Inhibition of 3-Methylcholanthrene-induced Skin Tumorigenicity in BALB/c Mice by Chronic Oral Feeding of Trace Amounts of Ellagic Acid in Drinking Water

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ABSTRACT

Chronic p.o. feeding of small amounts of ellagic acid, a naturally occurring dietary plant phenol, to BALB/c mice in drinking water afforded significant protection against skin tumorigenesis induced by 3-methylcholanthrene, a polycyclic aromatic hydrocarbon carcinogen. A significant increase in the latent period for the development of skin tumors by 3-methylcholanthrene was observed in the ellagic acid-fed group of mice (9 wk on test) as compared to the control group of animals (6 wk on test). The observed protection against tumor induction in the ellagic acid-fed group of animals may be due to the inhibition of the metabolic activation of the polycyclic aromatic hydrocarbon since epidermal aryl hydrocarbon hydroxylase activity was found to be significantly inhibited. Our results suggest that dietary supplementation with small amounts of ellagic acid may prove useful in reducing the risk of skin carcinogenesis induced by environmental chemicals.

INTRODUCTION

It is now widely accepted that the initiation of carcinogenesis by PAHs relates to their metabolic biotransformation into highly reactive molecules that subsequently bind to cellular macromolecules such as DNA causing structural alterations which may be mutagenic (1, 2). PAHs are themselves relatively inert environmental precarcinogens that must first be metabolized by mammalian enzymes to their biologically active ultimate carcinogenic forms (3, 4). Studies on the mutagenicity, tumorigenicity, and metabolism of the PAHs have indicated that bay-region diol-epoxides are the ultimate carcinogenic species and that binding of these diol-epoxides to DNA can explain their carcinogenicity (5, 6).

Knowledge that metabolic activation is a critical determinant of tumor induction by chemicals has led to a search for nontoxic inhibitors of this enzyme-mediated process. For example there has been growing interest in the identification of naturally occurring dietary factors as potential anticarcinogens (7). The increasing occurrence of dietary plant phenols, to BALB/c mice in drinking water afforded substantial protection against skin tumorigenesis induced by 3-methylcholanthrene, a polycyclic aromatic hydrocarbon carcinogen. A significant increase in the latent period for the development of skin tumors by 3-methylcholanthrene was observed in the ellagic acid-fed group of mice (9 wk on test) as compared to the control group of animals (6 wk on test). The observed protection against tumor induction in the ellagic acid-fed group of animals may be due to the inhibition of the metabolic activation of the polycyclic aromatic hydrocarbon since epidermal aryl hydrocarbon hydroxylase activity was found to be significantly inhibited. Our results suggest that dietary supplementation with small amounts of ellagic acid may prove useful in reducing the risk of skin carcinogenesis induced by environmental chemicals.

Materials and Methods

Chemicals. MCA, BP, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Ellagic acid dihydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals used were of highest purity commercially available.

Feeding of Ellagic Acid and Tumor Studies. One hundred male BALB/c mice, aged 6 wk, were obtained from Charles River Laboratory (Wilmington, MA). All mice were maintained on standard Purina chow ad libitum during the entire period of experimentation. The mice were divided into two groups of fifty each. One group of animals was supplied with double-distilled water for drinking whereas the other group of mice received ellagic acid (3 mg/liter) added to double-distilled water. At this concentration ellagic acid is soluble in distilled water. Ellagic acid in drinking water was prepared fresh on every Monday, Wednesday, and Friday. One hundred twenty days after initiating administration of ellagic acid, the mice were shaved using electric clippers and Nair depilatory was applied. Two days later the skin tumor experiment was begun. Forty mice from each group were further divided into two groups of 20 each and the remaining animals were used for metabolic studies. One group of 20 mice received 1.5 ¿imol MCA applied topically in 0.2 ml acetone as described earlier (13). The other group of mice received 0.2 ml acetone alone and served as controls. The treatments were repeated twice weekly for 16 wk, at which time the experiment was terminated. Skin tumor formation was recorded weekly and tumors greater than 1 mm diameter were included in the cumulative total only if they persisted for 2 wk or longer. No skin neoplasms occurred in any of the mice treated with acetone. The mean consumption of water in both groups of mice was similar with an average consumption of 6 ml water/day/animal. This water consumption is equivalent to approximately an 18 µg/mouse/day (0.8 mg/kg)-dose of ellagic acid. These doses of ellagic acid were well tolerated by the animals with no apparent signs of toxicity such as weight loss or mortality.

Enzyme Assays in Skin of Mice Fed Ellagic Acid. Prior to beginning the tumor experiment, six mice from each group receiving double-distilled water or ellagic acid in the drinking water were killed. Skin microsomal and cytosolic fractions were prepared according to established procedures in this laboratory (16). AHH activity was determined by a modification of the method of Nebert and Gelboin (17), the details of which have been described earlier (18). The partitioning of phenolic BP metabolites was based on comparison of fluorescence with a 3-OH BP standard. 7-Ethoxycoumarin O-deethylase activity was determined according to a slight modification of the procedure of Greenlee and Poland (19), the adaptations of which have been described earlier (18).

Epoxide hydrolase activity was assayed using BP-4,5-oxide as substrate according to the thin layer chromatographic technique of Jerina et al.

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3 Recipient of the Burroughs Wellcome Fund fellowship award from the Dermatology Foundation.

4 The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; BP, benz(a)pyrene; MCA, 3-methylcholanthrene, AHH, aryl hydrocarbon hydroxylase; GST, glutathione S-transferase; EH, epoxide hydrolase.
ELLAGIC ACID INHIBITION OF SKIN TUMORIGENICITY

RESULTS

Effect of Feeding Ellagic Acid on Skin Monoxygenase and GST Activities. Prior to initiating the tumor experiments metabolic studies were conducted on ellagic acid-fed mice to determine whether prolonged administration of this plant phenol in drinking water had inhibitory effects on the metabolism of PAHs. The data presented in Table 1 indicate that AHH, 7-ethoxycoumarin O-deethylase, and EH activities in skin of mice fed ellagic acid were significantly lower than the corresponding activities in the control animals. AHH and EH each participate in the metabolic activation of PAHs to diol-epoxides (2–6). Interestingly cytosolic GST activity in the skin of the ellagic acid-fed group was 44% higher than the enzyme activity in the control group. GST participates in the metabolic detoxification of PAHs metabolites produced by AHH and/or EH (3–4). The extent of binding of a radiolabeled PAH ([3H]BP) to epidermal DNA was 54% lower in the mice fed ellagic acid as compared to controls (data not shown). In summary these data suggest that p.o. feeding of ellagic acid results in diminished metabolic activation of PAHs.

Effects of Feeding of Ellagic Acid on Skin Tumorigenesis. The tumor induction studies were carried out on groups of BALB/c mice that were fed double-distilled water or trace amounts of ellagic acid in their drinking water for 120 days. The data presented in Fig. 1 represent the percentage of mice with tumors and cumulative number of tumors produced by topical application of MCA in each group. The animals pretreated with p.o. administered ellagic acid had significantly fewer skin tumors and the latent period prior to the onset of tumor development was considerably prolonged. The first tumor appeared after 10 wk of treatment with MCA in the ellagic acid-fed animals as compared to 6 wk in the corresponding control group receiving double-distilled water (Fig. 1A). After 10 wk of skin application of MCA only 10% of the mice in the ellagic acid-fed group demonstrated any neoplasms as compared to 90% of the animals in the control group (Fig. 1A). The data presented in Fig. 1B indicate that at each time point fewer tumors developed in the ellagic acid-fed group as compared to the mice receiving double-distilled water. The data in Table 2 represent skin tumors/mouse as a function of the number of weeks after carcinogen application. At each time point highly significant protection against MCA-induced skin tumor formation was evident in animals receiving ellagic acid in their drinking water. In aggregate these data indicate that feeding of small amounts of ellagic acid in drinking water to BALB/c mice has substantial antitumorogenic effects in the skin of these animals.

Table 1 Effect of chronic p.o. feeding of ellagic acid in drinking water on epidermal monoxygenase and GST activities

<table>
<thead>
<tr>
<th>Enzyme activities</th>
<th>pmol product/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Ellagic acid fed</td>
</tr>
<tr>
<td>AHH</td>
<td>0.89 ± 0.04* 0.64 ± 0.05*</td>
</tr>
<tr>
<td>7-Ethoxycoumarin O-deethylase</td>
<td>1.01 ± 0.05 0.59 ± 0.06*</td>
</tr>
<tr>
<td>EH</td>
<td>30 ± 2 22 ± 3*</td>
</tr>
<tr>
<td>GST</td>
<td>57 ± 3 82 ± 4*</td>
</tr>
</tbody>
</table>

* Mean ± SE of six individual values.

Fig. 1. Effect of p.o. feeding of ellagic acid on MCA-induced skin tumorigenesis. Male BALB/c mice (6–8 wk old) were fed ellagic acid in double-distilled water for 120 days. Controls received drinking water alone. After feeding of ellagic acid, mice were treated with topically applied MCA. •, normal drinking water; □, ellagic acid in drinking water. The percentage of mice with tumors (A) and the cumulative number of tumors (B) were plotted as a function of the number of weeks on test.

Table 2 Effect of feeding of ellagic acid in drinking water on skin tumorigenesis in BALB/c mice

<table>
<thead>
<tr>
<th>Wk after MCA application</th>
<th>Normal drinking water</th>
<th>Ellagic acid in drinking water</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.20 ± 0.15</td>
<td>NTF</td>
</tr>
<tr>
<td>7</td>
<td>0.60 ± 0.28</td>
<td>NTF</td>
</tr>
<tr>
<td>8</td>
<td>1.60 ± 0.40</td>
<td>NTF</td>
</tr>
<tr>
<td>9</td>
<td>3.90 ± 0.65</td>
<td>NTF</td>
</tr>
<tr>
<td>10</td>
<td>5.80 ± 0.75</td>
<td>0.40 ± 0.27*</td>
</tr>
<tr>
<td>11</td>
<td>6.90 ± 0.70</td>
<td>1.10 ± 0.49*</td>
</tr>
<tr>
<td>12</td>
<td>7.60 ± 0.57</td>
<td>1.50 ± 0.47*</td>
</tr>
<tr>
<td>13</td>
<td>8.90 ± 0.45</td>
<td>2.60 ± 0.54*</td>
</tr>
<tr>
<td>14</td>
<td>9.40 ± 0.51</td>
<td>3.20 ± 0.43*</td>
</tr>
<tr>
<td>15</td>
<td>9.50 ± 0.50</td>
<td>4.00 ± 0.42*</td>
</tr>
<tr>
<td>16</td>
<td>9.60 ± 0.48</td>
<td>5.25 ± 0.35*</td>
</tr>
</tbody>
</table>

* NTF, no tumor formed.

Values were significantly different (P < 0.05) from those obtained with normal drinking water (see "Materials and Methods").

DISCUSSION

In recent years it has become clear that a variety of naturally occurring substances has the ability to protect against certain types of environmentally induced cancers (7, 8, 24). Plant phenols are one important category of such naturally occurring chemopreventive agents (11, 14, 24). It has been estimated that some human individuals consume as much as 1 g of plant phenols/day in their diet (25). It is not clear, however, whether these individuals are at any diminished risk for developing cancer. We have been evaluating a series of plant phenols such as ellagic acid and its derivatives for their possible chemopreventive effects.
as ellagic acid for their potential as antagonist for PAH-induced carcinogenicity. Ellagic acid is a polyphenol which occurs largely as ellagitannins in woody dicotyledenous plants (10). It possesses a structural feature of a meta or para hydroxylated benzoic or cinnamic acid. Ellagic acid is present in tannins and in grapes, strawberries, black currants, raspberries, and nuts (10, 11). It possesses biological activity and has been shown to inhibit hemorrhage in animals (26, 27) and humans by activating the intrinsic blood coagulation system (28, 29). Ellagic acid is well tolerated by both experimental animals and humans. Rats fed ellagic acid at doses as high as 50 mg/day up to 45 days did not exhibit any signs of systemic toxicity (11). L.v. administered doses of ellagic acid (0.2 mg/kg) have been shown to be well tolerated by humans (30). Lesca (14) while evaluating the protective effects of ellagic acid on BP-induced lung tumor formation in A/J mice observed that p.o. administration of the plant phenol in the diet (daily dose of 100 mg/kg for 15 days) did not cause any toxicity. However he observed that i.p. administered ellagic acid resulted in severe toxicity and mortality after four injections of 100 mg/kg in sulfuroil oil. The dosage of ellagic acid fed to animals in our study were without any apparent toxicity.

PAHs such as BP are metabolized in mammalian cells to their corresponding epoxides by AHK, a membrane-bound cytochrome P-450-dependent mixed-function oxidase system (2–4). Studies on the mutagenicity, metabolism, DNA binding, and tumorigenicity of the PAHs carried out by numerous investigators in recent years have shown that one of the isomeric forms of the BP-7,8-diol-9,10-epoxide is the ultimate carcinogenic metabolite of BP (see reviews in Refs. 2–4 and references therein). Since the formation of this ultimate carcinogenic metabolite of BP is catalyzed by Ahh and Eh, these enzymatic pathways may play important roles in the generation of the ultimate carcinogenic moiety in target tissues. Reactive epoxides can also be detoxified by conjugation with glutathione, a reaction catalyzed by GST (31). In addition to these pathways of enzymatic activation and inactivation other processes such as the extent of carcinogen binding to DNA and the capability of DNA to repair structural damage caused by covalently bound carcinogens are also important determinants of the initiation of tumor formation by the PAHs (3, 32). The generation of a reactive metabolite is a prerequisite for DNA binding and therefore for the oncogenicity of these compounds. Since the metabolism of the PAHs is a prerequisite for their carcinogenicity, it is currently believed that one promising approach to reducing the risk of developing chemically induced cancers might be to modulate the activity of enzymes that are crucial for the metabolic activation and inactivation pathways.

The results of the present study indicate that chronic feeding of small amounts of the polyphenol ellagic acid in drinking water delays MCA-induced skin tumorigenicity. Very little information is available on the mechanism of the antitumorigenicity of ellagic acid. Studies by Del Tito et al. (33) have shown that topicaly applied ellagic acid is a potent inhibitor of epidermal microsomal AHK and of enzyme-mediated BP binding to calf thymus DNA in vitro and to epidermal DNA in vivo. Ellagic acid was also shown to inhibit the covalent binding of BP to mouse epidermal DNA in organ culture (34). The studies of Dixel et al. (35) have shown that ellagic acid inhibits BP and BP-7,8-diol metabolism and DNA binding in mouse lung explants. We have shown (36) that ellagic acid is a potent inhibitor of BP metabolism and its subsequent glucuronidation, sulfation, and covalent binding to DNA in cultured BALB/c mouse keratinocytes. Our recent studies have shown that following p.o. or i.p. administration of ellagic acid to BALB/c mice hepatic and pulmonary microsomal metabolism of BP is substantially inhibited (37). In this study we observed inhibition in the formation of diols (including BP-7,8-diol), as well as phenols and quinones. Our unpublished results indicate that ellagic acid topicaly applied to the skin of SENCAR mice is capable of inhibiting the binding of several PAHs to epidermal DNA. Although our studies have not identified a specific mechanism for the antitumorigenic effect of ellagic acid, Sayer et al. (38) postulated that the inhibitory effect of this compound is due to its direct and facile interaction with BP-diol-epoxide-2 to form an inactivated adduct. It is probable that this type of effect could explain our findings as well. In summary our data indicate that chronic p.o. feeding of low doses of ellagic acid to mice results in delayed tumor onset and affords substantial protection against PAH-induced skin tumorigenicity.

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REFERENCES


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