Sequential Phenotypic and Biochemical Alterations during Chemical Hepatocarcinogenesis

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Summary

Chronic exposure to chemical carcinogens induces in the target tissue a series of complex morphological and biochemical alterations that precede the appearance of overt cancer. Three types of experiments are described: (a) exposure of livers that had received subcarcinogenic doses of N-2-fluorenylacetamide to a subcarcinogenic dose of dimethylnitrosamine resulted in a 100% yield of neoplastic nodules and hepatocellular carcinoma; (b) neither normal hepatocytes nor those obtained from neoplastic nodules were agglutinated by any of the lectins tested. This finding was also true for slowly growing cells from carcinomas, while those of rapidly growing carcinomas were agglutinated by several lectins; (c) analysis of nonhistone proteins isolated from neoplastic nodules demonstrated the appearance of many new species in euchromatin when compared with normal liver. Carcinomas demonstrated an even greater number of new species and they were demonstrated in heterochromatin as well.

Results and Discussion

Subcarcinogenesis. The possibility that doses of carcinogens that are inadequate to induce cancer in the lifetime of the host might induce significant, persistent alterations in the target cells is extremely important. The no-threshold concept of carcinogenesis predicts that exposure to a carcinogen at any dose causes the onset of a chain of irrevocable events that can be considered to be carcinogenic. Most studies of chemical hepatocarcinogenesis in rats have concentrated upon morphological and biochemical alterations that were induced by increasing exposure to the agent, many of which required quite large doses (2, 13, 17, 18, 20, 24). Subcarcinogenic doses of 2-FAA are often sufficient to produce striking morphological and biochemical alterations of the liver similar to those of a fully carcinogenic dose. However, many of these alterations disappear following cessation of the carcinogen (14, 23). The question remained whether the alterations induced by subcarcinogenic regimens were a response to cell injury, a compensatory hyperplasia similar to that following chemical or surgical ablation of liver, or whether irrevocable molecular alteration of the cells had occurred.

The feeding of 2-FAA at a level of 0.065% for 3 cycles (1 cycle = 3 weeks of carcinogen) with 1 week of normal diet between each cycle produces extreme nodularity of the liver and striking biochemical alterations, many of which disappear within months after cessation of the diet (23). Subsequently, no neoplastic nodules can be identified nor do PHC result. A 4th feeding cycle induces large numbers of neoplastic nodules and PHC (23).

To determine whether the alterations induced by 3 feeding cycles were unrelated to the carcinogenic process as has been suggested, we challenged male ACI rats (Microbiological Associates, Walkersville, Md.) that had received this regimen with a single dose (5.5 mg/kg i.p.) 1 week after cessation of 2-FAA. It has been reported that (higher) doses of DMN, when administered during the response to 70% hepatectomy, induce moderate numbers of PHC after a long latent period (10, 11). Therefore, controls age-matched rats underwent 70% hepatectomy and received DMN at 20 hr, the time of maximal DNA synthesis. Table 1 reveals that neither carcinogen alone, nor the combination of regenerating liver and DMN resulted in the appearance of neoplastic nodules of PHC (4). Every rat exposed to the combina-

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2 The abbreviations used are: 2-FAA, N-2-fluorenylacetamide; PHC, primary hepatocellular carcinomas; DMN, dimethylnitrosamine; THC, transplantable hepatocellular carcinomas; Con A, concanavalin A; NHP, nonhistone proteins.
tion of 2-FAA and DMN demonstrated both lesions. Although this combined regimen is under more detailed analysis, in terms of the doses required and the duration of effect, it appears reasonable to state that doses of carcinogen which are themselves subcarcinogenic alter the hepatocyte in such a manner that sensitizes it to the further effects of other carcinogens. Obviously, this is the case with the 4th feeding cycle of 2-FAA. Previous experiments have also suggested the persistence of this susceptibility following subcarcinogenic exposure for long periods (9, 15). A recent paper has reported the presence of an interesting immunological alteration of the target hepatocytes that is induced by a 3-feeding cycle but disappears following cessation of 2-FAA (14). The same alteration became persistent after a 4th cycle (14).

**Lectin Agglutination.** Results such as those described above might be interpreted by many as resulting from sequential genetic damage, this accumulated damage then evoking malignant cells. Regardless of the correctness of this interpretation, it is clear that the eventual deleterious behavior of the malignant cell is a reflection of a pattern of abnormal phenotypic characteristics. Some suggestion has been made that the key alteration of such cells is to be found in the cell surface-host interface. Numerous reports have described alterations of the surfaces of malignant and transformed cells when compared with the normal cells from which they arose (12, 16, 19, 21). One of the most intriguing of these has been the demonstration that many malignant and transformed cells are agglutinated by various plant lectins (1, 8, 21). I have reported previously that the cells of a number of THC were agglutinated with Con A, while the normal rat hepatocyte was not (3). Although the findings were similar to those reported respectively for transformed and normal cultured cells (21), striking differences were noted. Thus, neither the dividing hepatocyte nor hepatocytes exposed to various proteases were agglutinated. Fetal hepatocytes were as fully agglutinated as were malignant cells. These findings raised the possibility that the agglutination of tumor cells by Con A was yet another manifestation of a return toward fetal phenotype.

It was of interest therefore to determine whether the neoplastic nodule (the putative premalignant lesion) demonstrated lectin agglutinability at any time in its life history. For this purpose, we have tested lectins that are known to have differing saccharide specificity and different cell agglutinability (22). Table 2 demonstrates the results of these experiments. Normal hepatocytes and those undergoing a mitotic cycle in response to 70% hepatectomy again demonstrated no agglutination, despite exposure to several lectins. Fetal hepatocytes demonstrated selective sensitivity with confirmation of previously reported Con A clumping.

Interestingly, the THC have demonstrated a widely varied pattern (fingerprint) of response to the different lectins. Although a slow tumor growth rate was most often associated with lack of responsibility, no readily identifiable pattern was demonstrated for rapidly growing cells. We are now attempting a computer analysis of these lectin patterns to determine whether any are related to other phenotypic manifestations. No neoplastic nodule taken at any time in its evolution (including livers bearing PHC) has demonstrated

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**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>No. of survivors at 1 hr</th>
<th>NN</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-FAA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMN&lt;sup&gt;f&lt;/sup&gt;</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>70% hepatectomy + DMN&lt;sup&gt;g&lt;/sup&gt;</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-FAA + DMN&lt;sup&gt;h&lt;/sup&gt;</td>
<td>12</td>
<td>9</td>
<td>40/9</td>
<td>12/9</td>
</tr>
</tbody>
</table>

<sup>* At onset of experiment.</sup>
<sup>† NN, neoplastic nodules; HC, hepatocytes.</sup>
<sup>‡ Number of nodules or hepatomas/number of livers in which these lesions appear.</sup>
<sup>d 0.06%, for 3 cycles.</sup>
<sup>f 5.5 mg/kg i.p.</sup>
<sup>g DMN administered at 20 hr.</sup>
<sup>h DMN administered 1 week following 3rd 2-FAA cycle.</sup>

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**Table 2**

<table>
<thead>
<tr>
<th>Lectin agglutination of hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>Hepatocytes</td>
</tr>
<tr>
<td>70% hepatectomy</td>
</tr>
<tr>
<td>Fetal hepatocytes</td>
</tr>
<tr>
<td>NN&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>THC 253</td>
</tr>
<tr>
<td>THC 252</td>
</tr>
<tr>
<td>THC 251c</td>
</tr>
<tr>
<td>THC 311c</td>
</tr>
</tbody>
</table>

<sup>* At 20 hr.</sup>
<sup>† At 20 days.</sup>
<sup>‡ NN, neoplastic nodules (12).</sup>
agglutinability of its cells (6).

We have recently reported that all PHC tested thus far demonstrated Con A agglutinability (6). It appears therefore that a significant alteration in surface character must occur in the cells of the hepatic nodule prior to, or at the time of, manifestation of malignant behavior. The failure of a small number of THC to demonstrate Con A agglutinability suggests either that the uniform response of PHC to date has been based on a statistical chance or that the characteristic is lost during transplantation. The former appears more likely.

**Chromatin Composition.** As I have described above, one of the striking features of the process of chemical carcinogenesis is the sequential alteration of the phenotypic pattern of the target cell.

Alterations ranging from morphological to isoenzymatic (13) to the production of the characteristic plasma proteins (5) have been reported. Although it is probable that many of these alterations are not related to the carcinogenic process in an obligatory manner, it is also obvious that others must be crucial in the development of the malignant cell.

A large body of experimental evidence has suggested that the crucial effect of chemical carcinogens is to alter DNA, therefore acting at the genetic level. The results of a combined carcinogen regimen could be construed as supporting this proposition. However, the consistency of surface alterations of malignant cells supports the concept that the final pathway of malignant behavior is in altered phenotypic expression.

Recent technology has begun to make possible the detailed analysis of the composition of mammalian chromatin, and with these developments, it may be possible to identify those proteins that regulate gene expression (7). The potential for describing crucial epigenetic alterations during carcinogenesis is increased. Our laboratory has utilized 2 approaches to increase the value of data obtained from analysis of the hepatocyte’s chromatin during exposure to 2-FAA. First, from past experience we are able to identify and isolate the putative premalignant lesion, the neoplastic nodule, and to compare its composition with that of background liver. Furthermore, by a method recently developed in our laboratory, we have accomplished the purest separation possible to date of eu- and heterochromatin from liver. By this approach, we are better able to define the alterations of chromatin into those that are involved in template activity, that is, transcription by active chromatin, and those that may be related to the repression of genetic expression. We can then analyze the NHP composition of each. At this preliminary juncture, it is only possible to report these results briefly and in tabular form.

As evidenced in our laboratory, whole-liver chromatin from normal rats demonstrates 24 NHP bands. Euchromatin demonstrates 8 NHP bands that range in molecular weight from 17,000 to 100,000 and that are present in the whole chromatin but not in heterochromatin. Heterochromatin demonstrated bands with molecular weights of 97,000, 185,000, and 200,000 that were not present in euchromatin. When these techniques were applied to neoplastic nodules, heterochromatin was found to be depleted of NHP at all molecular weights and no unique bands were found. Euchromatin from these nodules demonstrated a striking number of NHP bands at high molecular weight (many greater than 100,000), many of which were not present in normal liver.

To determine the relationship of these alterations to the carcinogenic process, we have begun to analyze the chromatin of THC of varying growth rates. Uniformly, in tumors induced by 2-FAA, a very large number of unique NHP bands were found in both eu- and heterochromatin. Many of these “new” bands were of molecular weights that approached the limits of resolution of the gels (300,000). Perhaps of equal interest, almost every tumor studied thus far has a pattern of NHP that appears to be unique; a fingerprint not unlike the finding with lectin agglutination.

**Conclusion**

It is clear that the complexity of the process of chemical carcinogenesis requires examination by a variety of techniques. The probability that many of the effects of these agents are extraneous to the carcinogenic process, as well as the ornate sequential patterns that are evident, make it difficult to focus upon a single class of alteration.

Although there is a high probability that genetic damage is a root cause of cancer, it remains unclear whether this is manifest by new phenotypic capabilities or by creating an imbalance of the normal patterns. Several conclusions can be drawn from the results of the experiments described above. It is obvious that we cannot equate the alterations induced by subcarcinogenic doses of 2-FAA with a response to injury. Whether at the genetic level or elsewhere in the cell metabolic processing, the dose of 2-FAA used sensitized the cell to the effect of DMN. Clearly, this effect is not mediated through the device of cell division before or after the fact. Seventy % hepatocytoma, which evokes a far greater mitotic response, did not result in PHC despite DMN administration at the peak of DNA synthesis.

The nature and significance of alterations in NHP during chemical carcinogenesis remain to be elucidated. At present all of those attempting such experiments are in the first stages of analysis of these complex proteins. However, their crucial role in normal genetic modulation and alterations such as those described above certainly suggest that further evaluation is warranted.

As is so often the case in tumor biology, the lectin agglutination of malignant cells is not uniform. Although all of the PHC tested thus far are agglutinated with Con A, several THC are not. The failure to agglutinate was evident with slow growth rates. Despite this finding, it remains of interest that no neoplastic nodule has demonstrated lectin agglutinability. This strongly suggests that in many (all?) instances, surface alterations occur at the moment when true malignant cells appear and raises the possibility that this alteration may be the best marker yet described to distinguish the onset of true cancer.

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References


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