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# PROTECTION AGAINST 3-METHYLCHOLANTHRENE-INDUCED SKIN TUMORIGENESIS IN BALB/C MICE BY ELLAGIC ACID

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Summary: Topical application of ellagic acid, a naturally occurring dietary plant phenol, to Balb/C mice resulted in significant protection against 3-methylcholanthrene (MCA)-induced skin tumorigenesis. Ellagic acid was found to be an effective inhibitor of tumor formation whether the tumor data are considered as percent mice with tumors, cumulative number of tumors, tumors per mouse or tumors per tumor bearing animal as a function of the number of weeks on test. By 8, 10, 12, 14, and 16 weeks of testing, the number of tumors per mouse in the group receiving MCA alone was 2.0, 3.4, 4.0, 4.9 and 5.3, respectively, whereas the corresponding numbers in the group receiving MCA plus 2$\mu$ mol ellagic acid were 0, 0.3, 0.4, 0.6 and 1.2, respectively. At the termination of the experiment (16 weeks) aryl hydrocarbon hydroxylase (AHH) activity in skin and liver and the extent of $^3$H-BP-binding to skin, liver and lung DNA were determined and both of these parameters were found to be significantly inhibited in the animals treated with ellagic acid. These results indicate that ellagic acid can inhibit the metabolism of polyaromatic hydrocarbons and modulate skin carcinogenesis induced by these chemicals.

Epidemiologic studies have clearly established that environmental factors such as diet, as well as chemical and physical exposures are important factors in the development of most human cancers (1). The diet contains a wide variety of naturally occurring mutagens and carcinogens and, in addition contain many natural antimutagens and anticarcinogens (2,3). The identification and characterization of such dietary inhibitors of carcinogenesis could lead to important new strategies for reducing the risk of human cancer.

PAHs are ubiquitous environmental pollutants which have long been implicated as carcinogens for human skin, lung, mammary and other tissues (4-7). The repeated topical application of PAHs such as MCA, 7-12-dimethylbenzanthracene, and BP to the skin of mice produces squamous cell carcinoma (8-10). In the

Abbreviations used: MCA, 3-methylcholanthrene; BP, benzo[a]pyrene; 3-OH-BP, 3-hydroxybenzo[a]pyrene; PAH, polycyclic aromatic hydrocarbon; AHH, aryl hydrocarbon hydroxylase.

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process of tumor induction by PAHs, it is now generally accepted that an essential step is the metabolic activation of the parent compound to chemically reactive bay region diol-epoxides, that can bind covalently to cellular macromolecules (6,7). The cytochrome P-450-dependent monooxygenase commonly known as AHH and epoxide hydrolase, are enzymes involved in the conversion of inert precarcinogenic PAHs into their ultimate carcinogenic species. Both are known to be present in mammalian skin (11-13).

Since the metabolism of PAHs is a prerequisite for their carcinogenicity, it is currently believed that one promising way to reduce the risk of developing chemically induced cancers might be to modulate the activity of enzymes that participate in the metabolic activation and inactivation pathways. This concept has led to the evaluation of a number of natural and synthetic compounds for their inhibitory effects on mutagenesis and carcinogenesis (14-16). Recently several naturally occurring plant phenols including ellagic acid were shown to inhibit the mutagenicity of bay region diol-epoxides of several PAHs in the Ames mutagen assay using S. typhimurium (17). It was therefore of great importance to evaluate the anticarcinogenic activity of these compounds. We report that ellagic acid is a potent inhibitor of PAH-induced skin carcinogenesis in Balb/C mice.

## MATERIALS AND METHODS

Chemicals: (G-$^3$-H)-BP (specific activity 25 Ci/mmol) was purchased from Amersham Searle (Chicago, IL). Protease (Type XI), m-cresol, 8-hydroxyquinoline, MCA, BP, NADPH, calf thymus DNA (Type I) and ribonuclease A (Type III-A) were purchased from Sigma Chemical Co. (St. Louis, MO). Ellagic acid dihydrate and 99% pure phenol were purchased from Aldrich Chemical Co., (Milwaukee, WI). All other chemicals used were of highest purity commercially available.

Animals: Eight weeks old female Balb/C mice were obtained from Charles River Laboratories (Kingston, MA).

Effect of ellagic acid on MCA-induced skin tumorigenesis in Balb/C mice: The mice were shaved with electric clippers and Nair depilatory was applied 1 day before the beginning of the experiment. Only those mice that were not in the hair regrowth cycle were selected for further studies. One hundred 8-week-old female Balb/C mice were divided into five groups of 20 mice each and received the following topical treatments: Group 1) 1.5 $\mu$moles MCA in 0.2 ml acetone as described earlier (18); Group 2) 2.0 $\mu$ mole ellagic acid in 0.2 ml of DMSO; Group 3) 1.5 $\mu$mole MCA, 1 hour following treatment with 1.0 $\mu$mole ellagic acid; Group 4) 1.5 $\mu$mole MCA, 1 hour following treatment with 2.0 $\mu$mole ellagic acid; Group 5) 0.2 ml acetone and DMSO (controls). The treatments were repeated twice weekly for 16 weeks, at which time the animals were killed and

the experiment terminated. Skin tumor formation was recorded weekly and tumors greater than 1 mm in diameter were included in the cumulative total only if they persisted for 2 weeks or more. No skin neoplasm occurred in any mice treated with acetone, DMSO, or ellagic acid alone (Groups 2 and 5). The latent periods were computed by the method of Shimkin and Andervont (19) as described earlier (18).

AHH determination in liver and skin of tumor mice with and without ellagic acid treatment: At the termination of the tumor experiment six mice from each group were killed and skin and liver homogenates were prepared according to established procedures in this laboratory (12). AHH activity in liver and skin homogenates was estimated according to our modifications of the fluorometric assay of Nebert and Gelboin (20) as described earlier (11). The quantitation of phenolic BP-metabolites was based on comparison with fluorescence of a 3-OH-BP standard.

$^{3}$H-BP-binding to DNA in tumor mice: At the termination of the tumor experiment 8 mice from each group received a single topical application of $^{3}$H-BP (1 nmol in 100 l acetone) 2 hours prior to sacrifice. Skin, liver and lung tissues from the animals were removed and DNA from each minced tissue was extracted essentially as described by Kates and Beeson (21), with an additional incubation step using protease K (0.5 mg/ml). A second extraction was performed using Kirby's phenol (22) before precipitation with cold 100% ethanol. The DNA was then digested with ribonuclease A (100 units/ml), washed 3 times with acetone and dissolved in 5-10 ml of 0.1 M sodium chloride, pH 7.0, and estimated by measuring its absorption at 260 nm. The purity of the DNA was assessed by the absorbance ratios A260/280 $\geqslant$ 1.98 and A260/230 $\geqslant$ 2.21 (23). Aliquots of the extracted DNA were counted on a Packard TriCarb 460 CD liquid scintillation spectrometer to determine the amount of $^{3}$H-BP bound to tissue DNA.

## RESULTS AND DISCUSSION

The tumor induction studies were conducted in groups of Balb/C mice receiving MCA simultaneously with or without the topical application of ellagic acid and the results are shown in Figure 1. Tumor data are presented in four different ways as the percent of mice with tumors (panel A), the cumulative number of tumors induced (panel B), the number of tumors per mouse (panel C) and the number of tumors per tumor-bearing mouse (panel D) as a function of number of weeks on test. In each case ellagic acid was found to be highly effective as an inhibitor of tumor formation by MCA. For example, it can be seen in Fig. 1A that by the 11th week of testing, the tumor incidence in positive controls, i.e. mice treated with MCA alone, was 100%, whereas the corresponding values for the MCA-treated groups receiving 1 $\mu$mol and 2 $\mu$mol of ellagic acid were 20% and 15%, respectively. The data in panel B clearly show that at the termination of the experiment after 16 weeks the total number of tumors in 20 mice receiving topically applied carcinogen alone was 108, whereas only 40 and 26 tumors were recorded in the groups receiving both carcinogen and

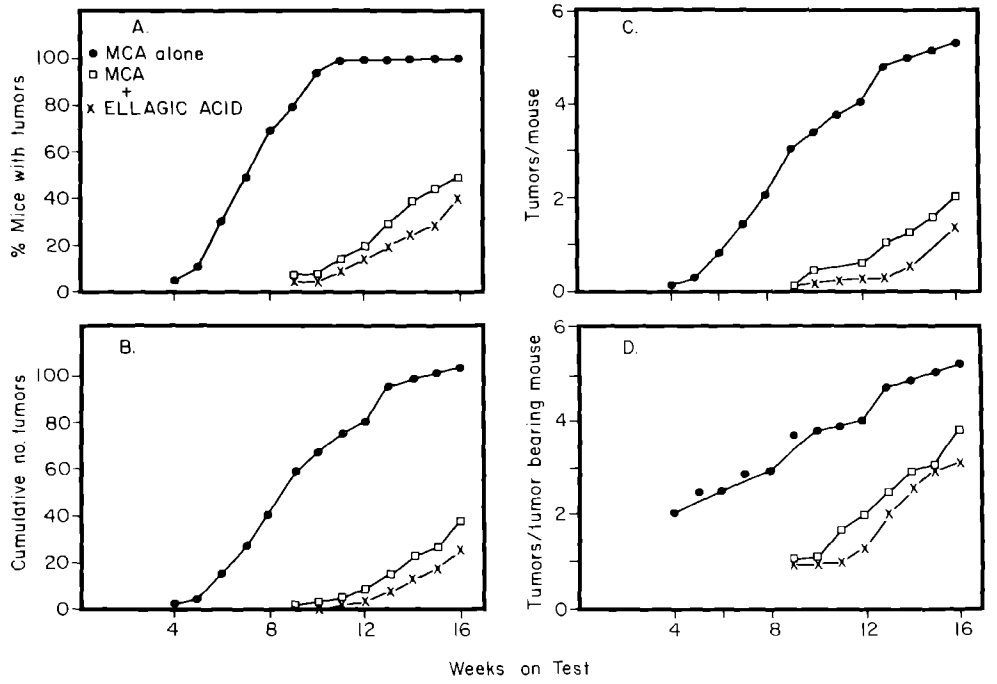


Fig. 1 The effect of ellagic acid on MCA-induced skin tumorigenesis in Balb/C mice.

The percent mice with tumors (panel A), the cumulative number of tumors (panel B), tumors/mouse (panel C), and tumors/tumor bearing mice (panel D) were plotted as a function of the number of weeks on test. The experiment clearly indicates that no matter how the data are expressed the mice treated with ellagic acid and MCA develop fewer tumors as compared to mice treated with MCA alone. ●——● mice treated with MCA alone (Group 1), □——□ , mice treated with ellagic acid ( 1 μmol) + MCA (Group 3), ×——× , mice treated with ellagic acid (2 μmol) + MCA (Group 4). Treatment and other details are provided under "Methods".

ellagic acid. These differences were more apparent at earlier times during the test period. When the skin tumor data are present as tumors per mouse versus time of test (panel C), the observed protection against tumor formation by ellagic acid becomes even more apparent. Thus by 8, 10, 12, 14, and 16 weeks of testing, the number of tumors per mouse in the group receiving MCA alone was 2.0, 3.4, 4.0, 4.9 and 5.3 tumors, respectively. The number of tumors per mouse in the corresponding group receiving 2 μmol ellagic acid was 0, 0.3, 0.4, 0.6 and 1.2 tumors, respectively. When skin tumor values are considered as tumors per tumor bearing mouse (mice which have no tumors were excluded for this presentation), it becomes clear that animals treated simultaneously with carcinogen and ellagic acid had fewer tumors as compared to the group receiving

the carcinogen alone. Overall these data suggest that ellagic acid is a strong inhibitor of MCA-induced skin carcinogenicity in mice.

Further studies were conducted in an effort to define the mechanism of the inhibitory effect of ellagic acid on MCA-induced skin tumor formation. At the termination of the tumor experiment, the metabolism of the PAH and its enzyme-mediated binding to DNA were assessed. The data shown in Table 1 clearly indicate that treatment of mice with topically applied ellagic acid resulted in profound inhibition of both skin and liver AHH activities and in the binding of BP to skin, liver and lung DNA. Thus it appears likely that ellagic acid inhibits tumor induction by MCA by inhibiting the metabolism of the hydrocarbon. In prior studies we had shown that ellagic acid is a potent inhibitor of BP metabolism and its binding to rat epidermal DNA (24). Thus treatment of the skin of mice with ellagic acid would be expected to result in reduced generation of reactive carcinogenic metabolites, resulting in diminished DNA-binding and in reduced tumor formation.

Ellagic acid is widely distributed in the human diet being present in fruits and vegetables, among them grapes, certain nuts and strawberry preserves

Table 1
Effect of topically applied ellagic acid on BP metabolism and its DNA-binding in MCA-induced tumor-bearing Balb/C mice

| | AHH Activity (pmol 3-OHBP/min/mg protein) | | $^{3}$H-BP-Bound to DNA (pmol/mg DNA) | | |
|---|---|---|---|---|---|
| Treatment of animals | Skin | Liver | Skin | Liver | Lung |
| Control (Group 5) | 0.69 ± 0.06 | 35.2 ± 2.1 | 0.65 ± 0.05 | 0.18 ± 0.02 | 0.12 ± 0.02 |
| Ellagic acid (Group 2) | 0.29 ± 0.03 | 23.4 ± 1.4 | 0.23 ± 0.04 | 0.12 ± 0.01 | 0.06 ± 0.01 |
| MCA (Group 1) | 1.31 ± 0.08 | 50.2 ± 3.1 | 0.95 ± 0.09 | 0.22 ± 0.03 | 0.18 ± 0.02 |
| MCA + Ellagic acid (Groups 3 and 4) | 0.63 ± 0.04 | 34.1 ± 2.1 | 0.40 ± 0.03 | 0.09 ± 0.04 | 0.07 ± 0.01 |

Data represent mean ± S.E.M. of three to four individual values. For each determination 2 mice were pooled as described in Methods. Since the data of mice for two ellagic acid treated groups (1.0 µmol and 2.0 µmol, groups 3 and 4) did not differ in terms of AHH activity and DNA-binding, they have been grouped together.

(25). Ellagic acid appears to be well tolerated by both experimental animals and humans. Rats fed ellagic acid at doses as high as 50 mg/kg per day up to 45 days did not exhibit any signs of systemic toxicity (26). Intravenously administered doses of 0.2 mg/kg ellagic acid have been shown to be well tolerated by humans (27). The data in this study demonstrate the considerable anticarcinogenic effect of ellagic acid when applied to the skin. Because this polyphenol is widely ingested in the human diet it is possible that when administered in this manner it could have anticarcinogenic effects in many different tissues including the skin. Studies are currently underway to evaluate this possibility.

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