An Early Event Associated with Liver Carcinogenesis Involving Loss of a Polypeptide That Binds Carcinogen

Gary R. Blackburn, John P. Andrews, K. V. Kesava Rao, and Sam Sorof
Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

ABSTRACT

This report describes an early and direct action of oncogenic agents and its apparent consequences. Chemical carcinogen has been found to interact principally with a specific polypeptide in livers of normal rats. Short-term ingestion of carcinogen causes marked reductions in the concentrations of both the carcinogen:polypeptide complex and the polypeptide itself. This action and its consequences are unique in several ways. (a) Chemical carcinogen is directly involved in the event. (b) Three kinds of liver carcinogens act in this way: the aromatic amide, N-2-fluorenylacetamide (2-acetylaminofluorene); the aminoazo dye, 3'-methyl-4-dimethylaminoazobenzene; and the amino acid analog, ethionine. (c) The interaction of chemical carcinogen with a specific polypeptide is involved. (d) Both the carcinogen:polypeptide complex and the polypeptide itself undergo marked reductions in concentration during hepatocarcinogenesis by the three types of carcinogens. (e) The consequences of the interaction are sensitive indicators unusually early during liver carcinogenesis. (f) The target polypeptide has a molecular weight of 14,700, similar to those of known polypeptide growth regulators.

INTRODUCTION

An important goal in present cancer research is the identification of the early molecular events that signal the onset of the carcinogenic process. A variety of biochemical, enzymatic, and immunohistochemical alterations have been found to be early indicators of preneoplastic putative initiated hepatocytes. Some of these markers are negative, appearing as losses of glucose-6-phosphatase, nucleotide polyphosphatase, glucuronidase, serine dehydratase, glycogen phosphorylase, RNases, and DNases. Other markers are positive, evident as increased levels of a-fetoprotein, preneoplastic antigen, y-glutamyl transpeptidase, DT-diaphorase, and chorionic gonadotropin (reviewed in Refs. 6 and 7). Unfortunately, all of these alterations bear no apparent direct or rational causal relationship to the oncogenic process.

We report here an early event during liver carcinogenesis that is thus far unique in that it directly involves an early action of oncogenic agents and its apparent consequences. Chemical carcinogen has been found to interact principally with a specific polypeptide in normal liver at the start of the hepatocarcinogenesis. Short-term ingestion of carcinogen brings about considerable reductions in the concentrations of both the resultant carcinogen:polypeptide complex and the polypeptide itself. Three kinds of liver carcinogens directly act this way, suggesting that the interactions and the consequences may be commonly involved in the oncogenesis.

MATERIALS AND METHODS

Rats Fed Carcinogens. Male Fischer 344 CDF rats (Charles River Breeding Laboratories, Wilmington, Mass.) were fed a grain diet (16) with 0.03% FAA or without carcinogen (control) for 3 weeks. The rats were then maintained on the control diet for 18 hr in order presumably to reduce endogenous stores of the carcinogen and its metabolites, administered intragastrically 10 μCi of [9-14C]FAA per 0.1 ml ethionine per 100 g body weight (46 to 52 mCi/mmol; New England Nuclear, Boston, Mass.; >99% radiochemical purity, previously confirmed), and sacrificed 48 hr later.

Male Sprague-Dawley Spd rats (ARS/Sprague Dawley, Madison, Wis.) were fed a semisynthetic diet (15) with 0.058% 3'-Me-DAB or without carcinogen (control) for 4 weeks. The rats were then administered [9-14C]FAA intragastrically at the above dosage and were fed their respective diets until sacrifice at 48 hr later.

Female Fischer 344 CDF rats were fed a semisynthetic diet (25) containing 0.3% DL-ethionine (Sigma Chemical Co., St. Louis, Mo.) or without carcinogen (control) for 4 weeks. The rats were then given [9-14C]FAA intragastrically at the above dosage and were maintained on their respective diets until sacrifice at 48 hr later.

In order to assess the effects of related noncarcinogens, rats of the above sexes and strains were similarly fed the respective equimolar concentrations of 0.022% fluorene for 3 weeks (26) or 0.048% 4-aminoazobenzene for 4 weeks (26) and then were processed as above.

Regenerating Livers. Male Sprague-Dawley rats, maintained on commercial stock diet, were partially hepatectomized (11), given the above standard dose of [9-14C]FAA 12 hr later, and sacrificed 16 hr after the administration of the dose.

Preparation of Liver Cytosols. Immediately following sacrifice of the rats, representative liver samples were removed for histological analysis. All subsequent operations were conducted at 1-4°C. The remainder of the livers were perfused portally with 0.25 M sucrose solution, minced, homogenized in 0.25 M sucrose (1:1, w/v) in a Potter-Elvehjem glass: Teflon homogenizer (BB size; Arthur H. Thomas Co., Philadelphia, Pa.), and centrifuged at 100,000 × g for 2 hr, all as reported previously (22). The resultant clear amber liver cytosols con-
Molecular Sieving of Liver Cytosolic Proteins. Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) was hydrated for 4 days (22°) and washed by sedimentation 3 times in 0.01 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl to remove fines. The gel was settled into columns and packed (approximately 197 x 2.5 cm) by elution of at least 500 ml of that buffer at a hydrostatic pressure of 20 cm at 1 to 4°. The constituents of 3.2 ml of the liver cytosols of individual rats were resolved according to molecular size by elution at approximately 10 ml/hr into fractions whose volumes were measured (approximately 5.5 ml). Protein concentrations were assayed by absorbances at 235 nm (22). Radioactivity in Aquasol 2 was determined by $\beta$-liquid scintillation spectrometry (Beckman Instruments, Inc., Palo Alto, Calif.).

The nomenclature of the components (S values) in the molecular weight profiles was based on sedimentation rates of the macromolecules present at component modes (22). The present study focused on the carcinogen:protein complex belonging to the 2S molecular weight class. The selectivity (specificity) of the formation of the 2S carcinogen:protein complex in the molecular size profiles was quantified in terms relative to the total bound radioactivity in liver cytosols.

Gel Electrophoresis of Liver Cytosolic Proteins. Rat liver cytosols were stored at -60° until used. The cytosols were incubated at room temperature overnight in 1 M urea, 1% sodium dodecyl sulfate, 2% $\beta$-mercaptoethanol, 10 KIU trasylool, and 0.01% Pyronine Y. Samples of 50 $\mu$g protein were electrophoresed in 15% polyacrylamide gels containing 0.025 M Tris, 0.2 M glycine buffer (pH 8.8), and 0.1% sodium dodecyl sulfate using the procedure of Laemmli (14). Electrophoreses were terminated when the Pyronine stain reached the bottom of the gel (14 cm long and 1.2 mm thick) at 16 to 18 hr (55 V). The gels were stained with 0.05% Coomassie Brilliant Blue R250 in methanol:acetic acid:water (5:1:5) and were scanned at 550 nm using a RFT scanning densitometer (Transidyne General Corp., Ann Arbor, Mich.). Molecular weights of cytosolic subunits were determined by use of 6 reference proteins (Fig. 1).

RESULTS

Loss of 2S Carcinogen:Protein Complex during Liver Carcinogenesis. This laboratory reported previously that the continued ingestion of the liver carcinogen FAA by rats results in the presence of a carcinogen:protein complex of ~150,000 daltons among the 7.5S class of liver cytosolic proteins. At 5 weeks on the regimen, this species is the principal carcinogen:protein complex in liver cytosol (23, 24). In extension of that finding, a series of experiments in the present study examined one of the changes in a complex that occurs at the start of the hepatocarcinogenesis by FAA.

Normal rats (fed commercial stock diet) and control rats (fed diet without carcinogen) given a tracer amount of labeled carcinogen form relatively large and similar amounts of a liver cytosolic carcinogen:protein complex of 2S macromolecular size. At 48 hr following a single dose of [9-14C]FAA to normal and control male Fischer 344 CDF rats, the 2S [14C]fluorenyl protein is the principal carcinogen:protein complex. This is evident in the molecular size distribution of the labeled carcinogen:protein complexes in the liver cytosols of such rats fed the control diet for 3 weeks. In contrast, rats fed the same diet with the liver carcinogen FAA for 3 weeks have little radioactive 2S carcinogen:protein complex (Chart 1). A small component of 2S complex is barely discernible. Table 1 indicates that the relative concentration of bound radioactive carcinogen in the 2S complex (based on all bound carcinogen in the FAA cytosols) is 25% of that in the control cytosols. However, the reduction of the 2S complex itself is actually more severe, inasmuch as the concentration of most complexes is lower in the FAA-fed rats (see Chart 1, ordinate). Rats of this sex and strain are highly susceptible to the hepatocarcinogenic action of FAA (26, 27). Histopathological examination of the livers of the FAA-administered rats revealed premalignant hepatocytic changes and ductal proliferation. The control livers were essentially normal. The conclusion therefore is that the loss of the labeled 2S carcinogen:protein complex is associated with the early cellular alterations during liver carcinogenesis by FAA.

Similarly, ingestion of the hepatocarcinogenic aminoazo dye 3'-Me-DAB lowers the liver concentration of the 2S [14C]fluorenyl protein. Male Sprague-Dawley Spd rats fed the control diet for 4 weeks make a relatively high level of the 2S [14C]fluorenyl carcinogen:protein complex at 48 hr following a single tracer dose of [9-14C]FAA. In contrast, such rats similarly fed the liver carcinogen 3'-Me-DAB have a very low level of 2S [14C]fluorenyl carcinogen:protein complex (Chart 2). The relative concentration of the labeled complex is 31% of that in control livers (Table 1). Actually, the drop in the content of the 2S complex itself is much greater, considering the lower level of most complexes in the carcinogen-fed rats (see Chart 2, ordinate). The azocarcinogen is strongly hepatocarcinogenic in rats of this sex and strain (2, 21, 26). Histopathological inspection of the livers revealed nodular hepatocytic regeneration, foci of early cholangiocarcinoma, and multinodular hepatocytic carcinoma. The control livers were normal. The loss of the 2S [14C]fluorenyl carcinogen:protein complex is thus an early event in the liver carcinogenesis by the azocarcinogen 3'-Me-DAB as well.

Likewise, feeding the hepatocarcinogenic amino acid analog ethionine causes a drastic reduction in the level of the 2S [14C]fluorenyl carcinogen:protein complex (Chart 3). At 4 weeks, the relative concentration of the 2S complex in the ethionine-fed rats is only 4% of that in the controls (Table 1). Again, the lowering of the amount of the 2S complex itself is considerably greater because of the general lowering of most complexes in the profiles of ethionine-fed rats (see Chart 3, ordinate). Female Fischer 344 CDF rats are quite susceptible to the liver carcinogenic actions of ethionine (4, 5, 26). Histological examination of the livers revealed foci of diffuse early malignant changes in hepatocytes and ductal proliferation. The control livers were normal. Thus, the loss of the 2S [14C]fluorenyl carcinogen:protein complex is an early event in the liver carcinogenesis by ethionine also.

Lack of Effect on the 2S Carcinogen:Protein Complex by Noncarcinogens and Partial Hepatectomy. Two noncarcinogenic analogs of these carcinogens fail to bring about a loss of the 2S [14C]fluorenyl protein complex. Neither the aminoazo dye 4-aminoazobenzene nor fluorene is hepatocarcinogenic in rats (26). The rats fed 4-aminoazobenzene for 4 weeks had histologically normal livers. The rats fed fluorene for 3 weeks had livers displaying some periportal degeneration. Neither of
these noncarcinogens (fed at concentrations equimolar with their carcinogenic analogs) caused a significant depression in the concentration of the 2S \([^{14}C]\)fluorenyl protein compared to that of their controls (Table 1).

The lowering of the concentration of the 2S carcinogen:protein complex by liver carcinogens probably does not result primarily from an increased rate of normal growth. Regenerating livers of rats 28 hr following partial hepatectomy contained two-thirds as much of the complex as did their sham-operated control livers (Table 1).

Properties of the 2S Carcinogen:Polypeptide Complex.

The bound carcinogen derivative is apparently covalently linked to the 2S protein. This conclusion derives from the 97% retention of the labeled derivative despite successive extractions of isolated 2S complex in methanol:ether (1:2), 95% ethanol, and ether. The association also withstands treatment with \(\beta\)-mercaptoethanol and gel electrophoresis in sodium dodecyl sulfate-containing buffer. The apparent covalency indicates that FAA is activated to a reactive state prior to the formation of the 2S complex.

The 2S \([^{14}C]\)fluorenyl carcinogen:protein complex contains one polypeptide, inasmuch as the molecular size of the complex is unchanged by dissociative conditions. Only one labeled protein is detectable in the 2S fraction treated with \(\beta\)-mercaptoethanol and electrophoresed in sodium dodecyl sulfate-containing buffer in polyacrylamide gel. The apparent molecular weight of the polypeptide is 13,000 as determined from liver cytosol (Fig. 1) but is 14,700 ± 600 (S.D.; 5 determinations) after isolation from polyacrylamide gels. These values are in agreement with the previously determined value of 10,000 as the molecular weight of the average undenatured 2S protein in liver cytosol (22).

Reduction in Amount of 14,700-Dalton Carcinogen-binding Polypeptide. Not only is the concentration of bound carcinogen in the 2S complex drastically decreased, but the level of the 14,700-dalton polypeptide itself is also markedly reduced during ingestion of the 3 carcinogens. The livers contained one-fifth to one-half of the concentration of the polypeptide present in the control livers. This reduction is demonstrated in comparisons of matched stained electrophoretic gels in Fig. 1 and their densitometric scans under standardized conditions (Table 2). In contrast, much less (or no significant) reduction was
brought about by the noncarcinogens, fluorene, and 4-aminoazobenzene.

The reductions in the liver concentrations of both the 2S carcino
gen:polypeptide complex and the 14,700-dalton polypeptide itself do not occur in response to all hepatocarcino-
gens. Exposure of male Sprague-Dawley Spd rats to diethyl-
nitrosamine in drinking water at 50 mg/liter for 8 and 16 weeks
failed to bring about these effects, even though the livers had
undergone histological premalignant alterations (data not pre-
sented).

Table 1

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Duration</th>
<th>Strain</th>
<th>Sex</th>
<th>No.</th>
<th>No. of analyses</th>
<th>Liver concentration of 2S [14C]carcinogen (^a)</th>
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<tbody>
<tr>
<td>Control</td>
<td>3 wk</td>
<td>CDF</td>
<td>M</td>
<td>1</td>
<td>4</td>
<td>28 ± 2.7 (^c) of control</td>
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<tr>
<td>FAA</td>
<td>3 wk</td>
<td>CDF</td>
<td>M</td>
<td>1</td>
<td>4</td>
<td>7 ± 2.3</td>
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<tr>
<td>Fluorene</td>
<td>3 wk</td>
<td>CDF</td>
<td>M</td>
<td>1</td>
<td>2</td>
<td>24 ± 3.9</td>
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<tr>
<td>Control</td>
<td>4 wk</td>
<td>Spd</td>
<td>M</td>
<td>1</td>
<td>4</td>
<td>29 ± 4.3 of control</td>
</tr>
<tr>
<td>3′-Me-DAB</td>
<td>4 wk</td>
<td>Spd</td>
<td>M</td>
<td>1</td>
<td>4</td>
<td>9 ± 1.1</td>
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<tr>
<td>4-Aminoazobenzene</td>
<td>4 wk</td>
<td>Spd</td>
<td>M</td>
<td>1</td>
<td>2</td>
<td>20 ± 0.6</td>
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<tr>
<td>Control</td>
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<td>CDF</td>
<td>F</td>
<td>1</td>
<td>3</td>
<td>45 ± 0.7 of control</td>
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<td>4 wk</td>
<td>CDF</td>
<td>F</td>
<td>1</td>
<td>4</td>
<td>2 ± 1.6</td>
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<tr>
<td>Sham-operated control</td>
<td>28 hr</td>
<td>Spd</td>
<td>M</td>
<td>2</td>
<td>1</td>
<td>27 ± 100</td>
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<tr>
<td>Regenerating liver</td>
<td>28 hr</td>
<td>Spd</td>
<td>M</td>
<td>3</td>
<td>1</td>
<td>18 ± 67</td>
</tr>
</tbody>
</table>

\(^a\) Content of ["C]carcinogen in the 2S component relative to the total macromolecular "C in molecular size profiles (Charts 1 to 3).

\(^b\) Number of rats used in each analysis.

\(^c\) Mean ± S.D.

Chart 2. Decrease in the level of the 2S ["C]fluorenyl carcino
gen:polypeptide complex during liver carcinogenesis by 3′-Me-DAB. Male Sprague-Dawley Spd rats were fed a semisythetic diet with 3′-Me-DAB (upper profiles) or without carcinogen (control; lower profiles) for 4 weeks. Details are provided in the legend of Chart 1 and in the text.
DISCUSSION

The presently described early event in liver carcinogenesis is unique in that it can be directly related to the oncogenic agent. The continued interaction of carcinogen with its principal polypeptide target, present in normal or control livers, brings about considerable reductions in the concentrations of the complex and the polypeptide itself. All early markers of carcinogenesis reported previously bear no apparent direct or rational relationship to the oncogenic process. They are apparently phenotypes of abnormal differentiation or the manifestations of abnormal levels of normal enzymes, proteins, or antigens (6, 7, 19). In contrast, the present early reductions in the concentrations of the complex and the polypeptide itself are apparently direct responses to the interactions of the polypeptide with the reactive forms of 3 carcinogens, demonstrated directly in the case of FAA and indirectly with 3'-Me-DAB and ethionine. A common mechanism is supported by the common ability of the 3 carcinogens to bring about these effects. The

Table 2

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Duration (wk)</th>
<th>No. of analyses</th>
<th>Concentration of polypeptide</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>6</td>
<td>61 ± 6^2</td>
<td>100</td>
</tr>
<tr>
<td>FAA</td>
<td>3</td>
<td>6</td>
<td>23 ± 10</td>
<td>38</td>
</tr>
<tr>
<td>Fluorene</td>
<td>3</td>
<td>2</td>
<td>45 (35, 55)^d</td>
<td>74</td>
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<tr>
<td>Control</td>
<td>4</td>
<td>3</td>
<td>47 ± 6</td>
<td>100</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>4</td>
<td>3</td>
<td>22 ± 10</td>
<td>47</td>
</tr>
<tr>
<td>4-Aminoazobenzene</td>
<td>4</td>
<td>2</td>
<td>41 (40, 41)^d</td>
<td>87</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>4</td>
<td>82 ± 13</td>
<td>100</td>
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<tr>
<td>Ethionine</td>
<td>4</td>
<td>3</td>
<td>17 ± 3</td>
<td>21</td>
</tr>
</tbody>
</table>

^ Each analysis relates to the liver of one rat.
^ Area of densitometric scans of gel electrophoretic bands stained with Coomassie Brilliant Blue R250. All determinations were carried out under matching conditions described in the legend of Fig.1.
^ Mean ± S.D.
^ Individual values.
Early Loss of Carcinogen-binding Polypeptide

Fig. 1. Reduction in content of the 14,700-dalton polypeptide in rat liver cytosols during liver carcinogenesis by 3 carcinogens. The arrow points to the band of the 14,700-dalton polypeptide. Molecular weights of standard (Std) proteins (left lane): phosphorylase b, 94,000 (top); bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 28,000; soybean trypsin inhibitor, 20,000; and lysozyme, 14,300. Shown are liver cytosols of rats fed: control (Con) diet, FAA diet, fluorene (F) diet; control (Con) diet, 3'-Me-DAB diet, 4-aminoazobenzene (AAB) diet; control (Con) diet, and ethionine (Eth) diet. Details are provided in the text.

lower levels of the complex and the polypeptide may be due to their increased rates of intracellular proteolytic degradation following the interactions with the 3 carcinogens, an effect similar to those observed with many derivatized proteins (8, 9).

Ketterer (12) and Ketterer et al. (13) have described previously an A protein in rat liver cytosol that has a molecular weight of 14,000 and binds long-chain fatty acid acyl-CoA and a number of lipophilic carcinogens. The properties of the protein are similar to those of the Z protein (17) and the fatty acid-binding protein (18) in rat intestinal mucosa, liver, myocardium, adipose tissue, skeletal muscle, and kidney. The possible relevance of these proteins to the presently described 14,700-dalton polypeptide is still to be elucidated.

The specificity of the present early event appears to reside with the 14,700-dalton polypeptide rather than with the carcinogens. This conclusion is supported by the diverse chemical natures of the 3 carcinogens and by the continued presence or even increase in the amounts of other species of carcinogen: protein complexes in liver cytosol (22-24) (see Charts 1 to 3).

The present observations that a polypeptide and the product of its interactions with 3 types of carcinogens act commonly in early events in liver carcinogenesis are suggestive that the interactions themselves may be involved in the oncogenic process by these carcinogens. It is intriguing to speculate that the 14,700-dalton polypeptide may normally have a growth regulatory function. Polypeptide growth regulators with molecular weights in the vicinity of that of the 14,700-dalton polypeptide are known (3, 10, 20).

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