Binding Patterns of 3-Methylcholanthrene to Replicating and Nonreplicating DNA of Preneoplastic Nodule of Mouse Mammary Gland

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SUMMARY

The binding of 3-methylcholanthrene (MC) to DNA and its relation to DNA synthesis in the mammary tumor virus-free hyperplastic alveolar nodule outgrowth of BALB/c mouse mammary gland were determined. As measured by tritium radioactivity, the carcinogen was found to bind to nodule DNA at 12 and 24 hr after a single intragastric feeding of 500 µg MC-3H to mice carrying the outgrowth. The binding of MC to DNA in vivo was slightly lower when DNA synthesis in the nodule cells was more active at 4 weeks compared to the period of reduced DNA synthesis at 8 weeks after fat-pad transplantation. In a separate experiment both 4- and 8-week-old outgrowths were incubated in vitro and DNA was double labeled with MC-3H and thymidine-2-14C. During the 6-hr incubation in a shaking water bath at 37°C, both MC-3H and thymidine-2-14C radioactivity was present in nodule DNA, but no direct relation to MC-3H and thymidine-2-14C radioactivity was present between the 4- and 8-week-old nodule outgrowth cells in vitro. When the nodule DNA, double labeled with 5-bromodeoxyuridine and MC-3H, was centrifuged to equilibrium in CsCl, the radioactive carcinogen was almost equally distributed between the preexisting and the newly synthesized, 5-bromodeoxyuridine-labeled heavier fractions of the nodule DNA. The results indicate that binding of MC to DNA of the preneoplastic nodule outgrowth of mouse mammary gland is not influenced by or dependent upon the rate of DNA synthesis in the tissue.

INTRODUCTION

Several hormone-induced, MTV2-free, hyperplastic alveolar nodules of BALB/c mouse mammary gland designated D1 to D8 (24, 28) are now maintained in our laboratory by serial fat-pad transplantation. In a separate experiment both 4- and 8-week-old outgrowths were incubated in vitro and DNA was double labeled with MC-3H and thymidine-2-14C. During the 6-hr incubation in a shaking water bath at 37°C, both MC-3H and thymidine-2-14C radioactivity was present in nodule DNA, but no direct relation to MC-3H and thymidine-2-14C radioactivity was present between the 4- and 8-week-old nodule outgrowth cells in vitro. When the nodule DNA, double labeled with 5-bromodeoxyuridine and MC-3H, was centrifuged to equilibrium in CsCl, the radioactive carcinogen was almost equally distributed between the preexisting and the newly synthesized, 5-bromodeoxyuridine-labeled heavier fractions of the nodule DNA. The results indicate that binding of MC to DNA of the preneoplastic nodule outgrowth of mouse mammary gland is not influenced by or dependent upon the rate of DNA synthesis in the tissue.

MATERIALS AND METHODS

The nodule outgrowth line D1 carried in BALB/c mice, obtained from the Cancer Research Laboratory, University of California, Berkeley, Calif., was maintained in our laboratory by serial fat-pad transplantation in young BALB/c females, with the standard procedure (11).

MC-3H Treatment of Nodule Outgrowth in Vivo. MC-3H (500 mCi/m mole) in benzene solution was supplied by Amersham/Searle Radiochemical Center, Arlington Heights, Ill. After the benzene was evaporated, the radioactive carcinogen was mixed with unlabeled MC (Calbiochem, Los Angeles, Calif.) and dissolved in olive oil. Each mouse carrying the outgrowth was given, i.g. feeding (25), 0.25 ml of the carcinogen solution containing 500 µg (250 µCi) MC-3H. The 500-µg dosage was given because the same dosage was highly tumorigenic in the D1 nodule outgrowth (25). Mice were killed by cervical dislocation; the inguinal fat pad containing the outgrowth freed from lymph nodes was removed and frozen.

Isolation of DNA

DNA from the nodule outgrowth tissue (318 to 775 mg) was extracted according to a modified procedure by Kirby (21). Nodule outgrowths were minced and homogenized in 5 ml 5% p-aminosalicylate: 1% sodium dodecyl sulfate, and the aqueous layer was extracted 3 times with 1 volume of phenol:8-hydroxyquinoline:m-cresol:water (500:0.5:70:55 by

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weight). DNA was precipitated with ethoxyethanol, collected on a glass rod, and dissolved in 0.001 M K2HPO4 buffer (pH 7). After incubation with RNase (20 μg/ml, RNase preheated at boiling temperature for 10 min) for 15 min at 37°, the DNA solution was reextracted with phenol and dissolved in the buffer. DNA was then hydrolyzed in 5% perchloric acid at 90° for 20 min, determined by UV absorbance in a Beckman Model DU spectrophotometer and also checked by the standard diphenylamine reaction. Tritium radioactivity in the hydrolysate counted in a Nuclear Chicago, Unilux II liquid scintillation spectrometer was used as a measure of carcinogen binding to DNA.

MC-3H and TdR-14C Double Labeling in Vitro. Pieces of 4- and 8-week-old nodule outgrowth tissue were suspended in a flask with 5 ml sterile Waymouth's medium containing MC-3H (30 μCi/ml [1.8 mCi/mg; Amersham/Searle] and TdR-14C (1 μCi/ml, 52.2 mCi/mole; New England Nuclear, Boston, Mass.) incubated at 37° in a shaking water bath. The tissue samples were weighed and frozen for subsequent chemical analysis; periodically, pieces of outgrowths were also fixed in acetic acid:ethanol (1:3, v/v) for histology and autoradiography.

Extraction of DNA from Double-labeled Tissue. The pieces of nodule outgrowth were pooled (71 to 134 mg) and homogenized in 2 ml Tris-HCl buffer (0.5 M Tris:27% sucrose (w/v):0.1 M EDTA, pH 8.4); the homogenate was incubated with Pronase (100 μg predigested) for 2 hr at 37° and extracted with phenol 3 times as described (14). The aqueous layer was then digested with RNase (100 μg, preheated) for 90 min, followed by Pronase (100 μg predigested) for another 90 min. DNA was precipitated by cold ethanol and collected by centrifugation (10,000 X g, 30 min). The pellet was dissolved in 1 X SSC, determined by UV absorbance and also checked by the diphenylamine method. MC-3H and TdR-14C radioactivity in CCl3COOH-insoluble material was counted by liquid scintillation spectrometry.

MC-3H and BrdU Labeling and Isopycnic Banding of DNA in CsCl. Pieces (2 to 4 mm) of D1 nodule outgrowth were incubated in a flask with 5 ml sterile Hanks' medium (without thymine) containing 6 X 10-6 M BrdU and penicillin at 37° for 8 hr in a shaking water bath. In 1 experiment, outgrowth tissue was incubated in the presence of BrdU and deoxyadenosine-3H (10 μCi/ml, 19.2 Ci/mole; New England Nuclear), and in a second experiment the incubation medium contained BrdU and MC-3H (25 μCi/ml, 500 mCi/mole; Amersham/Searle).

The outgrowth tissue was then homogenized in Tris-HCl buffer (0.1 M Tris:0.1 M NaCl:0.005 M EDTA, pH 8), and the homogenate was extracted 3 to 6 times with NaClO4 containing chloroform:isoamyl alcohol (10:1, v/v) as previously described (4, 27). Nucleic acid was precipitated by cold ethanol (−20°), removed on a glass rod, and dissolved in 0.1 X SSC. The mixture was treated with RNase (20 μg/ml) for 2 hr and also with Pronase (50 μg/ml) for another 2 hr. The solution was reextracted 2 to 5 times; DNA was precipitated by cold ethanol, collected on a glass rod, dissolved in 0.01 X SSC, and stored at 4°.

Isopycnic centrifugation in CsCl of the D1 outgrowth DNA was done as described (4, 13). CsCl solution was prepared in a volume of 4.5 ml by adding 4.3750 g optical grade CsCl (Harshaw Chemical Co., Solon, Ohio) to 3.4 ml 0.01 M Tris buffer (pH 8.2). The refractive index was read in an Abbe-3L refractometer (Bausch and Lomb) at 25° and the initial density range of the solution was 1.710 to 1.715 g/cu cm. The samples were centrifuged (Spinco L-2) in a 40-angle rotor at 33,000 rpm for 60 to 65 hr at 25°. Drop fractions were collected from the bottom and the UV absorbance of each fraction was measured in a Beckman DB-GT recording spectrophotometer. Radioactivity of DNA was determined after CCl3COOH precipitation by the filter paper methods (7) and the samples were counted in a liquid scintillation spectrometer.

RESULTS

Previously, we reported that, after transplantation into the cleared fat pad, DNA synthesis, including the thymidinemethyl-3H labeling index in the outgrowth, reached a maximal level at 4 weeks and then declined to a plateau at 8 weeks (1). Accordingly, these 2 time points after fat-pad transplantation were considered as periods of high and low DNA synthesis in the D1 outgrowth.

MC-3H Binding to D1 Nodule Outgrowth DNA in Vivo. Chart 1 shows that a substantial amount of MC-3H radioactivity was present in DNA of the D1 nodule outgrowth cells at 12 and 24 hr after 1 i.g. feeding of the labeled carcinogen. The radioactivity in the DNA of 8-week-old outgrowth in vivo was 36 and 38% greater at 12 and 24 hr, respectively, than that of 4-week-old outgrowth. Some amount of tritium was lost at 24 hr.

Results of the MC-3H and TdR-14C double labeling experiments are shown in Table 1. TdR-14C incorporation in the outgrowth in vitro progressively increased during the 6-hr incubation and, consistent with our earlier findings (1), tissue samples from 4-week-old outgrowth were more active in DNA synthesis. MC-3H radioactivity in DNA of both 4- and 8-week tissue in vitro also progressively increased during the 6-hr incubation. Binding of the carcinogen to DNA was slightly greater in the 8-week-old outgrowth, and an analysis of the ratio of MC-3H:TdR-14C radioactivity in 4- and 8-week outgrowths revealed that binding of the hydrocarbon to DNA

APRIL 1973 863
Table 1

<table>
<thead>
<tr>
<th>Hyperplastic alveolar nodule outgrowth age (wk)</th>
<th>Labeled compounds</th>
<th>DNA (cpm/100 μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr incubation</td>
<td>3 hr incubation</td>
</tr>
<tr>
<td>4</td>
<td>MC-3H</td>
<td>350.9 (1.9)</td>
</tr>
<tr>
<td></td>
<td>TdR-14C</td>
<td>178.6</td>
</tr>
<tr>
<td>8</td>
<td>MC-3H</td>
<td>487.7 (3.8)</td>
</tr>
<tr>
<td></td>
<td>TdR-14C</td>
<td>128.0 (10.8)</td>
</tr>
</tbody>
</table>

a Figures in parentheses indicate the ratio of MC-3H:TdR-14C radioactivity.

was not related to the rate of DNA synthesis in the respective tissues. The fact that TdR-14C incorporation progressively increased during the 6 hr in vitro indicates that the tissue was metabolically viable during the incubation period. This interpretation was further corroborated by autoradiography of histological sections of the tissue.

Chart 2 shows the buoyant density and radioactivity patterns of the DNA after nodule outgrowth tissue was incubated in the presence of BrdU and deoxyadenosine-3H. The UV absorbance pattern of the preexisting DNA was characteristic of mouse DNA, and virtually all deoxyadenosine-3H radioactivity was associated with the 2 heavier peaks, indicating that the DNA fractions which incorporated the BrdU constitute the newly synthesized DNA during the 8-hr incubation period. Centrifugation of BrdU- and MC-3H-labeled nodule DNA to equilibrium in CsCl showed that the carcinogen was bound to both the heavy and the light DNA peaks (Chart 3). The occurrence of more than 1 peak of heavy DNA may be due to the varying levels of thymine substitution by BrdU (9) in the newly synthesized nodule DNA during the 8-hr in vitro incubation.

DISCUSSION

After a single i.g. application, MC-3H was found to bind to DNA of the D1 nodule outgrowths in vivo. The overall pattern of binding of the carcinogen to DNA is consistent with earlier reports concerning various other tissues, including rat mammary gland (18, 20, 22, 26). MC-3H binds to DNA both at 4- and 8-week-old nodule outgrowths, and a slightly greater affinity for this DNA-carcinogen interaction is evident in the 8-week outgrowth in vivo, although DNA synthesis in the older outgrowth is one-half that of the 4-week tissue (1). These results thus indicate that the rate of DNA synthesis in the nodule tissue in vivo at the time of carcinogen treatment is not particularly related to binding of the carcinogen to DNA.

The above interpretation is further substantiated by the results of MC-3H and TdR-14C double labeling experiments. DNA synthesis in the nodule remained higher at 4 weeks than at 8 weeks during the 6-hr in vitro incubation, but MC-3H
radioactivity in DNA was essentially similar in the 2 outgrowths regardless of their TdR-\(^{14}\)C radioactivity. Analysis of the ratio of MC-\(^{3}\)H:TdR-\(^{14}\)C radioactivity in 4- and 8-week-old outgrowths after a 6-hr incubation in vitro failed to show a direct relation between MC binding to DNA and the rate of DNA replication in the nodule. Isopycnic density gradient analysis in CsCl of the BrdU- and MC-\(^{3}\)H-labeled DNA provides further evidence supporting the above contention. The fact that MC-\(^{3}\)H radioactivity is almost equally distributed between BrdU-labeled heavy and nonlabeled lighter DNA fractions clearly shows that the carcinogen binds to both replicative and nonreplicative DNA essentially to the same extent. Thus cells in DNA synthesis are not preferentially more susceptible to binding of the carcinogen to the cellular genome. Similar results were also obtained with the use of BrdU and DMBA-\(^{3}\)H labeling of mouse skin cells in tissue culture (32).

It is therefore reasonable to conclude that binding of MC to precancerous mammary nodule outgrowths DNA in vitro or in vivo is not influenced by or dependent upon the rate of DNA synthesis in the tissue. The interaction between the hydrocarbon and the nodule-native DNA may not be dependent upon the changing physicochemical characteristics of the DNA molecule, such as strand separation for replication or rewinding to form the double helix. In this context it may be mentioned that binding of DMBA to DNA does not impair the subsequent replicative ability of mouse skin cell DNA in tissue culture (6). Goshman and Heidelberger (16) also found that the binding of DMBA to DNA of mouse skin epidermis in vivo remained essentially constant regardless of the variable rate of DNA synthesis in the tissue. The present results as well as evidence in the literature thus appear to be in variance with the suggestion that carcinogenic hydrocarbon would selectively bind to replicative DNA, since the inhibition of DNA synthesis by hydroxyuracil may cause reduced binding (31). On the other hand, the present results show that MC possibly binds more efficiently to nodule DNA when it is less active in cell proliferative activity. Similar augmented carcinogenic hydrocarbon binding to DNA at the time of reduced DNA synthesis has been reported in hamster embryonic cells and mouse skin in vitro (2, 32). Furthermore, salmon sperm or calf thymus DNA are known to bind carcinogenic hydrocarbons when incubated in vitro in the presence of rat liver microsomes (15, 17).

In conclusion, the results strongly indicate that DNA replication is not essential for MC binding to mammary nodule outgrowth DNA, and hence increased susceptibility of the DNA-replicating cell to tumorigenic action of MC may not be related to augmented binding alone. However, since the rate of DNA replication in the tissue at the time of carcinogen treatment is important for tumorigenesis (1, 19), the possibility exists that binding of the hydrocarbon to replicative DNA may improve the chances of inducing aberrations in the newly made DNA molecule(s) and consequently leading to some altered genetic regulation in the daughter cells.

REFERENCES

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