Effect of dietary green tea extract and aerosolized difluoromethylornithine during lung tumor progression in A/J strain mice

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Chemoprevention strategies to prevent the development of lung cancer in at-risk individuals are a key component in disease management. In addition to being highly effective, an ideal chemopreventive agent will require low toxicity as patients are likely to require treatment for several years before their risk of cancer is lowered to background levels. In principle, a combination of safe agents that work through distinct mechanisms will improve efficacy while simultaneously maintaining a favorable safety profile. Here, we describe the use of the decaffeinated green tea extract Polyphenon E (Poly E) (1% in diet) and aerosolized difluoromethylornithine (DFMO) (20 mg/kg/day, 5 days/week) in a mouse lung cancer chemoprevention study using a progression protocol. Female A/J mice were injected with benzo[a]pyrene (B[a]P) at 8 weeks of age and precancerous lesions allowed to form over a period of 21 weeks before chemoprevention treatment for an additional 25 weeks. Poly E treatment did not significantly inhibit average tumor multiplicity but reduced per animal tumor load. Analysis of tumor pathology revealed a specific inhibition of carcinomas, with the largest carcinomas significantly decreased in Poly E-treated animals. Aerosolized DFMO did not have a significant effect on lung tumor progression. Magnetic resonance imaging of B[a]P-induced lung tumors confirmed the presence of a subset of large, rapidly growing tumors in untreated mice. Our results suggest a potential role for green tea extracts in preventing the progression of large, aggressive lung adenocarcinomas.

Background

Lung cancer is the leading cause of cancer mortality in both men and women in the USA. Whereas lifelong smokers have an increased risk of developing lung cancer, former smokers also remain at an elevated risk for developing disease for many years after smoking cessation (1,2). The goal of lung cancer chemoprevention is to prevent precancerous lesions present in at-risk populations, especially former smokers, from progressing to a cancerous state. Preclinical studies have demonstrated efficacy of many compounds in rodent models of lung cancer chemoprevention but translating these results has proven difficult as compounds that have been efficacious in preclinical models have frequently failed to provide efficacy in the clinical setting. Part of the reason for the failure of animal studies to translate into successful clinical trials is that most animal studies have been conducted during the tumor formation stage when chemopreventive treatment is more likely to be successful. Animal studies that examine chemopreventive efficacy in the progression stage of tumorigenesis are needed.

Green tea or its constituent compounds inhibit tumor formation in a variety of tissues, including the lung (3–6). Polyphenolic catechins are the active agents in green tea and function through a variety of mechanisms. The well-described antioxidant properties of tea catechins (7) may contribute to the antiproliferative and proapoptotic capacity of green tea by inhibiting AP-1 and NF-kB activity (8,9). Green tea catechins also inhibit DNA methyltransferases, and thus may function to reactivate tumor suppressor genes silenced by promoter hypermethylation (10). The inhibition of angiogenesis (11,12) and stimulation of apoptosis (11,13) by green tea has also been described.

Difluoromethylornithine (DFMO) is an irreversible inhibitor of the enzyme ornithine decarboxylase, the rate-limiting enzymatic step in polyamine synthesis (14). Intracellular and extracellular levels of the polyamines spermine, spermidine and putrescine are important regulators of cell proliferation and cell survival (15). Consistent with its growth inhibitory properties, DFMO prevented tumorigenesis in hamster upper respiratory tract (16,17). A limitation of oral administration of DFMO is the known ototoxicity of the compound (18). Aerosol drug delivery in lung cancer chemoprevention offers the advantage of delivering drug directly to the site of tumor formation and also potentially reducing systemic levels of drug, thus lowering the potential for toxicity. Aerosol delivery of DFMO to mice increased lung drug levels compared with administration by gavage (19) and is effective in preventing tumorigenesis in hamster upper respiratory tract (17).

One approach to improve the efficacy of chemoprevention is the use of treatment combinations, particularly combinations of agents that exert their effects via distinct mechanisms. In the current study, we examined the effect of dietary Polyphenon E (Poly E), a standardized green tea extract, and aerosolized DFMO on lung tumor progression. In the progression model, lung adenomas were allowed to form before initiation of chemoprevention treatment. The effect of the compounds on progression to adenocarcinoma was assessed. Tumor number, size and pathology were examined to determine the efficacy of each treatment. Dietary green tea extract inhibited lung adenocarcinoma growth whereas aerosolized DFMO had no effect. In particular, large tumors were less frequent in the green tea-treated group than the control group. As large tumors may represent the most aggressive tumor type, inhibition of this subclass of tumors has important implications for lung cancer chemoprevention and treatment. Our results demonstrate that Poly E inhibited the growth of large adenocarcinomas in this progression model and suggest that Poly E may be clinically useful in preventing lung adenocarcinoma progression.

Materials and methods

Reagents

DFMO and Poly E were obtained from the National Cancer Institute, Division of Cancer Prevention (Bethesda, MD). Benzo[a]pyrene (B[a]P) (>98% purity) and tricaprylin were purchased from Sigma–Aldrich (St Louis, MO).

Experimental design

Animal studies were conducted in accordance with approved protocols by the Institutional Animal Care and Use Committee at the University of Cincinnati and complied with Association for Assessment and Accreditation for Laboratory Animal Care policies. Female A/J mice 6–7 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate for 1 week before initiating experiments. Mice were fed and watered ad libitum and maintained on a 12 hour light/dark cycle with a temperature range of 22°C ± 1°C. Animals were randomized based on body weight into experimental groups. Mice were injected subcutaneously with benzo[a]pyrene (10 mg/kg) in tricaprylin (10 mg/kg) on days 0 and 7 and were subsequently randomized to treatment groups on day 0. One group was treated with Poly E (1%) in diet on days 0–21, while a second group was treated with aerosolized DFMO (20 mg/kg/day) on days 0–21. All treatment groups were maintained on a 12 hour light/dark cycle with a temperature range of 22°C ± 1°C. Animals were randomized based on body weight into experimental groups. Mice were injected subcutaneously with benzo[a]pyrene (10 mg/kg) in tricaprylin (10 mg/kg) on days 0 and 7 and were subsequently randomized to treatment groups on day 0. One group was treated with Poly E (1%) in diet on days 0–21, while a second group was treated with aerosolized DFMO (20 mg/kg/day) on days 0–21. All treatment groups were maintained on a 12 hour light/dark cycle with a temperature range of 22°C ± 1°C.

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Six weeks after treatment initiation, four mice from the absolute control, Poly E and combination groups were euthanized, blood obtained by cardiac puncture and plasma prepared via centrifugation of whole blood. Ascorbate buffer (0.1 volume of 0.4 M NaH₂PO₄, 20% ascorbic acid (wt/vol) and 0.1% ethylenediaminetetraacetic acid (wt/vol), pH 3.6) was added to plasma as an antioxidant and plasma stored at −20°C until analysis. Lungs from the same mice were weighed then frozen in liquid nitrogen. Frozen lung tissue was homogenized in ascorbate buffer (2 ml/g of lung tissue), centrifuged at 16 000g for 5 min and the supernatant transferred to a new tube. Samples were stored at −80°C until further analysis. Thirteen catechin levels were measured using an ocellar microimeter for calculation of tumor volumes using the equation $V = \frac{4}{3} \pi r^3$, where $r$ is tumor radius. Lung lobes were separated prior to paraffin embedding. Paraffin blocks were cut and stained using hematoxylin and eosin with sections in each level designated L0. Three additional 5 μm sections were obtained every 200 μm (L1, L2 and L3). Tumor histology (hyperplasia, adenoma, dysplasia and carcinoma) of hematoxylin and eosin-stained sections at each level was assessed by a pathologist blinded to the group identifications using previously described criteria (22). Digitized images of hematoxylin and eosin-stained sections were obtained and area of each tumor classification and total lung calculated using pixel counts as described previously (23).

Analysis of EGCG concentration

Six weeks after treatment initiation, four mice from the absolute control, Poly E and combination groups were euthanized, blood obtained by cardiac puncture and plasma prepared via centrifugation of whole blood. Ascorbate buffer (0.1 volume of 0.4 M NaH₂PO₄, 20% ascorbic acid (wt/vol) and 0.1% ethylenediaminetetraacetic acid (wt/vol), pH 3.6) was added to plasma as an antioxidant and plasma stored at −20°C until analysis. Lungs from the same mice were weighed then frozen in liquid nitrogen. Frozen lung tissue was homogenized in ascorbate buffer (2 ml/g of lung tissue), centrifuged at 16 000g for 5 min and the supernatant transferred to a new tube. Samples were stored at −80°C until further analysis. Thirteen catechin levels were measured using an ocellar microimeter for calculation of tumor volumes using the equation $V = \frac{4}{3} \pi r^3$, where $r$ is tumor radius. Lung lobes were separated prior to paraffin embedding. Paraffin blocks were cut and stained using hematoxylin and eosin with sections in each level designated L0. Three additional 5 μm sections were obtained every 200 μm (L1, L2 and L3). Tumor histology (hyperplasia, adenoma, dysplasia and carcinoma) of hematoxylin and eosin-stained sections at each level was assessed by a pathologist blinded to the group identifications using previously described criteria (22). Digitized images of hematoxylin and eosin-stained sections were obtained and area of each tumor classification and total lung calculated using pixel counts as described previously (23).

Statistical analysis

Means and standard deviations were calculated for analysis of tumor multiplicity and volume. The volume of tumor and the percent lung area covered by carcinoma were compared among groups using analysis of variance. The volume and percent area were also categorized (dichotomized as <40 or ≥40 mm³, <20 or ≥20 mm³ and <10 or ≥10 mm³ for volume and <10 or ≥10%, <5 or ≥5% and <3 or ≥3% for surface area) and analyzed using the chi-square or Fisher’s exact test, as appropriate. Further analysis involved volume and percent area categorized into quintiles. Volume and percent area between Poly E treated and untreated were compared within quintiles using a t-test.

Results

Serum and lung catechin levels

Poly E is a decaffeinated extract from green tea leaves with highly reproducible characteristics, consisting of ~65% EGCG with lesser amounts of other catechins. Before starting chemoprevention experiments, we determined plasma and lung tissue levels of tea catechins in mice given 0.5 or 1.0% (wt/wt) Poly E in diet compared with mice receiving 0.6% (wt/vol) Poly E in drinking water, a dose that has been used in previous chemoprevention studies (24,25). Plasma and lung tissue were collected after 4, 7, 14 and 28 days of Poly E diet and tea catechins measured by high performance liquid chromatography. As expected, EGCG was the most abundant catechin detected in plasma and lung tissue (Figure 1A). Plasma catechin levels were similar with all treatments at 4, 7 and 14 days, however, 1% Poly E in the diet produced the highest tissue and plasma catechin levels at the later time points (Figure 1C). Catechin levels detected in lung tissue were lower but paralleled the pattern observed in serum (Figure 1D). Plasma and lung tissue catechin levels were highest at 14 days, decreasing slightly at 28 days. Plasma levels obtained using these doses are similar to those reported in human I studies (26–28). Because 1% Poly E achieved higher serum and tissue levels of tea catechins, but mouse body weights were not significantly affected (data not shown), we chose this dose for our chemoprevention studies.

Chemoprevention study

AJ strain mice were injected with a single dose of B[a]P at 8 weeks of age to initiate lung tumorigenesis. Twenty weeks after B[a]P, mice were randomized into six groups. Group 1 was euthanized 21 weeks after B[a]P to establish a baseline for tumor multiplicity and tumor grades. Surface tumor multiplicity at baseline was 4.6 ± 0.7 (Table I). Pathological characterization revealed no carcinomas at baseline (n = 20 mice). Following randomization, the remaining groups were treated as indicated in Table I until 46 weeks post-B[a]P. Mice receiving aerosolized DFMO, alone or in combination, gained weight more slowly than the aerosol control group (P = 0.03). Although there was a trend toward lower body weight in the aerosol control group compared with the untreated control, the difference was not significant. There was no significant difference in body weight between the Poly E-treated and untreated groups.

Surface tumor number (multiplicity) and tumor diameter were measured in all groups after overnight fixation. The number of surface lung tumors increased by ~3-fold between 21 and 46 weeks without chemoprevention treatment (Table I, Group 1 versus Group 2). Poly E and DFMO, alone or in combination, did not have a significant effect on surface tumor multiplicity after chemoprevention treatment compared with untreated control or aerosol control. Tumor diameters were used to calculate a tumor load per mouse. Tumor load varied significantly between groups (overall P-value < 0.001 by analysis of variance). There was a significant reduction in tumor load for the a priori comparison between the Poly E-treated and absolute control groups (P = 0.0001), but not between aerosol control and the aerosol treatment groups. There was a significant reduction in tumor load in the aerosol control group compared with absolute control (P = 0.0013).

In addition, large tumors were more frequently observed in the absolute control group than in the Poly E treatment group. The proportion of surface tumors larger than 20 or 40 mm² was significantly lower in Poly E-treated groups compared with the absolute control (Fisher’s exact test P-value 0.05 and 0.01, respectively) (Table II and Figure 2A and B). The reduction in the proportion of large tumors...
after Poly E treatment was confirmed in an independent experiment using the identical dose of Poly E (data not shown). To more closely analyze the differences between treatment groups, we compared average tumor load per mouse in each quintile of the untreated control and Poly E-treated groups (Figure 2C). Average tumor size was significantly reduced in each of the upper three quintiles in Poly E-treated mice compared with the untreated control (P-values: third quintile < 0.04, fourth quintile < 0.001, fifth quintile < 0.002).

To determine if the chemopreventive agents affected tumor pathology, lungs were step sectioned and pathology grade determined at three different lung levels separated by 200 μm. Lesions were graded as hyperplasia, adenoma, dysplasia or carcinoma as described previously (22). The area (mm²) of each grade lesion was determined using pixel counts of digitized images. Poly E in the diet reduced progression to lung adenocarcinomas compared with the absolute control group as measured by the percent of sampled lung area occupied by carcinoma (P < 0.04) (Figure 3). Lung area occupied by lower grade lesions (i.e. hyperplasia, adenoma and dysplasia) was unchanged. The multiplicity of each tumor grade (tumors/cm² lung area) was unaffected by Poly E treatment (data not shown). Treatment with aerosolized DFMO did not have a significant effect on tumor progression compared with the aerosol control and the combination of Poly E and DFMO (P-value = 0.05).

Table I. Effect of chemoprevention on surface tumor characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Treatment</th>
<th>Tumor multiplicity (mean ± SEM)</th>
<th>Tumor load* (mean ± SEM, mm³)</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>None</td>
<td>4.6 ± 0.7</td>
<td>nd</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>None</td>
<td>12.6 ± 1.9</td>
<td>38.1 ± 11.4</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>Mock aerosol</td>
<td>12.2 ± 1.2</td>
<td>17.5 ± 2.7</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>Poly E (1% wt/wt in diet)</td>
<td>10.3 ± 0.8</td>
<td>14.0 ± 1.6</td>
<td>97</td>
</tr>
<tr>
<td>5</td>
<td>33</td>
<td>DFMO aerosol (20 mg/kg body wt)</td>
<td>11.5 ± 0.9</td>
<td>13.5 ± 1.4</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>34</td>
<td>Poly E + DFMO</td>
<td>11.8 ± 1.1</td>
<td>11.7 ± 1.5</td>
<td>97</td>
</tr>
</tbody>
</table>

*aAnalysis of variance P-value < 0.001. 
*bGroup 1 euthanized at 21 weeks, all other group at 46 weeks. 
P = 0.0001 compared with untreated control. 
*Not significant compared with aerosol control. 

Table II. Large tumors are significantly decreased after Poly E treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>&gt;40 mm³</th>
<th>&gt;20 mm³</th>
<th>&gt;10 mm³</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>21</td>
<td>6 (29%)</td>
<td>9 (43%)</td>
<td>16 (76%)</td>
<td>0.01</td>
</tr>
<tr>
<td>Poly E</td>
<td>32</td>
<td>1 (3%)</td>
<td>5 (16%)</td>
<td>20 (63%)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Significant P-values (Fisher’s exact test) are indicated in bold.
aerosolized DFMO and dietary Poly E did not have increased efficacy compared with either agent alone.

Similar to what was observed with surface tumors, large carcinomas were more frequent in untreated mice, compared with Poly E-treated mice. To quantitate this observation, we determined the proportion of mice in each treatment group with total carcinoma area per lung (% carcinoma) above a specific cutoff. In the absolute control group, 19% of mice had carcinomas comprising $>10\%$ of lung tissue analyzed, whereas no Poly E-treated mice met the same criteria ($P = 0.02$, Fisher’s exact test) (Table II, Figure 4). The proportion of lungs with $>5\%$ occupied by adenocarcinoma was also significantly reduced in the Poly E-treated group ($P = 0.004$). However, this difference was lost when lower cutoffs were used. We determined the average percentage of carcinoma area per mouse in each quintile and found a significant treatment effect from Poly E in the upper two quintiles ($P < 0.001$).

Tumor imaging in live mice

Because large tumors were observed more frequently in untreated mice compared with Poly E-treated mice, we examined a set of magnetic resonance imaging data for the presence of large, rapidly growing tumors in untreated mice. Magnetic resonance images from A/J mice treated with B[a]P at 8 weeks of age were used for the assessment. Mice were first imaged at 8 months post-B[a]P and again 3 months later. Tumors with a wide range of growth characteristics were observed, even within the same mouse (Figure 5). Lung tumors that grew rapidly and tumors that remained static were both observed over the 3 month period. The observation of rapid tumor growth in A/J mice is consistent with the presence of large tumors in our absolute control group.

Discussion

Halting the progression of existing precancerous lesions from developing into lung cancer is a critical approach to reduce the mortality associated with this disease. Whereas many preclinical studies have examined the ability of agents to prevent lung adenoma formation, few have examined the ability of agents to inhibit the progression of lung adenomas to adenocarcinomas. As a necessary first step, the majority of studies have examined the ability of single agents to prevent lung tumorigenesis in mice; however, it is probable that combinations of agents may improve chemopreventive efficacy compared with single agents.

In the current study, we determined the ability of dietary green tea extract and aerosolized DFMO, singly and in combination, to prevent lung tumor progression in female A/J mice. Chemopreventive agents were chosen that mediate their effects via distinct mechanisms, thereby improving the likelihood that combination therapy will improve efficacy. Additionally, both agents had known efficacy in animal models and a history of human usage. Aerosol delivery of DFMO was chosen to reduce or eliminate the toxicity observed with oral delivery of this drug (18) while simultaneously increasing lung deposition (19). Dietary Poly E (1\%) reduced carcinoma burden in A/J mouse lung after 25 weeks of treatment. A difference in the prevalence or extent of other grade lesions was not observed, nor was there a difference in tumor multiplicity. Previous studies that examined the ability of dietary Poly E to prevent adenoma formation found a reduction in tumor load per mouse with little or no effect on tumor multiplicity (29),
similar to what we observed in the progression model. In the present study, tumor pathology was carefully assessed and the burden of each pathological grade determined. The area of lung sections occupied by carcinomas was reduced but carcinoma multiplicity was unchanged, consistent with the concept that Poly E inhibited tumor growth but did not cause regression of existing tumors. This finding is consistent with the hypothesis that Poly E may be useful in limiting the growth of malignant lesions in the lung.

Large tumors were more frequently observed in control compared with Poly E-treated mice. Analysis of tumor size revealed a significant decrease in the frequency of large tumors using both surface tumor diameter and percent carcinoma as end points. Quintile analysis confirmed that overall differences in tumor burden were due to changes in the largest 40% of tumors. This was true when either surface tumor volumes or percent of lung occupied by carcinoma were used in the analysis and support the concept that the largest tumors observed on the lung surface are indeed carcinomas. In a set of B[a]P-induced lung tumors followed by magnetic resonance imaging over a period of 3 months, both aggressively growing and stable tumors were observed. Similar observations of rapidly growing mouse lung tumors 50 weeks after urethane treatment have been observed (30). The presence of a subset of tumors that grow aggressively late in the carcinogenic process is consistent with the subset of large tumors observed in our control group.

Inhibition of large tumors has important clinical implications. Large, aggressive tumors have been proposed to pose a greater risk to lung cancer patients compared with small, slow-growing tumors that may remain latent for long periods (31). In addition, larger tumor size is associated with reduced survival, even within stage IA and IB lung cancer (32–35). Thus, inhibiting the frequency of large, aggressive carcinomas with chemopreventive agents, even if small carcinomas remain, may improve patient outcome.

No significant effect of aerosolized DFMO on lung tumor progression was found in this study. Aerosol delivery was performed using
a system identical to that used previously to achieve robust and reproducible deposition in mouse lung (19). DFMO output from the aerosol apparatus was monitored weekly during the study and remained within expected parameters throughout. Detection of a chemopreventive effect for DFMO in the current study was confounded by a trend toward decreased tumor burden observed in the aerosol control group compared with the absolute control. The inhibitory effect of the control aerosol exposure may be due to the effects of handling and restraint associated with the aerosol exposure process. The stress associated with this handling may be reflected in the trend toward reduced weight gain throughout the study in the aerosol control group compared with the control group that did not receive aerosol. The relatively long exposure time (8 min/day) used in the current study was selected as it allows for production of a smaller aerosol particle to deliver the drug, thus increasing deposition in the distal lung where adenocarcinoma formation in the A/J model occurs. However, the longer exposure time may also increase stress to the animals and result in the observed decrease in tumor progression in the aerosol control group. As a result of this study, we have reduced daily exposure time in subsequent experiments.

A recent study by Lu et al. (36) examined the ability of Poly E to inhibit lung tumor progression in A/J mice induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Lung tumors were allowed to progress for 20 weeks before 0.6% Poly E was administered in drinking water for an additional 32 weeks. Poly E inhibited gross tumor burden by 3-fold higher using B[a]P in the current study compared with the study by Lu et al. (36) that used 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Increased multiplicity following B[a]P may reflect a more rapid progression in this model. Consistent with this, Lu et al. (36) reported carcinoma incidence of 65% in the control group at study end compared with 95–100% in the current study. Thus, while both studies began treatment at the same time after carcinogen, the B[a]P-induced tumors may have progressed farther at the initiation of treatment, potentially limiting the ability of Poly E to reduce tumor multiplicity or carcinoma development. Nevertheless, the ability of Poly E to inhibit carcinoma growth was maintained. The dietary delivery of Poly E used in the current study more closely reflects the method of oral administration currently being used in green tea clinical chemoprevention trials.

In summary, 1% dietary Poly E inhibited lung adenocarcinoma burden by ~50% compared with controls as measured by the percentage of lung tissue occupied by carcinoma. While aerosolized DFMO caused a slight decrease in the carcinoma burden, the decrease was not statistically significant compared with the aerosol control group. The combination of agents was not more effective than green tea extract alone. Green tea had a significant effect on preventing the growth of large adenocarcinomas in female A/J mice. Our data support the concept that green tea may be effective in limiting adenocarcinoma progression, particularly the growth of large tumors.

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References


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