Requirement of Cell Proliferation for the Initiation of Liver Carcinogenesis as Assayed by Three Different Procedures

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ABSTRACT

Experiments were designed to determine the role of cell proliferation in the initiation of liver carcinogenesis induced by chemicals. To investigate this, two methylating carcinogens, N-methyl-N-nitrosourea and 1,2-dimethylhydrazine, were used as the initiating carcinogens. The initiated hepatocytes were monitored by selectively stimulating them to grow into focal islands of presumptive preneoplastic hepatocytes. The experimental approach in brief consisted of the following. Rats received a nonnecrogenic dose of the carcinogen; at a time period when the carcinogen could no longer be detected in the system, they were subjected to either partial or sham hepatectomy. The initiated cells thus formed were selectively stimulated to grow into foci of preneoplastic hepatocytes using three different selection regimens: (a) feeding a diet containing 0.02% 2-acetylaminofluorene plus one administration of carbon tetrachloride (2 ml/kg body weight) intragastrically; (b) feeding a diet containing 0.05% phenobarbital; and (c) feeding a choline-deficient diet. The foci were quantitated by staining them for the presence of γ-glutamyltranspeptidase.

The results obtained indicated that irrespective of the type of selection procedure used foci of preneoplastic hepatocytes were seen only in rats that received the carcinogen coupled with a cell-proliferative stimulus such as partial hepatectomy. Very few or no foci were seen in rats that received the carcinogen plus sham hepatectomy. These results suggest that cell proliferation plays an important role in the initiation of liver carcinogenesis by chemicals.

INTRODUCTION

Cell proliferation has often been implicated in the carcinogenic process and in cell transformation induced by chemicals, radiation, and viruses (2, 5, 12, 14, 24, 33, 35). Compelling evidence to support the role of cell proliferation in the carcinogenic process has come from the observations that several carcinogens that normally do not induce liver cancer in adult animals become hepatocarcinogenic when given in a single dose but coupled with liver cell-proliferative stimulus such as PH4 (5, 12, 24, 35). However, the site and mechanism of its action in this process could not be determined with any accuracy largely because in these experiments cancer was used as the end point and many steps, especially the ones involving in this complex process, have not been completely identified. In addition, in most of the experiments, carcinogen was given following PH, and often a nonnecrogenic dose becomes necrogenic to liver when given to a partially hepatectomized rat. For example, a 10-mg/kg dose of dimethylnitrosamine, which is nonnecrogenic when given to an adult rat, becomes necrogenic to liver when given to a partially hepatectomized rat. Furthermore, in such experimental models, at the time of administration of the carcinogen, liver cells are at different stages of cell cycle, and the susceptibility of these different cells to carcinogenic attack is not yet established.

Recently, several procedures have been described (13, 20, 21, 23, 28, 31, 34) which selectively and rapidly stimulate the growth of the carcinogen-altered cells into foci of hepatocytes. These altered hepatocytes can be characterized using several enzyme markers including the presence of γ-GT (11, 18). Such selection procedures, especially those described by Solt and Farber (31) and Peraino et al. (20), following initial treatment with a carcinogen result in an increased cancer incidence (7, 22, 32). In addition, using the former procedure, a material continuity between the focal islands and liver cancer has been established by demonstrating the presence of cancer within the nodule (8). These results thus suggest that focal islands of hepatocytes may be preneoplastic and that the carcinogen-altered cells which can be grown into foci probably are initiated. Accordingly, in the present discussion, initiated cells are defined as those carcinogen-altered cells which can be stimulated to grow into foci. Since over 95% of the foci were positive for γ-GT activity (18), determination of the number of γ-GT-positive foci was considered as a valid assay for initiation.

Using foci of enzyme-altered hepatocytes as the end point and the selection procedure consisting of 2-AAF plus CCl4 (3), evidence was obtained to indicate that cell proliferation is an important factor in the initiation phase of carcinogenesis (3, 38). The qualitative and quantitative natures of the selection phenomenon, however, are not completely understood; also, it is not known whether the selection regimen consisting of 2-AAF plus CCl4 is selecting a unique or a fraction of initiated cell population. In order to establish the generality of the role of cell proliferation in the initiation phase, in the present investigation we used 2 carcinogens to induce initiation and 3 different selection regimens (3, 20, 28) to stimulate the growth of the initiated cells into foci of enzyme-altered hepatocytes.

The results obtained indicated that, regardless of the type of selection used, the foci of preneoplastic hepatocytes were seen only in rats that received the carcinogen coupled with a cell-proliferative stimulus. Very few foci if any were seen in rats that received carcinogen plus SH.

These results suggest that one of the sites of action of cell proliferation in carcinogenic process is at the initiation step.

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4 The abbreviations used are: PH, 67% partial hepatectomy; γ-GT, γ-glutamyltranspeptidase; 2-AAF, 2-acetylaminofluorene; SH, sham hepatectomy; PB, phenobarbital; MNU, N-methyl-N-nitrosourea; 1,2-DMH, 1,2-dimethylhydrazine; i.e., intragastrically.

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MATERIALS AND METHODS

Male F344 rats (Charles River Breeding Laboratory, Detroit, Mich.) weighing 120 to 140 g were maintained on Purina No. 5001 rodent laboratory chow diet and daily cycles of alternating 12-hr periods of light and darkness for 1 week before the start of the experiment. A semisynthetic high-protein diet (No. 101) containing 0.02% 2-AAF (31) or 0.05% PB, and a choline-deficient diet (28) were obtained from Bio Serv, Inc., Frenchtown, N. J. MNU obtained from K and K Fine Chemicals, ICN Pharmaceuticals, Inc., Cleveland, Ohio, was recrystallized from ethyl acetate and hexane. 1,2-DMH was obtained from Aldrich Chemical Co., Milwaukee, Wis.

Influence of PH on the Induction of Foci in Rat Liver by 1,2-DMH and Using 3 Different Selection Procedures. In Experiment 1, rats were given 1,2-DMH (100 mg/kg i.p.) in 0.9% NaCl solution containing EDTA (15 mg/100 ml; pH 7.0). Twelve hr after, a time period when the circulating carcinogen is no longer detectable (9, 10, 19), some of the rats were subjected to PH while the rest were subjected to SH. After a 2-week recovery period, the initiated cells were stimulated to grow into foci of presumptive preneoplastic hepatocytes using 3 different selection regimens (Chart 1). In one group, the animals were placed on a basal diet containing 0.02% 2-AAF for 2 weeks.

One week after the rats were on 2-AAF diet, they were given a single dose of CCl₄ (2 ml/kg body weight, in corn oil) i.g. (3). Following a total period of 14 days on the 2-AAF diet, the rats were placed on a basal diet for 1 week longer and then killed. Another group of rats were placed on a choline-deficient diet and were killed 6 weeks later, whereas the third group was placed on a diet containing 0.05% PB for 6 weeks before they were killed. A fourth group continued to be on basal diet for 4 more weeks. Liver samples were processed for histological and histochemical analyses. Section of liver was stained for y-GT histochemically.

In Experiment 4 (Chart 2), rats were given 1,2-DMH (100 mg/kg) but 18 hr after PH. The initiated cells were stimulated to grow into foci of preneoplastic hepatocytes by 3 different selection regimens as detailed above for Experiment 1.

Influence of PH on the Induction of Foci in Rat Liver by MNU and Using 3 Different Selection Procedures. In Experiment 2 (Chart 1), rats were given MNU (60 mg/kg) in 10 ml sodium citrate, pH 6.0, at a concentration of 5 mg/ml. Four hr later, a time period when the carcinogen is no longer detectable in both blood and liver (3), some rats were subjected to PH, while the rest were subjected to SH. The minimum level of detection of MNU using high-pressure liquid chromatographic technique is 5 to 10 ng/ml. After a 2-week recovery period, the initiated cells were selected to grow into foci of preneoplastic hepatocyte using 3 different selection regimens or the basal diet as detailed above, except that the rats on PB diet were kept for 10 weeks instead of 6 weeks as in Experiment 1.

In Experiment 5 (Chart 2), rats received MNU (60 mg/kg) 18 hr after PH. The initiated cells were stimulated to grow into foci by the above-mentioned 3 selection regimens as detailed in Experiment 2.

Influence of Selection Regimens after PH on the Induction of Foci in Control Rat Liver. In Experiment 3, rats were given 0.9% NaCl solution 4 hr prior to PH; 2 weeks later, the initiated cells, if any, were stimulated to grow by using the 3 different selection regimens as detailed in Experiment 2 (Chart 1).

Histology, Histochemistry, and Quantitation of y-GT-positive foci. At time periods when the carcinogen is no longer detectable in the system, the rats were subjected to either PH or SH. After a recovery period of 2 weeks, they were placed on 3 different selection regimens. Initiation was assayed by determining the number of y-GT-positive foci. Additional details are given in "Materials and Methods." CD, choline-deficient.
Liver Cell Proliferation and Initiation of Carcinogenesis

Influence of PH prior to the administration of the carcinogen on the induction of γ-GT-positive foci in rat liver

Table 2

<table>
<thead>
<tr>
<th>Carcinogen administered</th>
<th>Selection regimen</th>
<th>Experiment</th>
<th>γ-GT-positive foci</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number</td>
<td>Diameter (μm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No./sq cm</td>
<td></td>
</tr>
<tr>
<td>1,2-DMH 2-AAF + CCl₄</td>
<td>2-AAF + CCl₄</td>
<td>23.2 ± 4.7</td>
<td>819 ± 45 (60)</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>38.9 ± 6.4</td>
<td>420 ± 16 (112)</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>10.0 ± 1.4</td>
<td>225 ± 13 (85)</td>
</tr>
<tr>
<td>MNU 2-AAF + CCl₄</td>
<td>2-AAF + CCl₄</td>
<td>28.7 ± 5.1</td>
<td>682 ± 39 (56)</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>22.3 ± 2.3</td>
<td>398 ± 14 (60)</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>5.7 ± 0.9</td>
<td>196 ± 9 (51)</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
* Numbers in parentheses, number of animals.
* Numbers in parentheses, number of individual foci analyzed.
* CD, choline-deficient diet.

Table 1

Influence of PH given after the administration of the carcinogen on the induction of γ-GT-positive foci

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Carcinogen administered</th>
<th>Selection regimen</th>
<th>γ-GT-positive foci</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1,2-DMH 2-AAF + CCl₄</td>
<td>PH</td>
<td>17.1 ± 1.6 (14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SH</td>
<td>0.9 ± 0.4 (5)</td>
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<tr>
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<td></td>
<td></td>
<td>753 ± 87 (88)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>MNU 2-AAF + CCl₄</td>
<td>PH</td>
<td>8.6 ± 1.6 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SH</td>
<td>0.8 ± 0.5 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>591 ± 30 (57)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>0.9% NaCl solution</td>
<td>PH</td>
<td>8.6 ± 1.2 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SH</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>296 ± 21 (48)</td>
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<tr>
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<td>0.0</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
* Numbers in parentheses, number of animals.
* Numbers in parentheses, number of individual foci analyzed.
* ND, not determined; CD, choline-deficient diet.
The results obtained also permit one to postulate the scheme presented in Chart 3. According to the scheme, carcinogens induce several types of DNA damage. In an adult rat liver which is essentially a nonproliferating organ, most of the damage is repaired; however, if such damaged DNA replicates, the damage may be fixed in the daughter cell, and such a process may result in an initiated cell.

Several lines of evidence support such a scheme. For example, it has been demonstrated that carcinogens either by themselves or after being activated interact with DNA (16, 17, 26, 29) and that such DNA damage can be repaired in vivo (6, 26, 27). The results of the present study further strengthen the hypothesis presented in Chart 3 by providing evidence that cell proliferation is an important step in the induction of the initiated cell. Evidence was also obtained to indicate that it is the DNA replication during cell proliferation which is important for the induction of the initiated hepatocyte (36). In addition, recently, it has been shown that liver DNA with carcinogen-induced lesions replicates in vivo (1, 4, 25, 39) and that such newly made DNA synthesized on a carcinogen-damaged template is stable (39).

The results presented in Table 2, although pointing out the importance of cell proliferation in the induction of initiation, are difficult to interpret. The increased incidence of initiated cells obtained by giving the carcinogen after PH (Table 2) instead of SH (Table 1) may be caused by one or more of the following: (a) after the surgery, the liver is only one-third of the normal size and the administered dose of the carcinogen can become greater than that given to a control unoperated rat; (b) 18 hr after PH, hepatocytes are at different stages of cell cycle and cells at late G₁ and/or S phase may be more sensitive for carcinogenic attack (3, 15); and (c) during the proliferative stage of the cell, the removal of some critical DNA lesions may be inhibited. Indeed, the removal of O⁶-methylguanine and N⁴-3-methyladenine from DNA induced by dimethylnitrosamine (1), MNU, and 1,2-DMH was inhibited after PH. Similarly, the removal of some of the methylated purines induced by N⁴-methyl-N²-nitro-N-nitrosoguanidine in DNA was also inhibited during the S phase of 10T½ cells (30). If replication of DNA with carcinogen-induced critical lesions is important for the initiation process, the chances that such a process would occur are higher when the carcinogen is given after PH than in the model wherein the PH is performed after the administration of the carcinogen because, in the former model, comparatively fewer critical lesions will be removed prior to DNA replication.

Another observation that deserves consideration is that the number of foci per sq cm and their average size varied depending on the selection regimen used in spite of the fact that the initiating treatment is the same. This may mean that the potencies of the 3 selection regimens are different and probably cannot be compared at any one time point, because the mechanism by which these selection regimens stimulate the growth of the initiated cells may be different and/or the 3 selecting regimens are selecting different populations of initiated cells. It is also possible that one selection regimen may be selecting a population of initiated cells and the other may be selecting these plus other initiated ones. Nonetheless, irrespective of the mechanism by which the selection regimen stimulates the growth of the initiated cells and/or in spite of the possible occurrence of different types of initiated cells, their induction appears to require a cell-proliferative stimulus. The similarity between the induction of presumptive preneoplastic hepatocytes by chemicals and cell transformation by irradiation and viruses is noteworthy in that they both require at least one round of cell proliferation (2, 14, 33).

Although the mechanism by which cell proliferation exerts its unique effect in the initiation phase of carcinogenesis is not clearly understood, replication of DNA with carcinogen-induced lesions prior to repair offers an attractive mechanism by which carcinogen-induced critical damage may be appropriately transcribed and fixed into the newly made DNA and thus result in an initiated cell.

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REFERENCES


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