The Binding of 7, 12-Dimethylbenz(a)anthracene to Mammary Parenchyma DNA and Protein in Vivo 1

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SUMMARY

Sprague-Dawley female rats were sacrificed at various times after the intragastric administration of 7, 12-dimethylbenz(a)anthracene (DMBA)-3H. The abdominal-inguinal mammary glands were removed, and the parenchymal cells were separated. Parenchymal cell DNA and protein were isolated, and the DNA was purified by density equilibrium centrifugation in cesium chloride. Sixteen hr after carcinogen feeding, DMBA binding amounted to 47 pmoles/mg of DNA. Fifty % of the DMBA bound to DNA at 16 hr was present 14 days after administration, and 31% was still detectable at 42 days. The amount of DMBA binding to protein was less than one-half that observed bound to DNA. Furthermore, by 14 days, DMBA binding to protein had declined to just detectable levels. Increased parenchymal cell DNA content, due to cellular proliferation resulting from DMBA-induced tumorigenesis, was not responsible for the decreased DNA specific activity observed at 14 and 42 days. Thus, persistent binding of DMBA to rat mammary parenchymal cell DNA in vivo was demonstrated.

INTRODUCTION

During the last several years, there have been numerous reports indicating that a wide variety of chemical carcinogens are covalently bound to DNA of many different tissues (6, 12, 14, 22, 34, 35). DMBA 2 is a potent skin carcinogen (1, 4) and under specific conditions is capable of inducing 100% incidence of mammary carcinoma (19, 20) in female Sprague-Dawley rats. The binding of DMBA-3H to DNA, RNA, and protein of mouse skin has been reported (6, 14). More recent studies have been concerned with the binding of DMBA-3H to DNA in fetal mouse skin in culture (36), to DNA of other cells in culture (5, 10), and to DNA of presumably nonsusceptible tissues (25, 26, 32). However, it has been suggested that the binding of DMBA to DNA, at least in mouse skin, may be a necessary but not sufficient condition for carcinogenesis (5, 14). Since no information is available on the binding of DMBA-3H (or other carcinogens) to DNA and protein in the parenchymal cells of the rat mammary gland, a tissue which is highly susceptible to many carcinogens, such experiments were considered to be highly desirable for correlation of carcinogenicity with binding of specific chemical carcinogens to macromolecules in susceptible tissues.

MATERIALS AND METHODS

DMBA Binding. High-specific-activity DMBA-3H (Radiochemical Centre, Amersham, England; specific radioactivity, 17 Ci/mnmole) was freed of decomposition products, by being washed through a Florisil column (0.5 x 10 cm) with 25 ml of redistilled benzene, and was diluted with carrier DMBA similarly purified. The final specific radioactivity of the DMBA-3H was 32.0 mCi/mnmole. The radiochemical purity of the DMBA-3H was established by thin-layer chromatography on Silica Gel G, with benzene as a solvent. One-cm strips of the chromatograms were scraped into vials and counted in liquid scintillation solution composed of 10 ml of toluene, 40 mg of PPO, and 1 mg of POPOP. All of the radioactivity was recovered in a single spot with an Rf value identical with that of DMBA.

Sprague-Dawley female rats were fed Purina laboratory chow and water ad libitum. At 50 days of age, each animal received 20 mg of DMBA-3H (2.5 mCi) in 1 ml of sesame oil by gastric intubation. At 16 hr and at 3, 7, 14, and 42 days following DMBA-3H administration, the abdominal-inguinal mammary glands of 4 rats were removed, and mammary parenchymal cells were obtained by means of the method of Moon et al. (30). The mammary parenchymal cells obtained from 2 rats were pooled and homogenized in 8 ml of 1% sodium dodecyl sulfate in 0.015 M NaCl : 0.0015 M sodium citrate, pH 7.0. The homogenate was extracted twice with phenol (previously saturated with water), and nucleic acids were precipitated from the aqueous phase by the addition of 3 volumes of ice-cold ethanol. The phenol layer was saved for the isolation of protein, as described below. DNA fibers formed by the addition of ethanol to the aqueous phase were wound on a glass rod. This crude DNA was dissolved in 6.74 ml of 0.015 M NaCl : 0.0015 M sodium citrate, pH 7.0, and 8.0 g of CsCl were added. The resulting solution was centrifuged for 65 hr at 45,000 rpm at 25° in the Ti-50 angle head rotor of a Beckman Model L2-65B ultracentrifuge. Fractions of 0.5 ml each were collected from the bottom of the centrifuge tube. Each sample was diluted with 1.0 ml of 0.015 M NaCl : 0.0015 M sodium citrate, pH 7.0, and the absorbance at 260 nm was measured. Fractions containing the
peak absorbance were combined, and the DNA was precipitated with ice-cold ethanol. The DNA was reband in CsCl by density equilibrium centrifugation. Fractions were collected and diluted as before, and the absorbance at 260 nm was measured. The radioactivity of each fraction was determined by liquid scintillation counting in 15 ml of the Triton X-100 system (21), with a Packard Model 3375 liquid scintillation spectrometer. Counting efficiency was determined by means of the automatic external standardization ratio.

For determination of the quantity of DNA contained within the CsCl gradient fractions, a known amount of calf thymus DNA was added to those CsCl fractions with no absorbance at 260 nm. The absorbance of these fractions was measured and related to the amount of DNA added. The determined relationship was 68 µg of DNA/ml = 1 A260 unit.

Protein was precipitated by the addition of 3 volumes of ice-cold ethanol to the phenol layer. Precipitated protein was collected by filtration and washed extensively with ethanol and ether. The precipitate was dried to a constant weight under vacuum, and a 5-mg sample was digested in 1 ml of NCS solubilizer (Amersham/Searle, Chicago, Ill.). Radioactivity was measured by liquid scintillation counting in an appropriate scintillation solution (23).

**DNA Content.** Groups of 50-day-old female rats received either 20 mg of DMBA in 1 ml of sesame oil or 1 ml of the oil by gastric intubation. Animals were sacrificed at 1 hr and at 14 and 42 days following gastric intubation. The abdominal-inguinal mammary glands from the left side of each animal were treated to yield mammary parenchymal cells (30). Right abdominal-inguinal glands were removed and minced with scissors. The minced whole mammary gland or isolated parenchymal cells were homogenized in 10 volumes of cold 0.9% NaCl solution. Nucleic acids and proteins were precipitated by the addition of enough cold PCA to make the final solution 0.2 N with respect to PCA. The resulting precipitate was washed twice with 5 ml of ice-cold 0.2 N PCA and twice with 10 ml of chloroform : methanol (2 : 1; v/v). The precipitate was dissolved in 0.3 N KOH (8 ml for whole gland; 4 ml for cells) by heating at 37° for 1 hr. DNA and proteins were precipitated by the addition of enough ice-cold 3 N PCA to give a final concentration of 0.2 N PCA, and the precipitate was washed with 5 ml of cold 0.2 N PCA. DNA was extracted from the pellet by heating in 0.5 N PCA (10 ml for whole gland; 5 ml for cells) at 70° for 15 min. The pellet was rinsed with 5 ml of 0.5 N PCA, which was added to the supernatant. DNA was determined by the colorimetric method of Burton (7).

**RESULTS**

DNA isolated from rat mammary parenchymal cells after the intragastric administration of DMBA-3H was found to contain bound radioactivity. The relationship of measured absorbance at 260 nm to the determined radioactivity of a typical CsCl density gradient is shown in Chart 1. All of the radioactivity in the isolated DNA was associated with the DNA. This relationship was established for each CsCl density gradient centrifugation.

The pattern of binding of DMBA-3H to mammary parenchymal cell DNA and protein is illustrated in Chart 2. Sixteen hr following carcinogen administration, DMBA binding to DNA amounted to 47 pmoles/mg DNA. Between 3 and 14 days, the level of bound DMBA remained relatively constant, and 50% of the DMBA-3H to DNA at 16 hr was still present 14 days after carcinogen feeding. By 42 days, carcinogen binding had declined to 14 pmoles/mg of DNA. The amount of DMBA-3H linked to protein was less than one-half that observed for DNA. Only 22 pmoles of DMBA were bound per mg of protein at 16 hr postfeeding. The DMBA-protein complex remained at a constant level from Day 3 to 7 and thereafter declined to 2 pmoles/mg protein at Day 14 and 42 after administration.

**Chart 1.** Typical CsCl density gradient demonstrating the association of radioactivity with rat mammary parenchymal cell DNA isolated 16 hr after the administration of 20 mg of DMBA-3H (2.5 mCi) to each rat. DNA from the parenchymal cells obtained from 2 rats was dissolved in 6.74 ml of 0.015 M NaCl : 0.0015 M sodium citrate, pH 7.0, and 8.0 g of CsCl were added. The solution was centrifuged for 65 hr at 45,000 rpm at 25° in the Ti-50 angle rotor of a Beckman Model L2-65B ultracentrifuge.

**Chart 2.** Pattern of radioactivity bound to the DNA (o) and protein (•) isolated from rat mammary parenchymal cells following the intragastric feeding of 20 mg of DMBA-3H (2.5 mCi). Parenchymal cells separated from the mammary glands of 2 rats were pooled for the isolation and purification of DNA and protein. Each point represents the mean and range of at least 2 pooled samples.
The total DNA content of the mammary gland and parenchymal cells from rats sacrificed at 0, 14, and 42 days after administration of DMBA is shown in Chart 3. Mammary gland and parenchymal cell DNA content of control rats increased gradually over the 42-day period. Although the mammary gland and parenchymal cell DNA content of animals fed DMBA was greater than that of control rats, the increase in DNA content was not significant.

**DISCUSSION**

The present investigation demonstrates that a portion of the DMBA concentrated within the mammary parenchymal cell is bound to the DNA and protein of the cell. Significant levels of DMBA were bound to both DNA and protein within the first 16 hr, and the DMBA-DNA complex within the parenchymal cells was maintained at a relatively constant level from 3 to 14 days after carcinogen administration. A previous report indicated that DMBA was concentrated in the intracellular lipid of the parenchymal cell, was slowly released, and was apparently bound to some cellular component (24). Since carcinogenic hydrocarbons have been detected in the mammary fat of rats as late as 8 to 10 days following their p.o. administration (2, 3, 9), it is possible that the maintenance of the DMBA-DNA complex might result from a slow release of DMBA from the intracellular lipid, coupled with its subsequent metabolic activation.

Covalent binding of hydrocarbons to cellular constituents is thought to involve metabolic activation of the parent compound (29). Several investigators have provided evidence that a microsomal enzyme system may be responsible for the in vitro formation of covalently bound macromolecule-polycyclic hydrocarbon complexes (13, 15, 28). The binding of DMBA to the DNA, RNA, and protein of cells in tissue culture has been found to be higher in normal embryonic rodent cells than in transformed rodent cells (10). Furthermore, Diamond et al. (11) have shown that DMBA is metabolized to a greater extent in normal cells than in transformed cells. Recently, the K-region epoxide (DBA-5,6-oxide) of DBA has been found to be more active in the production of malignant transformations in hamster embryo cells than the parent hydrocarbon (16). In addition, Selkirk et al. (33) have shown that an epoxide is formed as an intermediate in the metabolism of DBA by liver microsomes. These studies have clearly implicated the need for metabolic activation of polycyclic hydrocarbons before they are bound to cellular constituents or are capable of producing transformations in rodent cells in culture.

DMBA-induced mammary carcinoma can be detected histologically 10 to 14 days after carcinogen feeding (reviewed in Refs. 8 and 18). The reported mean time of appearance of the first palpable tumor is approximately 42 (35 to 51) days postfeeding (reviewed in Refs. 8 and 18). Tumor formation between Days 14 and 42 could result in an increase in parenchymal cell DNA content large enough to cause the reduction in DNA specific activity observed during this time. However, a significant increase in the total DNA content of the mammary gland or the parenchymal cells at either 14 or 42 days after DMBA feeding was not observed. Thus, increased mammary gland DNA content, due to cellular proliferation resulting from DMBA-induced tumorigenesis, was not responsible for the decreased DNA-specific activity observed at Day 42. It is possible that DNA repair mechanisms may be responsible for the decline in specific activity.

The present investigation indicated that DMBA was bound to the DNA and protein of mammary parenchymal cells, which are highly susceptible to the tumorigenic properties of a number of compounds. Our experiments showed that DMBA was bound to the DNA to a greater extent than to the protein of the parenchymal cell. Furthermore, the DMBA-DNA complex persisted throughout the study, while DMBA binding to protein declined to barely detectable levels by 14 days following administration. Recently, the binding of DMBA to the DNA, RNA, and protein of tissues generally considered to be resistant to the carcinogenic activity of DMBA has been reported (25, 26, 32). DMBA was bound to the protein of liver, spleen, kidney, and lung to a greater extent than to the DNA of these tissues. The highly proliferating liver in newborn or in partially hepatectomized animals is susceptible to the carcinogenic activity of DMBA while the slowly growing, intact liver of the adult is resistant (27, 31). Marquardt et al. (26) reported that DMBA was bound to a greater extent to the DNA isolated from livers of immature rats or from those undergoing regeneration than to the DNA from intact livers of adult animals. In addition, the DMBA-DNA complex in regenerating liver was found to persist for at least 4 weeks after administration, while persistent binding to intact liver DNA was not observed (25).

Brookes and Lawley (6) have reported that various polycyclic hydrocarbons bind to mouse skin DNA and that the degree of binding to DNA is positively correlated with carcinogenic potency. The more potent polycyclic hydrocarbons, such as DMBA, were bound to DNA in greater amounts than were noncarcinogenic compounds, and the carcinogen-DNA complex persisted for longer periods. Furthermore, carcinogen binding to RNA and protein was not correlated with carcinogenic potency. The greater degree of DMBA binding to DNA of a susceptible tissue (mammary parenchyma) and the persistence of this complex demonstrated by the present experiments are consistent with the reports of Brookes and Lawley (6).
Goshman and Heidelberger (14) have reported that the extent of binding of DMBA to the epidermal DNA of Swiss mice and of a strain of hairless mice not susceptible to the tumorigenic properties of this hydrocarbon was almost identical. In addition, the time course and extent of binding of the noncarcinogen, dibenz-[a,c]anthracene, was found to be the same as that of the isomeric carcinogen, DBA. Furthermore, Heidelberger (17) has demonstrated a positive correlation between the binding of certain hydrocarbons to a specific protein fraction of mouse skin and carcinogenic potency. Thus, it has been suggested that the binding of DMBA to mouse skin DNA and/or protein may be a necessary but not sufficient condition for carcinogenesis (5, 14).

The present investigation concerning the binding of DMBA to mammary parenchyma DNA and protein does not answer the question as to the primary receptor of chemical carcinogens. However, our findings indicate that further investigation of the metabolism and mechanism of action of DMBA in the mammary parenchymal cell system may aid in the elucidation of the cellular events preceding the appearance of tumors.

REFERENCES

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