Identifying efficacious approaches to chemoprevention with chlorophyllin, purified chlorophylls and freeze-dried spinach in a mouse model of transplacental carcinogenesis

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The carcinogenic potential of dibenzo[a,l]pyrene (DBP) has been well characterized in numerous animal models. We have previously documented that a single dose of 15 mg/Kg DBP to pregnant mice late in gestation (GD 17) produces an aggressive T-cell lymphoma as well as lung and liver cancer in offspring. The current study examines the chemopreventive properties of chlorophyllin (CHL) and chlorophyll (Chl) in this transplacental carcinogenesis model. Pregnant B6129SF1 females, bred to 129S1/SvIm mice, received purified diets incorporated with either 2000 p.p.m. CHL, 2000 p.p.m. Chl or 10% freeze-dried spinach before, during and after gavage with DBP alone, no chemopreventive agent, or with controls dosed with DBP in tricaprylin (TCP) as the vehicle, with dietary CHL prior to a single carcinogenic treatment with AFB1 is ineffective (6). This mechanism should be essentially species independent and, therefore, effective in humans. Indeed, in a human clinical intervention trial in Qidong, China, where dietary AFB1 exposure is a serious concern (13), a dose of 100–300 mg of CHL, given with meals, for only 3 months was effective at reducing the urinary biomarker of AFB1-dependent DNA adduction by more than half (14). CHL costs pennies a day with no significant side effects being reported, making it extremely attractive for intervention due to the high rate of compliance.

It has been difficult to conduct cancer chemoprevention studies in vivo, mainly due to prohibitive costs and chemical instability. A counter-current chromatography method was recently reported (15), enabling the production of 23 g of highly pure Chl a/b from 90 Kg of spinach leaves in a single run. In addition to demonstrating chemoprevention against AFB1-dependent HCC, in both trout and rat (9,10), the Chl purified via this method markedly reduced AFB1 exposure in humans following oral coadministration (Jubert, Bench, Dashwood, Mata, Pereira, Tracewell, Turnbull, Williams, and Bailey, unpublished data), using 14C microdosing and accelerator mass spectrometry as the means of detection (16).

Our laboratory has developed a mouse transplacental cancer model (17,18,19,20) that has proven useful for chemoprevention studies. Incorporation of indole-3-carbinol (I3C, a major chemopreventive phytochemical from cruciferous vegetables) (20) or green tea (21) into the maternal diet or drinking water, respectively, provided marked protection for offspring with respect to development of dibenzo[a,l]pyrene (DBP)-dependent lymphoma and lung cancer. Although DBP-dependent lymphoma mortality is dependent upon fetal Cyp1b1 (18), chemoprevention by I3C was independent of the aryl hydrocarbon receptor (ahr) genotype (20). In the case of tea, it appeared that the major effect was from caffeine, as caffeine alone provided the greatest protection.

We now report that coadministration to pregnant mice of CHL by gavage with the potent polycyclic aromatic hydrocarbon (PAH), DBP, results in marked protection from mortality in offspring beginning at 3–6 months of age from an aggressive T-cell lymphoma and significantly reduced transplacental DBP-dependent lung tumor multiplicity as well. However, if CHL or Chl, either as pure compounds or as a component of freeze-dried spinach, was incorporated into the maternal diet before, during and after gavage with DBP alone, no chemoprotection toward the offspring was observed.

Introduction

Chlorophyllin (CHL) is a water-soluble derivative of chlorophyll (Chl) in which magnesium has been replaced with copper and the phytol chains lost. CHL has been safely used in human medicine (e.g. Derifil, primarily to control body odor in geriatric patients) for many years (1) and is available as a dietary supplement. Chl is present in our diet in green, leafy vegetables, reaching levels of 5.7% in spinach (2). Although the potential for CHL and Chl to act as antimutagens in vitro had been previously published (3), the cancer chemopreventive properties of CHL and Chl in vivo were first demonstrated in the aflatoxin B1 (AFB1) hepatocellular carcinoma (HCC) in rainbow trout model (4,5,6,7,8,9) and later in a rodent model (10). Physical complexation with the carcinogen reduces bioavailability to target organs (11,12), whereas extended preloading with dietary CHL prior to a single carcinogenic treatment with AFB1 is ineffective (6). This mechanism should be essentially species independent and, therefore, effective in humans. Indeed, in a human clinical intervention trial in Qidong, China, where dietary AFB1 exposure is a serious concern (13), a dose of 100–300 mg of CHL, given with meals, for only 3 months was effective at reducing the urinary biomarker of AFB1-dependent DNA adduction by more than half (14). CHL costs pennies a day with no significant side effects being reported, making it extremely attractive for intervention due to the high rate of compliance.

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Materials and methods

Chemicals and diets

CHL, tricaprylin (TCP) and dichloromethane were purchased from Sigma Chemical Co. (St Louis, MO). The chlorin content of CHL was based on the manufacturer’s assay of 4.5% copper and assertion that all copper was present as copper chlorins. DBP was provided by the National Cancer Institute sponsored Carcinogen Repository, at Midwest Research Institute (Kansas City, MO) and was at least 98% pure as determined by high-performance liquid chromatography. The semipurified diets, AIN93G and AIN93M, were purchased from Research Diets (New Brunswick, NJ). CHL was prepared as described below.

Preparation of CHL

The CHL used in this study was extracted from baby spinach purchased from a local organic grower. A detailed description of the extraction process can be found elsewhere (15). Briefly, after removal of stems, the leaves were washed with cold water, freeze dried, washed twice with petroleum ether (boiling point 30–60°C) and solids extracted twice using methanol:petroleum ether (3:1, vol/vol). Combined extracts were transferred to a separatory funnel and washed with saturated NaCl. A repeat wash of the aqueous layer with...
oil ether was reconstituted to give the final extract and again washed with saturated NaCl, filtered and evaporated in vacuo (<30°C). On average, 30 g of freeze-dried spinach yielded 300 mg of Chl. This Chl extract (90% pure by high-performance liquid chromatography) contained trace amounts of other pigments (carotenoids), as well as some oils, fats and waxes derived from the spinach leaves. Separation of these non-Chl fractions has revealed no protection against DBP carcinogenesis (Bailey et al., unpublished data).

**Preparation of test solutions**

Concentrated stocks (>5 mg/ml) of DBP were first prepared in dichloromethane and reconstituted to working concentrations in corn oil or TCP gavage vehicles. CHL is virtually insoluble in corn oil; thus, CHL solutions were prepared and diluted to the administered concentration in TCP gavage vehicle.

**Animals and treatment protocols**

Eight-week-old B6129SF1 female and 129S1/SvImJ male mice (Jackson Laboratories, Bar Harbor, ME) were housed at the Laboratory Animal Resource Center at Oregon State University. Mice were allowed to acclimate for 1 week at 20 ± 1°C and 50 ± 10% humidity, with a light–dark cycle of 12 h in microisolator cages (Life Products, Seaford, DE) with CareFRESH bedding. During breeding, gestation and lactation, mice were fed powdered AIN93G diet ad libitum. Upon breeding, gestation day 0 was established by the appearance of the vaginal plug. Beginning on the 9th day of gestation, pregnant mice were randomly assigned to one of the following feeding regimens: 2000 p.p.m. DBP (AIN93G). On the 17th day of gestation, pregnant mice were treated with TCP (0.01) as the vehicle. CHL was administered with DBP at a dose of 15 mg/kg by gavage (corn oil) on day 17 of gestation. The pregnant and nursing dams were administered powdered AIN93G diets supplemented with nothing [control (open squares)], 2000 p.p.m. CHL (filled triangles), 2000 p.p.m. Chl (inverted triangle-dashed line) or 10% freeze-dried spinach (inverted triangle-solid line).

**Histopathology**

At necropsy, the heart, thymus, lung, liver, spleen and kidney were removed, as well as other tissues if they appeared abnormal by gross pathology. The tissues were fixed in 10% formalin, stained with hematoxylin and eosin and analyzed by light microscopy. The previously identified T-cell lymphoblastic lymphoma produces high rates of mortality in this transplacental model (17,18,19). The lymphomas were very aggressive, resulting in invasion of numerous organs by transformed lymphocytes. In addition to lymphoma, mice surviving to 10 months of age developed lung tumors and most males had liver lesions, including foci, hepatocellular adenomas and, rarely, HCC. The lung lesions were initially scored by gross necropsy and a subset of each group submitted for histopathology. As identified previously, the lung lesions were diagnosed as hyperplasia, adenomas, adenoma with progression and carcinomas (17,18,19).

**Genotyping for ahb<sup>1</sup> and ahb<sup>10</sup> alleles**

At necropsy, an ear punch was collected and lyced overnight at 55°C in a solution of DirectPCR Lysis Reagent containing proteinase K (Viagen Biotech, Los Angeles, CA). The resulting lysate was briefly centrifuged prior to undergoing a polymerase chain reaction with allele-specific primers to permit one-true genotyping of the ahb alleles as described previously (17). Polymerase chain reaction products were separated and visualized on Novex 8% Tris-borate ethylenediaminetetraacetic acid gels (Invitrogen Technologies, Carlsbad, CA).

**Statistical analysis**

In statistical analysis of the offspring responses of various litter, we corrected for cluster (litter) effects if there was evidence of such an effect. This statistical approach is more completely described in (21).

**Survival curves**

To evaluate the survival curves, we used a log-rank test, also known as the Mantel–Haenszel or Mantel–Cox test (22). The survival curves of each group were evaluated (Figure 1); P-values that are significantly different at α = 0.05 from control A (diets, corn oil vehicle) and control B (co-gavage, TCP vehicle) are highlighted in Table I. In addition, in some cases it is relevant to adjust these P-values since multiple hypothesis tests are being performed.

**Multiplicity data**

To evaluate lung tumor data, we used a Wilcoxon rank sum test (alternatively known as the Mann–Whitney U test), which is a non-parametric version of a t-test for equal means. We used this because an initial evaluation of the multiplicity data for each group showed that, in many cases, the data could not be considered normally distributed. We compared the lung multiplicity information for all groups (Table II). Since we were performing multiple comparisons, which increases the likelihood of observing a significant comparison simply by chance, the P-value cutoffs for significance were modified by a Bonferroni correction.

### Table I. Effect of treatment and genotype on survival of offspring

<table>
<thead>
<tr>
<th>Maternal treatment</th>
<th>No. of offspring</th>
<th>Gender ratio</th>
<th>Genotype ratio</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male:female</td>
<td>b-1/d:d/d</td>
<td>Overall</td>
<td>b-1/d:d/d</td>
</tr>
<tr>
<td>Control-A</td>
<td>51 (9)</td>
<td>1.08:0.92</td>
<td>1.04:0.96</td>
<td>25.5 19.2 32.0</td>
</tr>
<tr>
<td>Control-B</td>
<td>60 (8)</td>
<td>0.52:0.2</td>
<td>0.93:1.07</td>
<td>50.0 41.4 58.1</td>
</tr>
<tr>
<td>Spinach</td>
<td>108 (14)</td>
<td>1.01:1.0</td>
<td>0.92:1.08</td>
<td>22.2 19.0 25.0</td>
</tr>
<tr>
<td>CHL</td>
<td>101 (13)</td>
<td>0.94:1.06</td>
<td>0.98:1.04</td>
<td>16.8 16.0 16.0</td>
</tr>
<tr>
<td>Chl</td>
<td>96 (12)</td>
<td>1.04:0.96</td>
<td>1.08:0.92</td>
<td>26.0 20.0 26.0</td>
</tr>
<tr>
<td>co-CHL</td>
<td>106 (15)</td>
<td>0.83:1.21</td>
<td>1.01:0.99</td>
<td>76.6 76.0 74.4</td>
</tr>
</tbody>
</table>

Day 0 of gestation was set as the first day a vaginal plug was observed. The numbers of dams for each group are indicated in parenthesis. Dams were placed on the listed dietary regimens beginning at gestation day 9 as their sole diet source. Dams designated in the following groups received DBP (15 mg/kg) as a single dose by gavage on day 17 of gestation (corn oil, spinach, CHL and Chl). The remaining dams (control-B and co-CHL) received DBP at identical levels in the TCP gavage vehicle. The group (co-CHL) in which the CHL (in TCP) was administered with DBP had a significantly higher survival rate than the control groups employing either corn oil (P < 0.001) or TCP (P < 0.01) as the vehicle.
Table II. Effect of treatment and genotype on lung tumor multiplicity

<table>
<thead>
<tr>
<th>Maternal treatment</th>
<th>Overall</th>
<th>Responsive (b-1/d)</th>
<th>Non-responsive (d/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-A</td>
<td>14.5 ± 2.6</td>
<td>15.2 ± 4.7</td>
<td>14.1 ± 3.0</td>
</tr>
<tr>
<td>Control-B</td>
<td>16.0 ± 1.3</td>
<td>13.8 ± 1.6</td>
<td>17.5 ± 1.8</td>
</tr>
<tr>
<td>CHL</td>
<td>14.8 ± 1.5</td>
<td>13.0 ± 1.5</td>
<td>16.3 ± 2.5</td>
</tr>
<tr>
<td>Chl</td>
<td>16.1 ± 1.2</td>
<td>16.5 ± 1.7</td>
<td>15.8 ± 1.8</td>
</tr>
<tr>
<td>Spinach</td>
<td>19.4 ± 1.6</td>
<td>16.5 ± 1.9</td>
<td>21.4 ± 2.2</td>
</tr>
<tr>
<td>Co-CHL</td>
<td>8.6 ± 0.7</td>
<td>8.9 ± 0.8</td>
<td>8.3 ± 1.0</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE for multiplicity (number of lung tumors per mouse), all groups are significantly different from co-CHL. Control-A, control-B, CHL, Chl and spinach are not significantly different from one another. Co-CHL b-1/d and co-CHL d/d are not significantly different from one another.

Results

CHL and Chl effects on DBP-dependent lymphoma mortalities

As previously documented by our laboratory, administration of a single dose of DBP on day 17 of gestation did not elicit acute maternal or fetal toxicities (number of pups/litter, weight at birth, sex ratio, etc.) and no gender differences were observed (17,18,19,20,21). Offspring born to mothers treated with DBP exhibited lymphoma-dependent mortality beginning at 10–12 weeks of age (Figure 1). When B6129F1 dams are crossed with 129 sires, half the offspring should have the ahr ‘responsive’ phenotype (genotype, ahr<sup>b-1/d</sup>) and half the ‘non-responsive’ (genotype, ahr<sup>d/d</sup>). Consistent with our previous findings, offspring identified as ahr responsive typically had lower rates of survival compared with their non-responsive littermates (Table I). Interestingly, administration of Chl or CHL, in the diet or by coadministration, eliminated that genotypic difference in offspring survival irrespective of any overall survival effect.

Maternal dietary exposure to freeze-dried spinach, CHL or Chl (in AIN93G) did not significantly alter the overall survival rates of offspring born to mothers treated with DBP (Figure 1). It should be noted that, as in previous studies with this model (17,18,19,20,21), there were no mortalities in offspring born to dams administered the corn vehicle (rather than DBP) among all the treatments (data not shown).

When dams were coadministered CHL with DBP, the protection against lymphoma mortality in the offspring was highly significant (P < 0.001, Figure 2A). The impact of coadministered DBP with CHL was apparent irrespective of offspring genotype (Figure 2B and Table I). To our surprise, mortality was lower in offspring born to mothers dosed with DBP alone using TCP instead of corn oil as the vehicle (Figure 2A). The difference was significant (P < 0.01), although less than in the mothers that were dosed with DBP in corn oil (P < 0.001). This result highlights the importance of including appropriate vehicle controls in transplacental chemoprevention studies, as performed here, since the TCP vehicle alone can alter DBP bioavailability to the fetus.

CHL and Chl effects on DBP-dependent transplacental lung cancer

As we have previously reported with this model, all mice exposed in utero to DBP and surviving to 10 months of age exhibited multiple lung lesions. The chemoprotective properties of I3C and green tea have been examined in this model and effectively reduced lung tumor burden by 35 and 32% in mice reaching 10 months of age (20,21). In the current study, maternal consumption of spinach, CHL or Chl in the diet did not provide any significant protective effects against DBP-dependent lung cancer. However, as with DBP-dependent lymphoma mortality, the ability of CHL to protect against lung tumor burden did prove significant if it was coadministered with DBP. Mice born to these mothers receiving the co-gavage of CHL and surviving to 10 months of age had ~51% fewer lung tumors (Table II, P < 0.01). Interestingly, although the vehicle used for delivery of DBP (corn oil versus TCP) had an impact on lymphoma mortality, mice surviving to 10 months of age had very similar levels of lung tumor multiplicity, 14.5 versus 15.2 for corn oil and TCP, respectively. Additionally, we also documented the time course of lung tumor multiplicity from 3 to 10 months of age. Coin treatment of CHL with DBP provided protection throughout the entire duration of the tumor study (Figure 3). If reduction in DBP bioavailability accounted for the extent of transplacental chemoprotection observed with corn oil versus TCP, one would have to account for the difference between fetal target tissues. The genotype of the offspring was not a significant factor in the degree of chemoprevention observed by coadministration of CHL (Table II). A potential explanation could lie in differences in DBP dose response among fetal target organs and end points in this model. As previously reported in a 10 000 animal dose–dose matrix experiment (8), DBP dose responses for liver tumor incidence and tumor multiplicity in the rainbow trout model were not linear, but instead reached a plateau or optimum at higher DBP doses. As a consequence, CHL chemoprevention in this organ was observed only at CHL doses sufficiently
Effects of coadministered CHL on lung tumor burden throughout the study. Offspring were euthanized due to lymphoma-dependent morbidity (3–9 months) or at the conclusion of the study (10 months) and lung lesions (predominantly adenomas) quantified by histopathology. Data indicate offspring born to mothers given 15 mg/kg DBP alone by gavage in TCP (open squares) or concurrent with 380 mg/kg CHL (in TCP) (inverted filled triangles). Numbers euthanized in the first group (<21 weeks) were 7 and 2. For the second group (>21 weeks and <44 weeks), 8 and 16 were euthanized. Numbers surviving to the end were 30 and 82 for control-B and co-CHL, respectively. The bars indicate ± SE.

Discussion

Exposure during pregnancy and lactation to chemicals in the environment, including tobacco smoke, has been associated with increases in incidences of disease, malformations or behavior in offspring (23,24,25,26,27,28,29,30,31). A number of chemicals have been shown to be transplacental carcinogens in rodent models (reviewed in refs 32,33) and epidemiology studies suggest that this phenomenon occurs in exposed human populations as well (23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38).

PAHs are formed from the incomplete combustion of organic materials including the burning of coal, petroleum products or tobacco (reviewed in ref. 39) and have been listed as human carcinogens by international agency for research on cancer (40). Increasing energy requirements, especially in countries such as China, are resulting in greater use of coal for energy production; indeed, China derives 70% of its energy from burning coal and the consumption is greater than in any other country (41). The burning of coal for energy production produces polycyclic aromatic hydrocarbons (PAHs), one of the major classes of PAHs. PAHs are formed from the incomplete combustion of organic materials including the burning of coal, petroleum products or tobacco (reviewed in ref. 39) and have been listed as human carcinogens by international agency for research on cancer (40). Increasing energy requirements, especially in countries such as China, are resulting in greater use of coal for energy production; indeed, China derives 70% of its energy from burning coal and the consumption is greater than in any other country (41). The burning of coal for energy production produces polycyclic aromatic hydrocarbons (PAHs), one of the major classes of PAHs.

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sensitivity rather than making it more complex. There is no statistical difference between CHL cotreatment with respect to response by the b-1/d and d/d ahr genotypes.

In summary, CHL, which is inexpensive and appears to lack toxicity in humans, was demonstrated to be effective in the reduction of transplacental cancer risk if given with the PAH carcinogen DBP. This protection was evident even with tumors that appeared well into adult life and is a further example of the ‘fetal basis of disease’. Cancer is the number two cause of death in children/young adults (accidents being number one) and lymphoma/leukemias are the most common of these cancers. Lung cancer is the major cause of cancer mortality in both sexes in the USA and has a relatively poor prognosis (5 year survival rate of 15%). For these reasons, chemopreventive strategies that begin early in development have the potential to reduce the suffering (as well as the health care dollars) associated with cancer, and perhaps other chronic diseases.

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References


Transplacental chemoprevention of cancer by chlorophylls

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