

Lung Cancer Inhibitory Effect of Epigallocatechin-3-Gallate Is Dependent on Its Presence in a Complex Mixture (Polyphenon E)

Huijing Fu,¹ Jun He,² Fan Mei,¹ Qi Zhang,² Yukihiro Hara,³ Seto Ryota,² Ronald A. Lubet,⁴ Ruth Chen,¹ Da-Ren Chen¹ and Ming You^{2,3}

Abstract

Green tea has been shown to exhibit cancer-preventive activities in preclinical studies. However, (–)-epigallocatechin-3-gallate (EGCG) alone was shown to be ineffective in preventing lung tumorigenesis in mice by aerosol administration. In this study, Polyphenon E and Polyphenon E without EGCG were administered by aerosol delivery to A/J mice 2 weeks after carcinogen treatment and continuing daily throughout the remainder of the study (20 weeks). An improved aerosol delivery system with a custom-built atomizer, an efficient solvent remove system, and a nose-only exposure chamber was used to provide aerosols with stable size distribution. There were no significant differences in the size distributions of Polyphenon E and Polyphenon E without EGCG. With a relatively low dose level (4.19 mg/kg), Polyphenon E decreased tumor multiplicity by 53%, whereas Polyphenon E without EGCG at the same dose failed to inhibit lung carcinogenesis. These results indicate that aerosol administration can be an effective approach in chemoprevention study, and aerosolized Polyphenon E can significantly inhibit pulmonary adenoma formation and growth in A/J mice. Furthermore, in aerosolized form, EGCG, which is thought to be the most active component of Polyphenon E, has to be present with other tea catechins to show chemopreventive activity on lung tumorigenesis.

Lung cancer is the most common form of cancer in the world. Despite improvements in therapy, 90% of lung cancer patients die from their disease (1). Chemoprevention could block the progression of lung cancer pathogenesis in current and former smokers, which is considered to be an important approach to decrease the incidence of lung cancer (1). To reach a good inhibition effect, higher dose of chemopreventive agent is often required, which may cause systemic toxicity or adverse effect. Targeting chemopreventive agents to specific areas within the body can result in better efficacy and lower toxicity (2).

Inhaled medications are widely accepted as the optimal route of administration in treating lung diseases. Directly delivering drugs to the lung could lead to a high concentration in target organ with a lower dose level compared with other means of administration. Aerosol delivery for therapy of lung

cancer in human has been reported to be effective without adverse effects (3, 4).

Experiments have been conducted in animal models by aerosol delivery for identifying effective agents or combinations of agents (5–9). Administration of isotretinoin by inhalation could reduce tumor multiplicity at a relatively low dose (5). Aerosol delivery of budesonide at low dose inhibited all stages of progression from hyperplasia formation to cancer in benzo(*a*)pyrene [B(*a*)P] induced mice lung carcinogenesis without systemic toxicity (6, 8, 9).

The cancer preventive activity of tea polyphenols has received more attention in recent years. The inhibitory activities of tea constituents against carcinogenesis at different organ sites have been shown in many animal models (10, 11). Polyphenon E is a well-defined pharmaceutical-grade mixture of polyphenols that contain at least five different catechins: epicatechin (EC), EC gallate, epigallocatechin (EGC), gallic acid, gallic acid gallate, and (–)-epigallocatechin-3-gallate (EGCG), with EGCG being the most abundant (11, 12). EGCG was thought to be the most active component in Polyphenon E (11). However, our previous results showed that aerosolized EGCG failed to show significant inhibition on both tumor multiplicity and tumor load, whereas at the same concentration, aerosolized Polyphenon E inhibited lung tumorigenesis significantly (13). This indicates that EGCG alone cannot show chemopreventive activity by aerosol administration, which means that either EGCG is not the active compound in Polyphenon E, or EGCG will show its antitumor activity only when other components in Polyphenon E

Authors' Affiliations: ¹Department of Energy, Environmental and Chemical Engineering, Washington University of St. Louis and ²Department of Surgery, Campus Box, The Alvin J Siteman Cancer Center, Washington University School of Medicine, St. Louis, Missouri; ³Mitsui Norin Co., Ltd., Shizuoka, Japan; and ⁴Chemoprevention Agent Development Research Group, National Cancer Institute, Bethesda, Maryland
Received 10/12/08; revised 12/22/08; accepted 1/5/09; published OnlineFirst 5/26/09.

Note: D-R. Chen and M. You contributed equally to this work.

Requests for reprints: Ming You, Department of Surgery and Alvin J. Siteman Cancer Center, Washington University in St. Louis, 660 South Euclid Avenue, Campus Box 8109, 10130 Wohl Clinic, St. Louis, MO 63110. Phone: 314-362-9294; Fax: 314-362-9366; E-mail: youm@wudosis.wustl.edu.

©2009 American Association for Cancer Research.

doi:10.1158/1940-6207.CAPR-08-0185

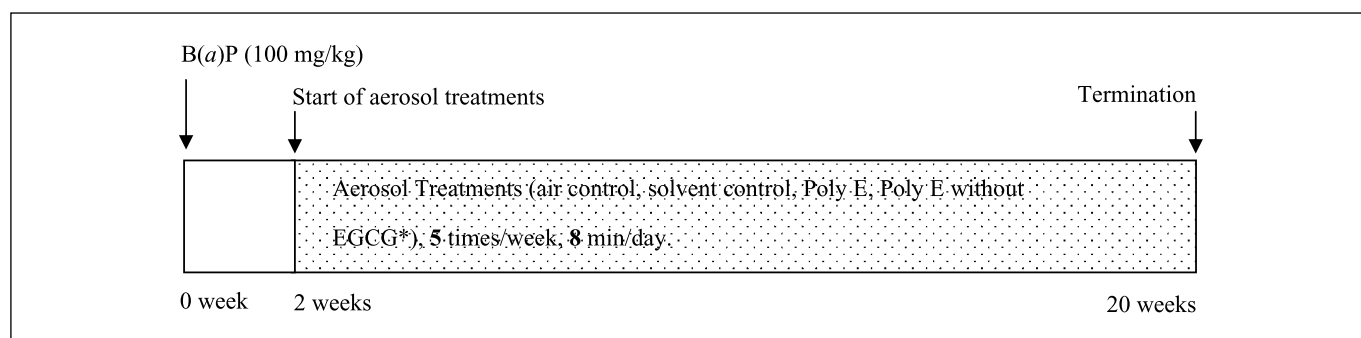


Fig. 1. Protocol of aerosol treatment. Two weeks after the i.p. injection of B(a)P, all mice were subjected the aerosol delivery treatment for 8 min per day, 5 d per week. The total treatment duration continued for 18 wk. For Polyphenon E without EGCG group, 2.8 of 8 min were treated with Polyphenon E without EGCG, only air was introduced into chamber at other times.

are present. The capacity of other tea catechins to prevent lung tumorigenesis needs to be further investigated to understand the role of EGCG in chemoprevention of lung cancer. This work was initiated to compare the inhibitory activities of Polyphenon E and Polyphenon E without EGCG by aerosol administration.

Materials and Methods

Chemicals and animals

Ethanol, acetonitrile, ammonium acetate, and methanol were purchased from Sigma-Aldrich. All organic solvents were high-performance liquid chromatography (HPLC) grade, and the water was deionized. B(a)P (99% pure) and tricapyrylin were purchased from Sigma-Aldrich. B(a)P were prepared just before use by dissolving in tricapyrylin. The chemopreventive agents Polyphenon E and Polyphenon E without EGCG were obtained from Mitsui Norin Co., Ltd. Female A/J mice at 6 wk of age were obtained from The Jackson Laboratory. The use of animals was approved by the Washington University's Institutional Animal Care and Use Committee.

Animal experiments

Female A/J mice were given a single i.p. dose of B(a)P (body weight, 100 mg/kg) in 0.2 mL of tricapyrylin. The mice were housed at a constant temperature and humidity and received a standard diet and water. Two weeks after B(a)P injection, the mice were randomly divided into 4 groups with 16 mice per group: (a) air control group (to account for stress factors during mouse handling procedures in aerosol delivery), (b) solvent control group (25% ethanol water solution), (c) Polyphenon E without EGCG group (15 mg/mL in 25% ethanol water solution), and (d) Polyphenon E group (15 mg/mL in 25% ethanol water solution). Treatments by aerosol delivery was begun 2 wk after B(a)P, and then continued for 18 wk (8 min/d and 5 d/wk; Fig. 1). All solutions were prepared just before use and stored on ice. All solutions were prepared fresh daily and stored on ice until use. Because 65 weight percent (w.t.%) of Polyphenon E is EGCG, the daily delivery time for group 3 was 2.8 min/d with Polyphenon E without EGCG and 5.2 min/d with only air. The total exposure time of Polyphenon E without EGCG group was also 8 min/d, which was the same as other groups. Therefore, mice in groups 3 and 4 were exposed to similar amount of tea catechins other than EGCG. All solutions were prepared just before aerosol treatment. The inhalation exposures were conducted using a custom-built nose-only exposure chamber. The mice were exposed singly to aerosol by placing their noses into the cone of the apparatus. The mice in the air control group were placed in the chamber for 8 min without aerosol treatment to control for potential stress factors affecting tumorigenesis. The body weights of the mice were measured every week for the duration of

treatments. Mice were sacrificed 20 wk after exposure to B(a)P by CO₂ asphyxiation. Lungs from each mouse were fixed in Tellyesniczky's solution (14) overnight, followed by 70% ethanol. The fixed lungs were evaluated under a dissecting microscope to obtain fixed surface tumor count and individual tumor size. Tumor volume (*V*) was calculated using tumor diameter (*r*) based on the following formula: $V \text{ (mm}^3\text{)} = 4\pi r^3/3$ (14). The total tumor volume in each mouse was calculated by the sum of all tumors. Tumor load was determined by averaging the total tumor volume of each mouse in each group.

Aerosol procedure

Polyphenon E or Polyphenon E without EGCG was dissolved in 25% ethanol water solution and atomized into droplets by atomizer. The flow rate was 2.27 liters/min. Aerosol flow was then passed through two diffusion dryers containing silica gel to remove water from droplets and a scrubber with active carbon that was used to remove ethanol. The resulting dry aerosol flow with only desired chemicals was then introduced into the nose-only exposure chamber from the top inlet. Effluent aerosol was discharged from an opening at the bottom of the chamber. The schematic diagram is shown in Fig. 2.

The size distribution of the aerosol was determined by Scanning Mobility Particle Sizer spectrometer, which includes a Electrostatic Classifier (TSI model 3080), a Differential Mobility Analyzer (TSI model 3081), and a Condensation Particle Counter (TSI model 3025). Geometric median diameter, mass median aerodynamic diameter (MMAD), geometric SD, and particle concentration were obtained. Online samples were taken inside the chamber right at the place where mice were supposed to inhale aerosols. Different sample points were used to monitor the concentration uniformity inside the chamber.

The mass of inhaled drug was calculated as follows:

$$M_{\text{inhaled}} = [C]_{\text{aerosol}} \cdot RMV \cdot t$$

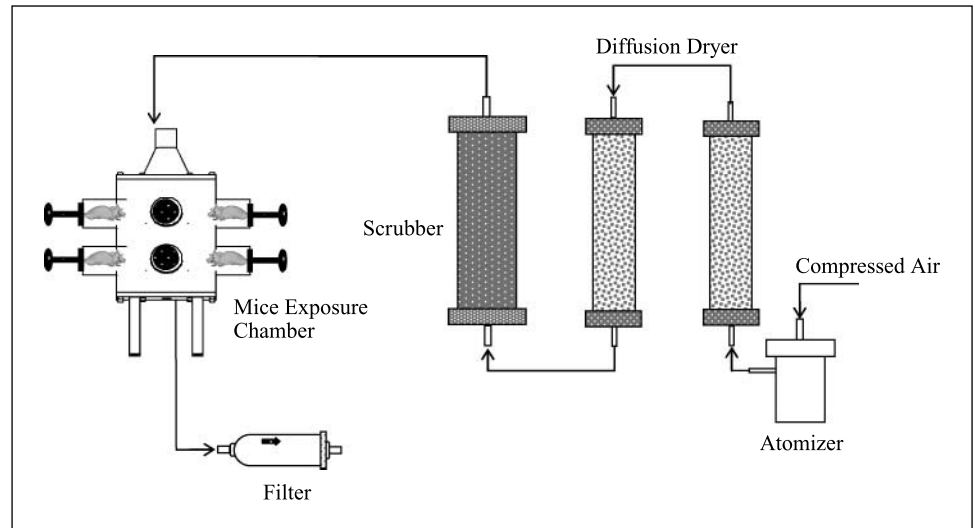
where $[C]_{\text{aerosol}}$ is the aerosol concentration of drugs (mg/liter), *RMV* is the respiratory minute volume of the mouse [0.025 liters/min, based on Guyton's formula *x*], and *t* is the exposure time. The deposition ratio of aerosol within the lung was estimated by the following equation:

$$\% \text{Deposition} = \frac{M_{\text{tissue}}}{M_{\text{inhaled}}} \times 100\%$$

where M_{tissue} (mg) is the mass of drug that deposited in lung.

A preexperiment was carried out to determine the dosed mass in the lung. Mice were exposed to the drug aerosols (Polyphenon E or Polyphenon E without EGCG) for 10 min. After exposure, the animals were sacrificed by cervical dislocation at designated time points with the end of the exposure marked as time zero. Blood was obtained by cardiac puncture, and collected into plastic centrifuge tubes. Vc-EDTA buffer that consisted of 20% ascorbic acid and 0.1% EDTA sodium salt

Fig. 2. Schematic diagram of aerosol delivery system. A custom-built atomizer was used to generate droplet containing Polyphenon E or Polyphenon E without EGCG. Aerosol flow was then passed through two diffusion dryers containing silica gel to remove water from droplets and a scrubber with active carbon that was used to remove ethanol. The resulting dry aerosol flow with only desired chemicals was then introduced into the nose-only exposure chamber from the top inlet. Effluent aerosol was discharged from an opening at the bottom of the chamber.



in 0.4 mol/L NaH_2PO_4 buffer at pH 3.6 was added into serum sample at a ratio of 20 $\mu\text{L}/\text{mL}$ blood. The lung was severed at the carina, the esophagus and trachea were removed. All samples were stored in liquid nitrogen until assayed.

Tissue assay method

Drug concentrations in lung and serum samples were determined by HPLC. The HP 1100 series HPLC system consisted of an autosampler, a binary pump, a thermostatted column compartment, and a diode array detector (Agilent Tech). The HPLC column was 4.6 \times 75 mm Zorbax SB-C18 3.5 μm column, and the wavelength was 244 nm. Stock solutions were prepared by dissolving Polyphenon E and Polyphenon E without EGCG separately in 25% ethanol water solution with

20 $\mu\text{L}/\text{mL}$ Vc-EDTA buffer. The mobile phase consisted of ammonium acetate/acetonitrile (80:20). The column temperature was maintained at 40°C. The flow rate of the mobile phase was 1 mL/min. The largest peak of Polyphenon E and Polyphenon E without EGCG was used for quantification, respectively. EGCG peak was used for Polyphenon E and EC peak was used for Polyphenon E without EGCG.

Lung tissues were weighed and homogenized in 0.2 mL of ice-cooled Vc-EDTA buffer, and 0.8 mL of 25% ethanol water solution. Then the sample was vortexed for 2 min and centrifuged at 13,000 rpm for 15 min at 4°C. Supernatants were transferred and dried under nitrogen gas stream. Residues were reconstituted in 1 mL 25% ethanol water solution and centrifuged, the supernatants was tested by HPLC. The serum was extracted as above for lung tissue homogenate.

Fig. 3. Chromatogram of Polyphenon E and Polyphenon E without EGCG. Eight different catechins were identified in Polyphenon E, with EGCG being the most abundant (65 w.t%). For Polyphenon E without EGCG, EC (44%) and EGC (28%) account for >50% of all catechins, whereas small amount (1.4%) of EGCG was also detected.

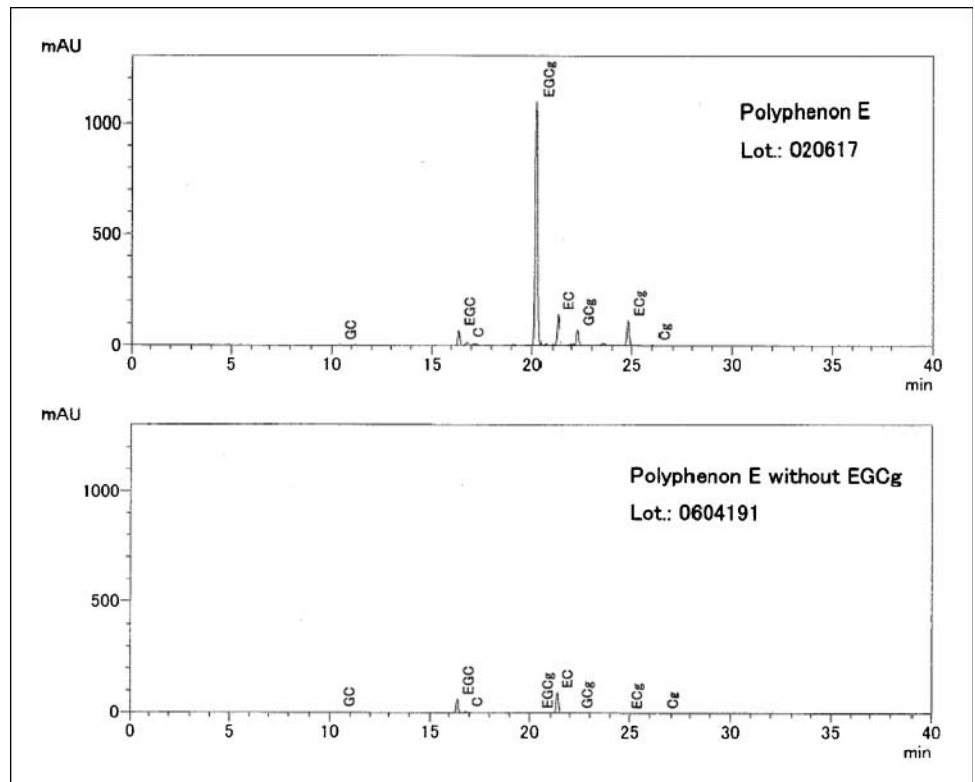


Table 1. Composition of Polyphenon E and Polyphenon E without EGCG

	Abbreviation	Polyphenon E (w.t.%)	Polyphenon E without EGCG (w.t.%)
Caffeine	—	0.7	0.8
(+)-Gallocatechin	(+)-GC	0.2	1.7
(-)-Epigallocatechin	(-)-EGC	3.8	28.2
(+)-Catechin	(+)-C	1.0	3.3
(-)-Epigallocatechin gallate	(-)-EGCG	65.0	1.4
(+)-Epicatechin	(+)-EC	9.1	43.9
(-)-Gallocatechin gallate	(-)-GCG	3.5	0.1
(-)-Epicatechin gallate	(-)-ECG	6.6	0.3
(-)-Catechin gallate	(-)-CG	0.2	0.2
Total catechins	—	89.4	79.1

NOTE: Data provided by Mitsui Norin Co., Ltd. (Japan).

Statistical analysis

Tumor multiplicity and tumor load were analyzed by two sided Student's *t* test using Microsoft Excel 2002 SP-3 (Microsoft) to determine differences in the number and in the size of lung tumors per mouse between groups. In all *t* tests, the level of statistical significance was set at *P* value of <0.05.

Results

Composition analysis for Polyphenon E and Polyphenon E without EGCG

The chromatogram of Polyphenon E and Polyphenon E without EGCG are comparatively shown in Fig. 3. Eight different catechins were identified in Polyphenon E, with EGCG being the most abundant. EGCG accounted for 65 w.t.% of Polyphenon E, whereas no other catechins accounted for >10 w.t.%. The concentrations of the various catechins in Polyphenon E without EGCG were as follows: EC, 44%; EGC, 28%; and the other 6 catechins, 7%. Small amount (1.4%) of EGCG was also detected in Polyphenon E without EGCG. The composition of Polyphenon E and Polyphenon E without EGCG are given in Table 1. Although the ratios among different catechins were different, the main difference between Polyphenon E and Polyphenon E without EGCG was in the amount of EGCG.

Aerosol characteristics

The typical size distributions of Polyphenon E inside chamber are given in Fig. 4. Polyphenon E and Polyphenon E without EGCG (data not shown) yielded similar particle size distribution with geometric median diameter of 0.13 μm and geometric SD (geometric SD) of 1.6. The MMAD of Polyphenon E and Polyphenon E without EGCG was around 0.3 μm . Two sample points were chosen inside the exposure chamber to measure the size distribution. No difference in geometric median diameter, MMAD, and geometric SD was found among measurements at each sample point.

Deposition dose

In Fig. 5, the concentrations of EGCG in Polyphenon E and EC in Polyphenon E without EGCG in the lung were given as a function of time after aerosol exposure. The concentration of EGCG or EC immediately after the exposure was taken as

time 0. Because the catechins in Polyphenon E other than EGCG are in really small mass ratio (<10%) and the dose mass of aerosol was much lower than that achieved by other administrations such as oral, the concentrations of other catechins in Polyphenon E were lower than the detection limit. For Polyphenon E without EGCG, EC was detected and used for quantification. Because EGCG is 65% of Polyphenon E in mass and EC is 44% of Polyphenon E without EGCG in mass, the concentrations of Polyphenon E and Polyphenon E without EGCG were estimated. The concentration of Polyphenon E was 0.042 $\mu\text{g}/\text{mg}$ in lung at time 0, and decreased with time slowly. After 50 minutes, the concentration was 0.023 $\mu\text{g}/\text{mg}$. The half-life of elimination of Polyphenon E from the lung, $t_{1/2,\text{lung}}$, was estimated to be 48 minutes. The concentration of Polyphenon E without EGCG showed similar trend as Polyphenon E. The dosed mass and delivery ratio were summarized in Table 2. The concentration of all the tea catechins in Polyphenon E and Polyphenon E without EGCG in the serum was much lower (<0.04 $\mu\text{g}/\text{mL}$) for both cases.

Using an average body weight of 0.020 kg, the inhaled dose of was calculated to be 4.19 mg/kg for Polyphenon E and 1.83 mg/kg for Polyphenon E without EGCG. The dose level was

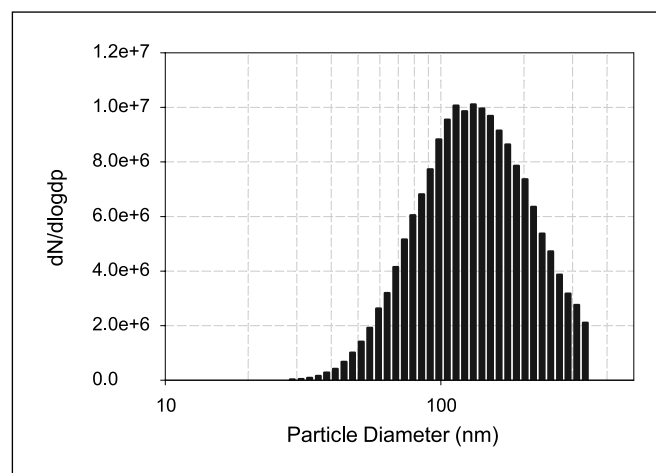


Fig. 4. Size distributions of Polyphenon E in exposure chamber. The mean size was 0.13 μm , with the geometric standard derivation around 1.65.

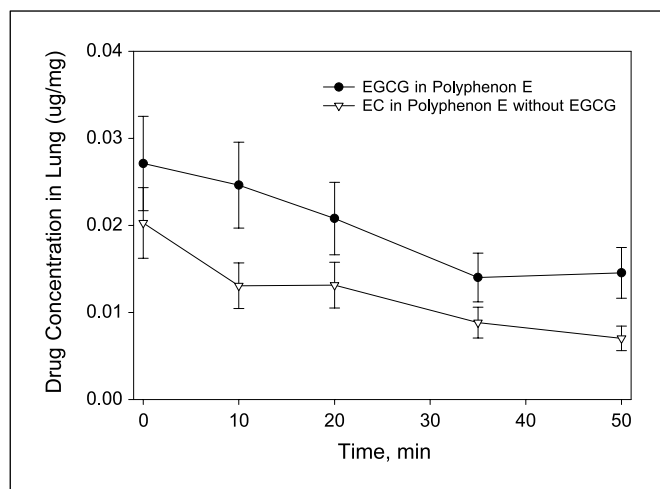


Fig. 5. Concentration of EGCG in Polyphenon E (●) and EC in Polyphenon E without EGCG (▽) in the lung after aerosol inhalation. The concentration of EGCG in Polyphenon E and EC in Polyphenon E without EGCG in mice lung decreased with time slowly.

much lower than oral administration previously reported (15). The deposited mass in lung was calculated from assayed lung concentration, given as deposited mass versus body weight. The deposition ratios of Polyphenon E and Polyphenon E without EGCG group were 5.2% and 5.4%, respectively (Table 2).

Pharmacokinetic parameters of EGCG in Polyphenon E and EC in Polyphenon E without EGCG are given in Table 3. The following parameters were estimated as described earlier (16): maximum lung concentration, the mean area under the lung concentration-time curve (AUC), the area arising from the last measured time point to infinity (AUC_{∞}), the mean resident time, and the lung half-life. The residence time of EC in Polyphenon E without EGCG was shorter than that of EGCG in Polyphenon E, which showed that EGCG has a longer half-life than EC when EGCG was in mixture form.

Chemoprevention effect

Mice were treated by aerosol for 18 weeks after the injection of B(a)P. During the experiment, all mice showed great tolerance to treatment with either Polyphenon E or Polyphenon E without EGCG. No significant difference in body weight was observed (data not shown).

B(a)P induced an average of 5.69 ± 3.30 ($n = 16$) tumors per mouse in air control group and 5.88 ± 2.19 ($n = 16$) tumors per mouse in solvent control group. Results in tumor multiplicity and tumor load showed no significant difference in air control group and Solvent control group. Mice treated with Polyphenon E showed a significant decrease in both tumor multiplicity [53%; 2.79 ± 1.37 ($n = 14$)] and tumor load (62%; 0.66 ± 0.65) when compared with the solvent control group ($P < 0.001$), whereas Polyphenon E without EGCG treatment exhibited statistically nonsignificant reduction in tumor multiplicity [20%; 4.73 ± 2.46 ($n = 15$)] and tumor load (22%; 1.36 ± 0.94 ; Table 2).

Discussion

Green tea has been shown to be able to inhibit the development of lung cancer in several animal models (14, 17–20).

Polyphenon E, which is a mixture of polyphenols containing at least five tea catechins, is believed to be the effective agent. Among tea catechins, EGCG, which is believed to have the highest antioxidant activity, is the most abundant catechin in Polyphenon E. However, our previous results showed that Polyphenon E could decrease tumor load by 59%, whereas at the same concentration, EGCG did not show any significant inhibition on tumor multiplicity and tumor load (13). Results also showed that inhalation of EGCG did not modulate tobacco smoke-induced tumorigenesis (21). Herein, by aerosol administration, Polyphenon E without EGCG was compared with Polyphenon E on their chemopreventive efficacies under the same condition. The results showed that Polyphenon E inhibited lung tumorigenesis, which agreed to our previous results, whereas Polyphenon E without EGCG failed to inhibit lung tumor growth.

Chemoprevention results

In this study, the aerosolized Polyphenon E inhibited tumor multiplicity by 53% and tumor load by 62%, respectively (Table 2). The results show that by aerosol delivery, Polyphenon E could both cause the regression of the tumorigenesis once the tumor progression was initiated by carcinogen, and inhibition of tumor growth during tumor progression. This indicates that Polyphenon E is preventive at all stages of carcinogenesis, which agrees with previous results (22). However, at the same concentration as catechins besides EGCG in Polyphenon E, Polyphenon E without EGCG did not show statistically significant effects in both the tumor multiplicity (20%) and tumor load (22%; Table 2).

Based on HPLC analysis, the main difference between the chemical composition of Polyphenon E and Polyphenon E without EGCG was the content of EGCG. Sixty-five percent of Polyphenon E was EGCG, whereas in Polyphenon E without EGCG, there was only 1.4% EGCG left, compared with 44% of EC and 28% of EGC (Table 1). Although there were differences in the ratios among other tea catechins in Polyphenon E and Polyphenon E without EGCG, the difference became minor when compared with the difference in the amount of EGCG. The difference in tumor multiplicities between Polyphenon E group and Polyphenon E without EGCG group was attributed to the presence of EGCG.

It is believed that EGCG is the main active compound in tea catechins. However, in our previous study, aerosol administration of EGCG alone at the same concentration for 18 weeks in a similar way as this study did not show significant effects in the tumor load (13). Combined previous results without this study, EGCG in purified form and Polyphenon E without EGCG both showed no significant effect on lung tumorigenesis, whereas Polyphenon E inhibited tumor multiplicities by 53%. This indicates that EGCG or other tea catechins alone are not as active as the combined form, Polyphenon E, in lung tumor inhibition. Both EGCG and other tea catechins need to be present to show inhibition effect on lung cancer. One possible reason might be that the presence of other tea catechins may affect the absorption (23), biological activity, or other properties of EGCG. Because tea catechins have similar chemical structure, they compete for any resources they need, such as binding sites, and thus, the metabolic conversion of EGCG is retarded and the residence time of EGCG increases when compares to the case in which pure EGCG was administrated.

Table 2. Effects of aerosolized Polyphenon E and Polyphenon E without EGCG on lung tumorigenesis in female A/J mice

Group	Solution	Deposited mass* ($\mu\text{g}/\text{kg}$)	Deposition* (%)	Final body weight* [†] (g)	Tumor multiplicity*	Inhibition [‡]	Tumor load* (mm^3)	Inhibition [‡]
Air control	None	—	—	22.63 \pm 1.86	5.69 \pm 3.30	—	1.78 \pm 1.31	—
Solvent control	25% ethanol	—	—	22.25 \pm 1.84	5.88 \pm 2.19	—	1.74 \pm 0.83	—
Polyphenon E without EGCG	15 mg/mL in 25% ethanol	80 \pm 22	5.4 \pm 1.6	23.20 \pm 1.78	4.73 \pm 2.46	20.0% [§]	1.36 \pm 0.94	21.8% [§]
Polyphenon E	15 mg/mL in 25% ethanol	222 \pm 51	5.2 \pm 1.3	23.13 \pm 1.74	2.79 \pm 1.37	52.5%	0.66 \pm 0.65	62.1%

*Data shown as Mean \pm SD.[†]Average body weight after 18 wk of treatment [20 wk after B(a)P injection].[‡]Compared with solvent control group.[§] $P > 0.05$.^{||} $P < 0.001$.

Comparison of pharmacokinetic properties of both decaffeinated green tea and purified EGCG in rat administrated by i.v. injection showed that other components in decaffeinated green tea could affect the plasma concentration and elimination of EGCG (24). In our study, the half-life of EGCG in Polyphenon E in lung after aerosol administration was longer than that of EC in Polyphenon E without EGCG (Table 3). Detailed information on EGCG and Polyphenon E administrated by aerosol is not available thus far. More studies are expected in the future.

Aerosol generation

Compared with other methods of administration, aerosol inhalation could reach higher efficacy at the primary target sites in the lung with relatively low dose level (16). In this study, an improved aerosol delivery system was used to give a stable size distribution with smaller particle mean size and SD.

Particle size is always a key factor that affects lung deposition of an aerosol. In general, for humans, aerosols with an

MMAD of $<3 \mu\text{m}$ have a higher chance of reaching the lower airways and being deposited in the alveoli (25). Because of the smaller size of respiration tract, the optimal particle size for mice inhalation study is much smaller. Study showed that for CF1 mice, $0.27 \mu\text{m}$ particles reached a higher deposition ratio in lung (45.4%) than larger particles ($d_p, >1.09 \mu\text{m}$; deposition ratio, $<9.7\%$; ref. 26). Most of previous studies on aerosolized chemopreventive agent used different types of nebulizer to generate aerosol. Although nebulizer has a high mass throughput, it usually generates particles in a relatively wide size range with large geometric SDs (5, 13, 16). In this study, we used a custom-built atomizer, which gave a much narrower particle size distribution. The MMAD of Polyphenon E and Polyphenon E without EGCG were both around $0.3 \mu\text{m}$, which was more favorable in mice inhalation study. Besides, both Polyphenon E group and Polyphenon E without EGCG group used the same concentration (15 mg/mL), which assured that the MMAD were similar for both cases.

In this study, diffusion dryer and scrubber were used to remove water and ethanol from droplets formed by atomizer before aerosol flow entering the exposure chamber, which guaranteed that mice were exposed to only the desired agents and air. Each diffusion dryer contained two concentric cylinders formed by an inner wire screen cylinder and an acrylic outer cylinder. The annular volume between cylinders was filled with silica gel. As aerosol flowed through the inner cylinder, water vapor diffused through the wire screen and into the silica gel. Scrubber worked similarly with diffusion dryer, except that scrubber had four inner wire screen cylinder to enhance removal efficiency. In diffusion dryer, particle loss was minimized because the particles did not come into contact with the silica gel or active carbon. Compared with diffusion dryer, the evaporation-condense method, which was commonly used in previous studies (8, 9, 13, 16), introduced extra particle loss because of thermophoresis (27), which reduced the deposition ratio in lung.

The *in situ* size distribution measurement showed that the inhalation system provided aerosol with stable size distribution. In this study, the Scanning Mobility Particle Sizer system was used to provide the high-resolution size distribution in

Table 3. Pharmacokinetic parameters of EGCG in Polyphenon E and EC in Polyphenon E without EGCG for aerosol administration

Parameters	EGCG in Polyphenon E	EC in Polyphenon E without EGCG
C_{max} ($\mu\text{g}/\text{g}$)	27.3 \pm 2.6	20.2 \pm 2.2
AUC ($\mu\text{g}/\text{g h}$)	16.2 \pm 1.9	9.8 \pm 1.3
AUC ₀₋₂₄ ($\mu\text{g}/\text{g h}^2$)	28.0 \pm 2.5	14.1 \pm 1.2
MRT ₀₋₂₄ (h)	1.15 \pm 0.11	0.64 \pm 0.07
$t_{1/2}$ (min)	48.1 \pm 7.2	34.7 \pm 5.2

NOTE: Data shown as mean \pm SD.Abbreviation: C_{max} , maximum lung concentration; MRT₀₋₂₄, the mean resident time; $t_{1/2}$, the lung half-life.

the exposure chamber. Through the sample hole at the bottom of the chamber, two sample points were selected. Each sample point was at the height where mice nose would be. Thus, the exact size distribution information of the aerosol in the chamber could be collected. No significant difference in size distributions indicates that the aerosol inside exposure chamber was uniform and stable. All mice were exposed to particles with the same size distribution. Furthermore, mice had been put into the chamber randomly throughout the treatment. So, mice in the same group could be considered to receive similar treatment.

In summary, the delivery by aerosol of Polyphenon E has been shown to be a novel method for providing the powerful efficacies in chemoprevention of lung tumorigenesis. The inhalation system used in this study is able to provide aerosol with stable size distribution, which was suitable for chemoprevention study in mice as well as in men. This study

showed that Polyphenon E was an effective chemopreventive agent on inducing tumor regression and inhibiting tumor growth, whereas Polyphenon E without EGCG was ineffective. EGCG is thought to be the most active component in Polyphenon E, but it has to be with other tea catechins to show chemopreventive activity on lung tumorigenesis in aerosolized form. The aerosol delivery of Polyphenon E is an effective method of chemoprevention, which could be considered for further studies in animal models as well as clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Yukihiko Hara of Mitsui Norin Co., Ltd. for supplying Polyphenon E and Polyphenon E without EGCG and providing the composition analysis.

References

- Minna JD, Roth JA, Gazdar AF. Focus on lung cancer. *Cancer Cell* 2002;1:49–52.
- Lippman SM, Lee JJ, Sabichi AL. Cancer chemoprevention: progress and promise. *J Natl Cancer Inst* 1998;90:1514–28.
- Tatsumura T, Koyama S, Tsujimoto M, Kitagawa M, Kagamimori S. Further study of nebulisation chemotherapy, a new chemotherapeutic method in the treatment of lung carcinomas: fundamental and clinical. *Br J Cancer* 1993;68:1146–9.
- Verschraegen CF, Gilbert BE, Loyer E, et al. Clinical evaluation of the delivery and safety of aerosolized liposomal 9-nitro-20(s)-camptothecin in patients with advanced pulmonary malignancies. *Clin Cancer Res* 2004;10:2319–26.
- Dahl AR, Grossi IM, Houchens DP, et al. Inhaled isotretinoin (13-cis retinoic acid) is an effective lung cancer chemopreventive agent in A/J mice at low doses: a pilot study. *Clin Cancer Res* 2000;6:3015–24.
- Estensen RD, Jordan MM, Wiedmann TS, Galbraith AR, Steele VE, Wattenberg LW. Effect of chemopreventive agents on separate stages of progression of benzo(a)pyrene induced lung tumors in A/J mice. *Carcinogenesis* 2004;25:197–201.
- Koshkina NV, Waldrep JC, Knight V. Camptothecins and lung cancer: improved delivery systems by aerosol. *Curr Cancer Drug Targets* 2003;3:251–64.
- Wattenberg LW, Wiedmann TS, Estensen RD, Zimmerman CL, Steele VE, Kelloff GJ. Chemoprevention of pulmonary carcinogenesis by aerosolized budesonide in female A/J mice. *Cancer Res* 1997;57:5489–92.
- Wattenberg LW, Wiedmann TS, Estensen RD, et al. Chemoprevention of pulmonary carcinogenesis by brief exposures to aerosolized budesonide or beclomethasone dipropionate and by the combination of aerosolized budesonide and dietary myoinositol. *Carcinogenesis* 2000;21:179–82.
- Ju J, Lu G, Lambert JD, Yang CS. Inhibition of carcinogenesis by tea constituents. *Semin Cancer Biol* 2007;17:395–402.
- Clark J, You M. Chemoprevention of lung cancer by tea. *Mol Nutr Food Res* 2006;50:144–51.
- Chang PY, Mirsalis J, Riccio ES, et al. Genotoxicity and toxicity of the potential cancer-preventive agent polyphenon E. *Environ Mol Mutagen* 2003;41:43–54.
- Yan Y, Cook J, McQuillan J, et al. Chemopreventive effect of aerosolized polyphenon E on lung tumorigenesis in A/J mice. *Neoplasia* 2007;9:401–5.
- Zhang Z, Liu Q, Lantry LE, et al. A germ-line p53 mutation accelerates pulmonary tumorigenesis: p53-independent efficacy of chemopreventive agents green tea or dexamethasone/myo-inositol and chemotherapeutic agents taxol or adriamycin. *Cancer Res* 2000;60:901–7.
- Yan Y, Wang Y, Tan Q, et al. Efficacy of polyphenon E, red ginseng, and rapamycin on benzo(a)pyrene-induced lung tumorigenesis in A/J mice. *Neoplasia* 2006;8:52–8.
- Liao XM, Liang W, Wiedmann T. Lung distribution of the chemopreventive agent difluoromethylornithine (DFMO) following oral and inhalation delivery. *Exp Lung Res* 2004;30:755–69.
- Liao J, Yang GY, Park ES, et al. Inhibition of lung carcinogenesis and effects on angiogenesis and apoptosis in A/J mice by oral administration of green tea. *Nutr Cancer* 2004;48:44–53.
- Mimoto J, Kiura K, Matsuo K, et al. (–)-Epigallocatechin gallate can prevent cisplatin-induced lung tumorigenesis in A/J mice. *Carcinogenesis* 2000;21:915–9.
- Yang CS, Yang GY, Landau JM, Kim S, Liao J. Tea and tea polyphenols inhibit cell hyperproliferation, lung tumorigenesis, and tumor progression. *Exp Lung Res* 1998;24:629–39.
- Cao J, Xu Y, Chen J, Klaunig JE. Chemopreventive effects of green and black tea on pulmonary and hepatic carcinogenesis. *Fundam Appl Toxicol* 1996;29:244–50.
- Witschi H, Espiritu I, Ly M, Uyeminami D, Morin D, Raabe OG. Chemoprevention of tobacco smoke-induced lung tumors by inhalation of an epigallocatechin gallate (EGCG) aerosol: A pilot study. *Inhal Toxicol* 2004;16:763–70.
- Wang ZY, Huang MT, Ho CT, et al. Inhibitory effect of green tea on the growth of established skin papillomas in mice. *Cancer Res* 1992;52:6657–65.
- Yang CS, Chen LS, Lee MJ, Balentine D, Kuo MC, Schantz SP. Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. *Cancer Epidemiol Biomarkers Prev* 1998;7:351–4.
- Chen LS, Lee MJ, Li H, Yang CS. Absorption, distribution, and elimination of tea polyphenols in rats. *Drug Metabol Dis* 1997;25:1045–50.
- Labiris NR, Dolovich MB. Pulmonary drug delivery. Part I: Physiological factors affecting therapeutic effectiveness of aerosolized medications. *Br J Clin Pharmacol* 2003;56:588–99.
- Raabe OG, Al-Bayati MA, Teague SV, Rasolt A. Regional deposition of inhaled monodisperse coarse and fine aerosol particles in small laboratory animals. *Ann Occup Hyg* 1988;32:53–63.
- Zheng F. Thermophoresis of spherical and non-spherical particles: a review of theories and experiments. *Adv Colloid Inter Sci* 2002;97:255–78.