Selective Inhibition of MethylbenzylNitrosamine-induced Formation of Esophageal O6-Methylguanine by Dietary Ellagic Acid in Rats

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INTRODUCTION

Epidemiological studies have identified a number of environmental factors including certain foods which are associated with a reduction in the incidence of carcinoma in humans (1-4). Animal models have examined a number of these foods and have identified a variety of anticarcinogenic compounds, including the naturally occurring plant phenol, ellagic acid (1). Dietary ellagic acid has been shown to reduce the incidence of a number of carcinogen-induced tumors (5-7) including MBN1-induced esophageal carcinoma in the rat (8). MBN is naturally occurring carcinogen associated with the increased incidence of human esophageal carcinoma of China (9-11). MBN has been shown to be carcinogenic to esophageal cells in culture (12), and rats treated with MBN develop squamous cell carcinoma.

MATERIALS AND METHODS

Animal Care. Weanling male Sprague-Dawley rats were purchased from Charles River, Inc. (Region 10; Portage, MI). Animals were maintained at the American Association for the Accreditation of Laboratory Animal Care-approved Biological Resource Laboratory of the University of Illinois at Chicago. The lights were on for 12 h each day, and the animals received the control or ellagic acid diets and deionized water ad libitum. The custom-formulated control and ellagic acid diets were prepared by Teklad, Madison, WI. These diets were nutritionally complete and identical (32) except for the addition of ellagic acid (0.4 g/kg of diet) to the ellagic acid diet. This dose of ellagic acid has previously been shown to reduce the incidence of MBN-induced esophageal carcinoma by 30 to 50% (8). The animals were housed in individual suspended stainless steel cages and weighed weekly.

In Vivo Methylation Studies. After 3 wk on the diets, animals were weighed and given injections i.p. with a single dose of MBN (Ash Stevens, Inc., Detroit, MI), 2.0 mg/kg of body weight (32-34), and then sacrificed with carbon dioxide 1 h after the MBN injection. The esophagi were then rapidly removed, and the esophageal mucosa was isolated by physically shearing the outer muscle-submucosal layers away from the mucosa. The resulting mucosa was completely intact and contained no muscle or submucosal contamination as determined by histological examination. All subsequent steps were performed at 4°C. The mucosa from five to eight animals was combined to provide of the esophagus which is histologically very similar to human esophageal carcinoma (13, 14). The proposed mechanism of MBN-induced esophageal carcinoma is through oxidative metabolism of MBN to form benzaldehyde and an activated metabolite which methylates DNA. MBN is known to methylate DNA in vivo forming O6-mGua and m7Gua adducts (15, 16). The highest concentration of these MBN-induced adducts is found in the esophageal DNA of the rat (15, 16), the target tissue of this carcinogen (13, 17-19). Formation of O6-mGua adducts is strongly associated with carcinogenesis and mutagenesis in a variety of animal models, while m7Gua does not appear to be related to tumor induction (20-24). O6-mGua adducts can lead to guanine to adenine point mutations (25-29) by misreading the O6-mGua as if it were an adenine. Such guanine to adenine point mutations have been shown to be directly responsible for methylbenzylamino-induced mammary carcinoma in the rat (30, 31). The mechanisms through which dietary ellagic acid reduces the incidence of MBN-induced esophageal carcinoma remain to be determined.

In this paper, we describe experiments which demonstrate that dietary ellagic acid leads to a significant reduction in the in vivo formation of MBN-induced esophageal O6-mGua without reducing the formation of esophageal m7Gua. Examination of this effect in an in vitro methylation assay demonstrated that dietary ellagic acid did not reduce the ability of esophageal microsomes to methylate purified calf thymus DNA; however, pretreatment of the calf thymus DNA with ellagic acid selectively reduced the MBN-induced formation of O6-mGua by microsomes from both ellagic acid-fed and control animals without altering the in vivo formation of m7Gua. These results suggest that ellagic acid bound to DNA selectively blocks methylation of the O6-position of guanine without inhibiting the activation of MBN or the ability of MBN to methylate DNA.

ABSTRACT

Ellagic acid is a naturally occurring plant phenol which has been shown to reduce the incidence of a number of carcinogen-induced tumors including methylbenzylNitrosamine (MBN)-induced esophageal carcinoma in the rat. The postulated mechanism of MBN-induced esophageal carcinogenesis is through oxidation of MBN to form benzaldehyde and an activated metabolite which methylates DNA forming a variety of methylated DNA adducts including O6-methylguanine (O6-mGua) and 7-methylguanine (m7Gua). O6-mGua adducts have been shown to induce DNA mutations which can lead to cancer, while m7Gua adducts do not appear to be related to tumor induction. In this study, we examined whether the decreased incidence of MBN-induced esophageal carcinoma observed with dietary ellagic acid was associated with a decrease in the in vivo and in vitro formation of MBN-induced DNA adducts and whether this reduction was specific to O6-mGua or due to a reduction in total methylation. Weanling male Sprague-Dawley rats were fed a nutritionally complete diet with and without the addition of 0.4 g of ellagic acid per kg of diet. This dose of dietary ellagic acid has previously been shown to reduce the incidence of MBN-induced esophageal carcinoma by 30 to 50%. After 3 wk on the diets, rats were given injections of a single dose of MBN (2.0 mg/kg of body weight i.p.) and sacrificed 1 h after injection. Dietary ellagic acid significantly reduced the MBN-induced in vivo formation of esophageal O6-mGua, without significantly reducing the formation of esophageal m7Gua. Examination of this effect in an in vitro methylation assay demonstrated that dietary ellagic acid did not reduce the ability of esophageal microsomes to methylate purified calf thymus DNA; however, pretreatment of the calf thymus DNA with ellagic acid selectively reduced the MBN-induced formation of O6-mGua by microsomes from both ellagic acid-fed and control animals without altering the in vitro formation of m7Gua. These results suggest that ellagic acid bound to DNA selectively blocks methylation of the O6-position of guanine without inhibiting the activation of MBN or the ability of MBN to methylate DNA.
sufficient DNA for each analysis. Eight animals from each dietary group were sacrificed without exposure to MBN to determine basal levels of O'-mGua and m'Gua in the esophageal DNA of the animals raised on the test diets.

Isolation of Esophageal DNA. Esophageal DNA was isolated by a modification of the method of Margison (21, 35). The mucosa was placed in ten volumes of 6% sodium p-aminosalicylate/2% NaCl solution and homogenized on ice using a Brinkmann Polytron (three cycles of 10 s with 30 s between cycles). An equal volume of phenol:methylcresol:8-hydroxyquinoline:water (100:15.0:1:11) was added to the homogenate, and the sample was vigorously shaken for 30 min. After centrifugation (2400 × g) for 10 min at 4°C, the aqueous layer was extracted twice more with the phenol mixture and twice with an equal volume of chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated with 2.5 volumes of absolute ethanol (−20°C) containing 3% potassium acetate and collected by centrifugation (26,000 × g) at 4°C for 30 min. The pellet was then washed twice with 3% potassium acetate at 70°C ethanol and resuspended in 5 ml of TE buffer with the addition of pancreatic RNase A (previously heated for 10 min at 100°C) to a final concentration of 10 µg/ml. The mixture was then rocked gently overnight at 4°C. Proteinase K (Sigma) was added to a final concentration of 100 µg/ml, and the mixture was rocked at 4°C for 6 h. The DNA was then precipitated in 2.5 volumes of absolute ethanol (−20°C) containing 3% potassium acetate, pelleted, and washed as above.

Quantitation of Methylated Bases. DNA was then resuspended in 0.5 ml of 5 mM Na2HPO4, pH 7.0, and an aliquot was removed to determine DNA content. Quantitation of DNA by UV absorption and by HPLC determination of total guanine per esophagus revealed similar levels of DNA in the esophagi of the ellagic acid and control groups. Esophageal levels of m'Gua and O'-mGua were measured to determine if alterations in the esophageal content of O'-mGua were due to changes specific to the formation and repair of O'-methylolation, or were due to a nonspecific effect on total methylation. The DNA was then heated at 100°C for 20 min to release the m'Gua without hydrolyzing the other more stable purines off the DNA (36). A neutral buffer was used to prevent the loss of m'Gua that occurs as the result of ring hydrolysis at an alkaline pH. The samples were then cooled on ice, and 0.05 ml of 1 M HCl was added to precipitate the DNA. The supernatant, containing the m'Gua, was removed following centrifugation (12,000 × g) at 4°C for 10 min, and 0.05 ml of 0.6 M ammonium phosphate in water (pH 2.5) was then added to each supernatant. If the m'Gua is not extracted separately from the other purines, then the HPLC peak of the m'Gua may be lost on the shoulder of the much larger adenine peak. The DNA pellet was then dissolved in 0.5 ml of 1 M HCl and heated for 30 min at 80°C to release the methylated purines (including O'-mGua, guanine, and adenine) from the DNA. The O'-mGua samples were then centrifuged (5000 × g) for 5 min. Ammonium phosphate (0.05 ml of a 0.6 M solution in water, pH 2.5) was then added to each supernatant, and the mixture was titrated to pH 2.5 with the addition of 10 µl of NaOH using a 1- to 10-µl positive displacement pipettor (Drummond Scientific, Broomall, PA). The supernatants of the O'-mGua and m'Gua samples were then analyzed by high-pressure liquid chromatography, using an Alltech Adsorbosphere SCX column (Alltech Associates, Deerfield, IL), a Beckman Model UV153 HPLC UV detector (254 nm), and a Shimadzu Model RF-530 HPLC fluorescence detector (exciting at 286 nm with a motor-driven Teflon and glass homogenizer. Homogenates of esophageal mucosa from 10 animals were pooled to yield the amount of microsomal protein required for the methylation assays. Individual livers were homogenized in three volumes of homogenizing buffer with a motor-driven Teflon and glass homogenizer. Homogenates were centrifuged (12,000 × g) at 4°C for 30 min, and the supernatant was centrifuged (105,000 × g) at 4°C for 60 min to isolate the microsomal pellet. Microsomes were resuspended in storage buffer (50 mM Tris-HCl:20% glycerol, pH 7.4) and stored in liquid nitrogen. Comparisons of assays on the same samples before and after freezing showed less than a 10% change in activity with freezing. Microsomal protein concentrations were determined by the method of Lowry et al. (40).

Preparation of Calf Thymus DNA for the in vitro Methylation Assay. Purified calf thymus DNA (Sigma) was prepared by first sonicating the DNA in TE buffer (20 mg in 5 ml sonicated for 5 min using a Heat Systems, Inc., Model W220 sonicator at 20% output) to break the DNA into smaller, more soluble pieces. The DNA was then precipitated in 2.5 volumes of absolute ethanol (−20°C) containing 3% potassium acetate, pelleted, and washed as described above for the esophageal DNA. The calf thymus DNA was resuspended in TE buffer, and the concentration of DNA was quantitated by UV absorbance at 260 nm. A portion of this DNA was then incubated with 200 nm ellagic acid in a shaking water bath at 37°C for 30 min (41). The ellagic acid-treated DNA was then precipitated, washed, and resuspended in TE buffer 3 times prior to use in the metabolic assay to remove unbound ellagic acid. Higher concentrations of ellagic acid in solution have been shown to alter the in vitro microsomal metabolism of MBN (42, 43). Samples of the ellagic acid-treated DNA and the untreated DNA were then hydrolyzed, and DNA content was calculated from total guanine as determined by HPLC. This method of DNA quantitation was used because ellagic acid absorbs in the UV range.

Incubation Conditions. The microsomal incubation mixtures contained 3.0 mM methylbenzylisotroisamine, 20 mM semicarbazide, 2 mM NADP+, 10 mM glucose-6-phosphate, 1.2 units of glucose-6-phosphate dehydrogenase, 10 mM MgCl2, 50 mM Tris-HCl (pH 7.4), 0.5 mg of esophageal microsomal protein, 6 mg of hepatic microsomal protein in storage buffer, and 1.6 mg of calf thymus DNA in 0.4 ml of TE buffer. Total assay volume was 1.0 ml. To determine the effects of calf thymus DNA and ellagic acid-treated calf thymus DNA on the microsomal metabolism of MBN to benzaldehyde and on the MBN-induced in vitro methylation of DNA, duplicate assays were performed with 1.6 mg of untreated calf thymus DNA in 0.4 ml of TE buffer, 1.6 mg of calf thymus DNA pretreated with ellagic acid in 0.4 ml of TE buffer, or 0.4 ml of TE buffer without DNA. NADP+ (sodium), glucose-6-phosphate (monophosphat), glucose-6-phosphate dehydrogenase (type XV), and purified calf thymus DNA were purchased from Sigma Chemical Co. (St. Louis, MO).

Methylbenzylisotroisamine was purchased from Ash Stevens, Inc. (Detroit, MI). Semicarbazide was added to the assay system to block nonenzymatic degradation of benzaldehyde to benzoic acid (44). Assay samples were incubated for 45 min at 37°C under air with shaking. Assay mixtures were then centrifuged at 23,000 × g for 20 min, and the reaction mixtures were then centrifuged at 105,000 × g for 60 min to isolate the microsomal pellet. Microsomes were resuspended in storage buffer (50 mM Tris-HCl:20% glycerol, pH 7.4) and stored in liquid nitrogen. Comparisons of assays on the same samples before and after freezing showed less than a 10% change in activity with freezing. Microsomal protein concentrations were determined by the method of Lowry et al. (40).
in the dark at -20°C until analyzed. Freezing was necessary to prevent nonenzymatic formation of benzaldehyde prior to analysis. The DNA was then extracted from the remaining 0.75 ml for quantitation of the MBN-induced in vitro methylation of DNA. This portion of the reaction mixture was incubated with proteinase K (final concentration, 100 μM) for 45 min at 37°C after which an additional 2.0 ml of the TE buffer were added (to increase the sample volume), and then an equal volume of phenol:m-cresol:8-hydroxyquinoline:water (100:15:0.1:11) was added to the sample. The DNA was then extracted, and methylated DNA adducts were quantitated as described above. O⁶-mGua formation in this assay was linear with time for at least 45 min and linear with protein concentration to 1.0 mg of esophageal microsomal protein and to 6.0 mg of hepatic microsomal protein per sample. Benzaldehyde formation in this assay was also linear with time for at least 45 min and linear with protein concentration to 1.0 mg of esophageal microsomal protein and to 6.0 mg of hepatic microsomal protein per sample.

**Benzaldehyde Analysis.** The supernatant solutions for the quantitation of microsomal metabolism were thawed and immediately chromatographed on a Beckman high-pressure liquid chromatography system in an Alttech Model C18 liquid chromatography column eluted isocratically with 17% (v/v) acetonitrile/3% (v/v) acetic acid in water, pH 2.6, at a flow rate of 2.0 ml/min as previously described (32). The eluate was monitored at 254 nm, and metabolite formation was quantitated by peak area analysis. Controls were reaction mixtures incubated at 37°C without microsomal protein or without NADPH. These reactions yielded only the low background levels of benzaldehyde, which were present in the MBN substrate.

**Evaluation of Results: Statistical Considerations.** All data are expressed as mean ± SE. Means were compared using the Student t test, with n equal to the number of independent preparations of esophageal DNA or the number of independent preparations of microsomes and not equal to the number of animals used.

## RESULTS

After 3 wk on the diets, the average weight of the animals was 195.2 ± 2.9 g in the ellagic acid group and 198.8 ± 3.2 g in the control group. Weight of esophageal mucosa was 50.6 ± 3.7 mg per rat in the ellagic acid group and 51.3 ± 8.3 mg in the control group. Quantitation of esophageal mucosal DNA by UV absorption and by HPLC determination of total guanine and control groups (P < 0.05). Quantitation of esophageal m⁷Gua (defined as O⁶-mGua adducts/10⁶ guanine bases per mg of DNA or the number of independent preparations of microsomes and not equal to the number of animals used.

**Fig. 1.** O⁶-Methylguanine adducts and 7-methylguanine adducts in the esophageal mucosal DNA of rats sacrificed 1 h after a single i.p. injection of methylbenzylxanthine (2.0 mg/kg of body weight). Columns, mean; bars, SE. * significant difference in the levels of O⁶-methylguanine between the ellagic acid and control groups (P < 0.05).

**Fig. 2.** In vitro formation of MBN-induced O⁶-methylguanine adducts and 7-methylguanine adducts of untreated calf thymus DNA by esophageal mucosal microsomes from the ellagic acid-fed and control animals. Expressed as methylated adducts per 10⁶ guanine bases per mg of microsomal protein with 1.6 mg of DNA per assay. Columns, mean; bars, SE. There were no significant differences in the levels of O⁶-methylguanine or 7-methylguanine between the ellagic acid and control microsomes.

**Fig. 3.** In vitro formation of MBN-induced O⁶-methylguanine adducts and 7-methylguanine adducts of untreated calf thymus DNA by hepatic microsomes from the ellagic acid-fed and control animals. Expressed as methylated adducts per 10⁶ guanine bases per mg of microsomal protein with 1.6 mg of DNA per assay. Columns, mean; bars, SE. There were no significant differences in the levels of O⁶-methylguanine or 7-methylguanine between the ellagic acid and control microsomes.

**Fig. 4.** In vitro formation of MBN-induced O⁶-methylguanine adducts and 7-methylguanine adducts of ellagic acid-treated calf thymus DNA by hepatic microsomal microsomes from the ellagic acid-fed and control animals. Expressed as methylated adducts per 10⁶ guanine bases per mg of microsomal protein with 1.6 mg of DNA per assay. Columns, mean; bars, SE. * significant differences in the formation of O⁶-methylguanine observed between the in vitro methylation of untreated DNA and ellagic acid-treated DNA by the hepatic microsomes from both the ellagic acid-fed and control groups (P < 0.05).

**Fig. 5.** In vitro formation of MBN-induced O⁶-methylguanine adducts and 7-methylguanine adducts of ellagic acid-treated calf thymus DNA by esophageal mucosal microsomes from the ellagic acid-fed and control animals. Expressed as methylated adducts per 10⁶ guanine bases per mg of microsomal protein with 1.6 mg of DNA per assay. Columns, mean; bars, SE. * significant differences in the formation of O⁶-methylguanine observed between the in vitro methylation of untreated DNA and ellagic acid-treated DNA by the hepatic microsomes from both the ellagic acid-fed and control groups (P < 0.05). O⁶-mGua were due to specific changes in the formation of O⁶-mGua or were due to a nonspecific effect on total methylation. Quantitation of esophageal m⁷Gua (defined as m⁷Gua adducts/10⁶ guanine bases per mg of DNA or the number of independent preparations of microsomes and not equal to the number of animals used.)
Ellagic acid is a naturally occurring plant phenol which has been shown to reduce the incidence of a variety of carcinogen-induced tumors including methylbenzylideneamine-induced esophageal carcinoma in the rat (8) and polycyclic aromatic hydrocarbon-induced skin and lung tumors in the rat and mouse (5–7). It has been demonstrated that ellagic acid reduces the enzymatic activation of the polycyclic aromatic hydrocarbons (45, 46), but examination of the effects of ellagic acid on benzo(a)pyrene-DNA adduct formation demonstrated a greater reduction in DNA-adduct formation than could be explained by ellagic acid inhibition of benzo(a)pyrene activation (46). Following these observations, it was suggested that the binding of ellagic acid to DNA (47, 48) might prevent the attachment of activated carcinogens to DNA.

In conclusion, dietary ellagic acid results in a significant reduction in the MBN-induced formation of esophageal O⁶-mGua, without significantly affecting the formation of MBN-induced esophageal m⁷Gua. These results provide a possible mechanism for the decreased incidence of MBN-induced esophageal carcinoma observed with dietary ellagic acid.
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