Alteration of Hepatocytes by Subcarcinogenic Exposure to N-2-Fluorenylacetamide\textsuperscript{1}

Frederick F. Becker

Department of Pathology, New York University School of Medicine, New York, New York 10016

SUMMARY

These experiments examined the effects of a single, subcarcinogenic dose of dimethylnitrosamine or N-hydroxyfluorenylacetamide when administered after a subcarcinogenic dietary regimen of N-2-fluorenylacetamide. Control rats that received either carcinogen diet alone or a single dose of carcinogen demonstrated neither hepatic nodules nor hepatocellular carcinomas. Those animals that received dimethylnitrosamine subsequent to carcinogen diet demonstrated many persistent hepatic nodules and 100% hepatocellular carcinomas.

These data support the concept that the nodules produced by subcarcinogenic ingestion of N-2-fluorenylacetamide are not composed simply of normal hepatocytes undergoing compensatory regeneration but consist of cells that have been altered by the carcinogen. One manifestation of this alteration is an increased susceptibility to further carcinogenic evolution.

INTRODUCTION

The results of several studies suggest that chemical carcinogenesis is a multiphasic process (1, 7, 11, 15). The validity of this concept is supported by the sequential cellular alterations induced in the liver by many carcinogens that result in the development of hepatic nodules. These nodules are aggregates of morphologically and biochemically altered hepatocytes from which malignant tumors arise, providing that the cumulative dose of carcinogen is sufficient (12, 14, 18, 20). Hepatic nodules that have a significant relationship to the development of hepatocellular carcinomas persist following the cessation of carcinogen, while those that are induced by subcarcinogenic exposure do not persist (13, 18, 20). Despite the major contrast in characteristics of persistence and malignant potential between these 2 types of nodules, few functional differences between them have been demonstrated (4, 12).

It has been suggested that the disappearance of nodules following cessation of carcinogen indicates that their component hepatocytes were normal and were undergoing compensatory regeneration. If this supposition were true, it would have significant implications in the area of carcinogenic exposure. These experiments were aimed at further characterizing the cells of these "reversible" nodules in terms of their response to a further challenge with carcinogens.

MATERIALS AND METHODS

Fifty male, ACI rats (Microbiological Associates, Walkersville, Md.) weighing 125 g were begun on carcinogen diet at approximately 7 weeks of age. 2-FAA\textsuperscript{2} (Eastman Organic Chemical Co., Rochester, N. Y.), mixed in standard laboratory meal at a level of 0.06%, was administered for 3 weeks; a week of normal diet followed. Three such feeding cycles were administered (20). At the end of this exposure, the 50 surviving rats were returned to normal diet and were given further treatment. One week after cessation of the carcinogen, 12 2-FAA-fed rats received a single i.p. dose of N-OH-FAA, 25 mg/kg in dimethyl sulfoxide (Group 1). Ten rats received the solvent alone (Group 2). Eighteen rats received a single i.p. dose of DMN (Eastman) in 0.9% NaCl solution, 5.5 mg/kg (Group 3). To determine whether this dose of DMN induced necrosis in 2-FAA-fed rats, 2 rats from Group 3 were sacrificed at 24, 48, and 72 hr after administration of DMN and sections of their livers were processed for histological examination. Ten rats received 0.9% NaCl solution alone (Group 4).

In addition, 12 age-paired rats that had not received 2-FAA received an identical dose of N-OH-FAA (Group 5) and 12 others received DMN (Group 6). All subsequent deaths occurred during the next 3 months, prior to the expected appearance of tumors (Table 1). The surviving rats in each group were sacrificed at 1 year. Blood was collected from the inferior vena cava at sacrifice, and the \( \alpha_1F \) level was determined by a sensitive radioimmunooassay as previously described (19). Complete autopsies were performed, and multiple sections of the livers were prepared for histological analysis.

Four hepatocellular carcinomas that were sufficiently large were analyzed for chromosome composition. The technique for determining the karyotype of tumor cells was previously reported (2).

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\textsuperscript{2}The abbreviations used are: 2-FAA, N-2-fluorenylacetamide; N-OH-FAA, N-hydroxyfluorenylacetamide; DMN, dimethylnitrosamine; \( \alpha_1F \), \( \alpha \)-fetoprotein.
Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats*</th>
<th>Survivors 1 year</th>
<th>Nodules*</th>
<th>Hepatocellular carcinomas*</th>
<th>α₁F (ng/ml)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2-FAA + N-OH-FAA</td>
<td>12</td>
<td>6</td>
<td>3/2</td>
<td>1/6</td>
<td>62 ± 15</td>
</tr>
<tr>
<td>2. 2-FAA + dimethyl sulfoxide</td>
<td>10</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>47 ± 12</td>
</tr>
<tr>
<td>3. 2-FAA + DMN</td>
<td>12</td>
<td>9</td>
<td>40/9</td>
<td>12/9</td>
<td>89 ± 15</td>
</tr>
<tr>
<td>4. 2-FAA + 0.9% NaCl</td>
<td>10</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>43 ± 10</td>
</tr>
<tr>
<td>5. N-OH-FAA</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>6. DMN</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>28 ± 5</td>
</tr>
</tbody>
</table>

Control rats
No treatment | 6 | 20 ± 4 |

* At onset of experiment.
* Number of nodules or hepatomas/Number of livers in which these lesions appear.
** Mean ± S.D.

RESULTS

Morphology. DMN at 5.5 mg/kg did not induce necrosis in either nodules or nonnodular parenchyma.

The number and distribution of nodules and hepatocellular carcinomas are described in Table 1. Three persistent nodules were noted in 2 livers of Group 1 rats. One of these livers also demonstrated an hepatocellular carcinoma. Every liver in Group 3 demonstrated multiple nodules for a total of 40 nodules and at least 1 hepatocellular carcinoma.

The livers of rats in Groups 5 and 6 were not remarkable. Some nuclear atypia was noted in those receiving DMN alone.

Cysts in the livers of all 2-FAA-fed rats were typical of those derived from ductal elements as previously described (18). In all of the 2-FAA-fed rats, ductular proliferation of slight to moderate degree formed septa or abortive septa. The hepatic nodules of Groups 1 and 3 were identical to those described previously (12, 18, 20). In addition, in 1 of the livers in Group 1 and in all of those in Group 3, there was a variety of hepatic nodules in various stages of involution that demonstrated fatty change or focal degeneration.

All of the hepatocellular carcinomas could be classified histologically as either well differentiated or very well differentiated. However, all demonstrated invasion and destruction of the adjacent hepatic parenchyma; and all demonstrated areas of cellular pleomorphism and nuclear irregularity, foci of mitosis, and focal areas of necrosis.

Chromosome Composition. Four of the hepatocellular carcinomas of Group 3 were large enough to be analyzed for chromosome composition. The cells of 3 of these tumors demonstrated a diploid karyotype with an occasional tetraploid cell. The 3rd tumor, although predominantly diploid, revealed approximately 5% cells with 43 chromosomes.

α₁F. The α₁F level of Group 1 was 62 ± 15 (S.D.) ng/ml, while that of its control, Group II, was 47 ± 12 ng/ml (Table 1). The α₁F level of Group 3 was 89 ± 25 ng/ml, while that of Group 4 was 43 ± 10 ng/ml. The minimal elevation of α₁F in the presence of these carcinomas was in keeping with our previous report (5) that diploid hepatocellular carcinomas usually fail to produce this fetal protein. The somewhat elevated α₁F levels of Groups 2 and 4 were commensurate with those that were detected several months after cessation of 2-FAA (6). Neither Group 5 nor Group 6 differed significantly from age-paired rats that had not been exposed to 2-FAA, 20 ± 7 ng/ml.

DISCUSSION

It has been suggested that chronic exposure to chemical hepatocarcinogens induces sequential alterations in target hepatocytes which result in malignant evolution (1, 11, 15, 18, 20). To date, it is unknown whether these progressive effects are additive mutations, as occur in some spontaneous cancers (16), or other cell alterations. It has been demonstrated previously (2, 4, 20) that feeding 3 cycles of 2-FAA, as used in these experiments, results in the development of many hepatic nodules. Ninety-five % or more of these nodules disappeared after cessation of the carcinogen diet and very few hepatocellular carcinomas appeared (20). It was possible, therefore, that the hepatocytes of the reversible nodules represented normal cells undergoing regenerative cell division in response to carcinogen-induced cell loss and were not otherwise altered. These experiments indicate that these cells are altered by the subcarcinogenic exposure to 2-FAA in a manner that distinguishes them from normal hepatocytes. Thus, a single subcarcinogenic dose of DMN, which induced a 100% yield of hepatocellular carcinomas in such livers, induced none in either quiescent or regenerating normal livers (10). This result bears similarity to that reported when the carcinogen 3'-methyl-4-dimethylaminoazobenzene is administered at some time after an initial subcarcinogenic exposure, that is, a cellular alteration results from the primary exposure, which does not resolve (8). Similar results were also reported with a 2-carcinogen system consisting of radiation and carbon tetrachloride (9). Whether all of these results can be grouped under the broad concept of initiation-promotion is as yet unclear, especially.
in view of the use of a known carcinogen in the role of a "promoting agent."

The basis for the susceptibility of the nodules to a subcarcinogenic dose of DMN is also unknown. It is possible that this phenomenon is related to the active cell division that is demonstrated by the hepatocytes of these nodules (3, 12). It has been reported (10) that subcarcinogenic doses of DMN can produce hepatocellular carcinomas if administered during the regenerative response of residual hepatocytes after 70% hepatectomy. However, the yield of hepatomas in this study far exceeded that reported when DMN is administered during liver regeneration; the lag time was much shorter, and the dose was below that reported to be effective. This occurred despite a much greater, total mitotic activity during the postoperative regenerative process. Once again, it would appear that the hepatocytes of the nodules are altered in a manner that makes them more susceptible to DMN than normal cells. Regardless of the mechanism underlying this synergistic activity by 2 carcinogens, it is certain that cells of these nodules retain the capacity to activate DMN metabolically, since alteration of this agent is required for its carcinogenicity (17).

The addition of a 4th feeding cycle of 2-FAA has been reported (20) to result in a high percentage of persisting hepatic nodules and a proportionate yield of hepatocellular carcinomas. The combination of DMN and 2-FAA that was used in these experiments yielded many persistent nodules as well as carcinomas. The 2-FAA-treated control rats and the majority of Group 1, demonstrated no nodules at autopsy and no carcinomas. It is, therefore, unlikely that the association between persistent nodularity and the presence of hepatocellular carcinomas in Group 3 is coincidental. Statistically, this association is highly significant and the rats were selected randomly at the conclusion of the 3 feeding cycles. The mechanism that underlies the persistence of nodules after cessation of the carcinogen is unknown, but it strongly suggests that the persistent nodule is neoplastic in nature.

We cannot explain the failure of the N-OH-FAA to give rise to carcinomas when administered subsequently to 2-FAA. This may represent a defect in a selected metabolic pathway, i.e., esterification, a deficiency in dose, or other factors at the level of macromolecular interaction. However, it is also possible that this was the result of the small number of rats surviving or the duration of follow-up.

All of the hepatocellular carcinomas that resulted from this combined regimen were histologically well differentiated, and 4 were diploid. From the results of our previous studies, the well-differentiated histological appearance of the remaining 9 carcinomas and the low levels of circulating $\alpha_F$ in their presence indicate that they too were predominantly diploid (5). This uniformity of characteristics differs from the variety of tumors produced by the 4-cycle, 2-FAA regimen. Those hepatocellular carcinomas were often aneuploid and poorly differentiated and produced high levels of $\alpha_F$ (2, 4, 5).

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REFERENCES

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