be exploited therapeutically.

We also examined whether LL2-LUC cells contributed to the large (24.1-fold) increase in Ptx3 expression within the tumor samples, using an in vitro–stimulated cell expression model. LL2-LUC cells were treated with 0.1 or 10 ng/ml of IL–1β, a known inducer of Ptx3 expression (33), and expression levels were determined by RT-PCR and enzyme-linked immunosorbent assay (Figure 3). LL2-LUC cells had undetectable (<0.2 ng/ml) levels of secreted Ptx3 as determined by a murine Ptx3 enzyme-linked immunosorbent assay (R&D Systems, data not shown). Although basal levels of Ptx3 expression were detectable in LL2-LUC cells by quantitative RT-PCR, treatment with 10 ng/ml IL–1β did not increase Ptx3 expression. Conversely, treatment with 10 ng/ml treatment of IL–1β did increase expression of Ptx3 in murine primary dermal fibroblasts (Figure 3).

To further elucidate possible mechanisms for enhanced expression of Mt3 and/or Ptx3 in the tumor microenvironment of mice receiving the pPLC conjugate feed, we examined changes in murine inflammatory cytokines and receptors again using a RT*Profiler™* PCR array. Analyses revealed upregulation of four cytokines tested on the array (Table III). These findings suggest that pPLC treatment may significantly alter the inflammatory profile of the tumor microenvironment and further influence tumor cell growth. However, further experiments are necessary to determine the types of inflammatory cells that may be recruited to pPLC-treated tumors and if this indeed influences tumor growth.

**Discussion**

The National Cancer Institute recognizes cancer preventive therapy as the reduction of cancer mortality via reduction in the incidence of cancer. Cancer prevention involves lifestyle changes, such as smoking cessation, reduced alcohol consumption, avoiding exposure to known carcinogens, and enhanced surveillance to allow early detection and surgical resection of precancerous lesions. Cancer prevention also includes chemoprevention that was demonstrated over 30 years ago, as well as the more recent concept of angioprevention (16,34,35). Chemoprevention and angioprevention involve dietary or pharmacologic interventions that disrupt the carcinogenic and/or angiogenic processes associated with cancer development and progression.

In this study, we examined the antitumorigenic activity of a compound phytochemical agent pPLC using a rapidly growing syngeneic murine model of lung carcinoma. The aim of these studies was to use this fast growing, highly aggressive murine lung cancer model to rapidly determine the antitumor activity of pPLC before initiating more complex prevention studies. Our results demonstrate that pPLC reduced tumor-induced angiogenesis and enhanced tumor cell apoptosis, which are consistent with the reported effects of the soy sterols, ferulic acid

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<th>Gene name</th>
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Table II. Fold change in gene expression in tumors from mice fed a pPLC diet compared to tumors from control mice using the Murine Hypoxia Signaling Pathway RT*Profiler™* PCR Array
pPLC decreases tumor growth and angiogenesis

and lipoic acid constituents of pPLC. However, it is presently unclear if these effects are related, in that reduced blood flow to the tumor further enhances hypoxic conditions that promote tumor cell apoptosis.

Rapidly growing tumors and tumors with reduced angiogenesis often encounter hypoxic conditions. Our quantitative RT-PCR analysis of differential gene expression demonstrated enhanced levels of Mt3 and Ptx3. Mt3 is a cysteine-rich cytosolic protein that regulates intracellular metal atom homeostasis. Low cytoplasmic Mt3 levels are associated with poor patient outcome in non–small cell lung cancer patients (36). Mt3 expression is reduced in gastric and esophageal carcinomas by methylation of key CpG islands in the promoter region (37). In contrast, Mt3 levels are increased in breast cancer tissues but not normal breast tissue (38). Apo-forms of Mt3 can bind zinc where oxidized zinc-binding cysteine residues of Mt3 will cause a release of zinc leading to cell death (13,16). Therefore, the oxidative status can influence whether Mt3 acts as a zinc buffer or induces zinc cytotoxicity within cells. Augmented Mt3 gene expression in pPLC-treated lung tumors may initially act as an antioxidant, but increased oxidative stress that may be associated with reduced tumor angiogenesis would increase intracellular zinc concentrations contributing to enhanced tumor cell apoptosis.

The elevation of Ptx3 levels in non-tumor cells was confirmed in vitro using IL-1β-stimulation and in vivo using immunohistochemistry. Ptx3 is a soluble pattern recognition receptor, also referred to as tumor necrosis factor–stimulated gene 14 and is part of the long pentraxin subfamily (11,39). Ptx3 is synthesized at sites of inflammation by mononuclear phagocytes, myeloid dendritic cells, fibroblasts, adipocytes, granulosa cells, mesangial cells, smooth muscle cells and endothelial cells (9,12). Extensive studies on potential Ptx3 ligands indicate that Ptx3 binds complement component C1q; apoptotic cells;

Fig. 2. Photomicrographs of Ptx3 immunostaining 21 days posttumor cell inoculation. An increase in the number of distinct, solitary Ptx3 positive cells was observed in LLC tumors from mice fed the pPLC conjugate diet compared with vehicle controls (A–D, upper and middle panels). The bar graphs represent quantification of staining assessed by calculating the mean number of Ptx3 positive cells in 10 high-power (×200) fields per animal in each group (Control versus pPLC; E and F, lower panel). Graph pad Prism 6 t-test was used to compare mean values. * indicates P < 0.05.
to examine pPLC effects on the tumor-associated cellular infiltrates. Results of additional gene expression profiling experiments indicate that pPLC dietary supplementation alters the cytokine production and that this may alter the composition of the cellular compartment in the tumor microenvironment, leading to decreased tumor growth.

Future studies are needed to investigate the long-term effects of pPLC on prevention of tumor initiation, growth and angiogenesis, as well as the cellular composition of the tumor microenvironment. In conclusion, using a rapid growing in vivo, syngeneic murine lung cancer model (Lewis lung carcinoma), we demonstrate that pPLC has a significant effect in slowing tumor growth, reducing tumor-associated angiogenesis and increasing apoptosis. These effects are consistent with the reported effects of the soy sterols, ferulic acid and lipoic acid present in pPLC. Furthermore, the effects of pPLC are mediated by decreased tumor angiogenesis and a direct effect on tumor cell gene expression (Mt3). These compounds present in pPLC have little or no reported toxicity, which makes pPLC an excellent candidate for long-term administration as a cancer preventive agent via its combined chemopreventive and angiopreventive activities.

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References


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pPLC decreases tumor growth and angiogenesis