Histochemical Profile of Mouse Hepatocellular Adenomas and Carcinomas Induced by a Single Dose of Diethylnitrosamine

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ABSTRACT

In continuation of earlier studies on murine neoplastic liver lesions, we characterized by histochemical methods the phenotype of hepatocellular adenomas and carcinomas induced by single injections of diethylnitrosamine (1.25, 2.5, or 5.0 μg/g of body weight) in 15-day-old C57BL/6 x male C3H F1 mice.

The hepatocellular adenomas were composed predominantly of basophilic cells but stored excessive amounts of fat and glycogen in large portions of the tumors. Irrespective of the carcinogenic dose, the adenomas showed a consistent histochemical pattern. Glycogen synthase and phosphorylase were highly active in the hepatocytes that stored glycogen. In cells poor in, or free of, this polysaccharide, these enzymes were only moderately active or even inactive. In glycogen-storing portions of the adenomas, the activity of adenylate cyclase was reduced compared with male C3H FI mice.

In contrast, adenylate cyclase, glucose-6-phosphatase, glyceraldehyde-3-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase showed an increased activity in all adenomas compared with preneoplastic foci, which in turn exhibited a higher glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase activity than the surrounding parenchyma or the liver of untreated controls.

The hepatocellular carcinomas showed remarkable histochemical changes compared with adenomas. The levels of fat and glycogen and the activities of glycogen synthase, phosphorylase, and in most cases also that of glucose-6-phosphate dehydrogenase, were reduced significantly. In contrast, adenylate cyclase, glucose-6-phosphatase, glyceraldehyde-3-phosphate dehydrogenase, and also alkaline phosphatase showed a striking elevation in developing carcinomas. Similar, although more pronounced, histochemical changes were seen in the advanced hepatocellular carcinomas. These observations indicated that progression from adenomas to hepatocellular carcinomas was associated with a change in the activity of several enzymes involved in cell membrane function, glycogen metabolism, the oxidative pentose phosphate pathway, and glycolysis.

INTRODUCTION

Several studies (1, 2) showed that low, single doses of DEN administered to infant mice resulted in a sequential emergence of preneoplastic hepatic foci, hepatocellular adenomas, and hepatocellular carcinomas. The frequency of developing lesions, as well as the time of their emergence, were dose-dependent. The preneoplastic foci increased progressively until their number reached the maximal response to a given dose. Subsequently, their number decreased concurrently with the emergence of the hepatocellular adenomas. The hepatocellular carcinomas began to develop either within existing adenomas or within the hepatocellular parenchyma. Hepatocellular carcinomas grew rapidly, involved surrounding tissue, and metastasized to lungs (1). The temporal relationship between foci, adenomas, and carcinomas clearly pointed to time-dependent tumor progression, a phenomenon well established in several experimental systems and observed in human carcinogenesis.

In a previous paper (3), the early focal lesions induced in mice by a single dose of DEN were characterized histochemically. The main feature of these lesions was a decrease in G6Pase and an increase in both G6PDH and GAPDH, indicating a shift from the gluconeogenic situation predominating in normal liver to a glucose consumption by pentose phosphate pathway and glycolysis. The focal hepatic lesions also showed increased labeling indices as well as an increase in their diameters and volumes with time. Thus, it was concluded that the changes mentioned above acted in concert, manifesting the acquired functional and replicating potential of focal lesions. The present article reports on the histochemical characteristics of the subsequently emerging hepatocellular adenomas and carcinomas.

MATERIALS AND METHODS

Tumors were induced in 15-day-old male C57BL/6 x C3H F1 mice by single injections of DEN (1.25, 2.5, or 5.0 μg/g of body weight) as described earlier (4). The DEN-treated and nontreated control animals were killed 44 and 68 weeks following the administration of the carcinogen. The objective was to sample primarily hepatocellular adenomas and carcinomas for histochemical characterization.

Histology. Preneoplastic lesions in DEN-treated mouse livers were classified as intermediate cell foci as defined earlier (3). Neoplastic lesions were classified as hepatocellular adenomas and frank hepatocellular carcinomas (1). Hepatocellular carcinomas that were observed as emerging within the hepatocellular adenomas were referred to as early carcinomas. No such distinction was observed in the “late” carcinomas.

Histochemistry of Enzymes and Metabolic Products. Pretreatment of tissues and histochemical procedures used were identical to those in the preceding study (3). Briefly, pieces from shock frozen liver tissue of normal (one specimen) and carcinogen-treated (3 specimens, 1 for each dose) mice were frozen onto the same tissue holder, and serial sections of all 3 pieces (0.15 cm2 each) were cut simultaneously in a Cryostat (Jung, Nussloch, Federal Republic of Germany). The sections were mounted onto the same slide or membrane and incubated according to the respective histochemical reaction. With this technique, it was possible not only to produce sections of the same thickness but also to treat them simultaneously under identical conditions for the specific histochemical assays. The thickness of the sections was varied (6 to 14 μm) in accordance with the reaction intensity of the enzyme to be demonstrated. The activities of the following enzymes were demonstrated: SYN, PHO, G6Pase, G6PDH, ATPase, γ GT, and AC Pase. In addition, 2 other enzymes, ADC and ALKPase, were studied in preneoplastic and neoplastic lesions since they play a pivotal role in the regulation of cell metabolism (5) and transport processes (6, 7), respectively. Details of the histochemical assays for ADC are given by Mayer.
et al. (8) and those for ALKPase are given by Lojda et al. (9).

Furthermore, sections were stained for basophilia with toluidine blue and for the presence of neutral lipids with Fettrot B. Others were treated with either the periodic acid-Schiff reaction or Lugol's iodine to demonstrate glycogen.

Evaluation of Histochemical Reactions. The histochemical reactions were evaluated under a comparison microscope (Leitz, Wetzlar, Federal Republic of Germany), which allowed exact correlation of the various histochemical parameters with specified focal lesions in the serial sections. The intensity of the histochemical reactions was estimated semiquantitatively using 5 grades (no change, increase, strong increase, decrease, strong decrease) as compared with the reaction in the concurrently processed tissue of the untreated control animals.

RESULTS

At 44 weeks, a large number of hepatocellular adenomas were observed in addition to the intermediate cell foci described earlier. At 68 weeks, the samples studied contained both hepatocellular adenomas and carcinomas (Table 1).

The histological appearance and the histochemical pattern of the intermediate cell foci that were observed at 44 weeks did not differ from foci detected at earlier time points (3). The foci were active in SYN and PHO. In comparison with normal liver parenchyma, ADC was reduced, and ALKPase and γGT did not show a change. G6Pase and ACPase activities were decreased. However, G6PDH and GAPDH were increased in comparison with the surrounding liver tissue and the parenchyma of the untreated animals.

The histochemical patterns of the adenomas, early carcinomas, and the late carcinomas (fully developed carcinomas) are summarized in Table 2. For comparison, the histochemical patterns of intermediate cell foci are presented in Table 2.

### Table 1 Number of preneoplastic and neoplastic lesions studied histochemically at different stages of the experiment

<table>
<thead>
<tr>
<th>DEN (μg/g body wt)</th>
<th>Animals investigated</th>
<th>Intermediate cell foci</th>
<th>Adenomas</th>
<th>Early hepatocellular carcinomas</th>
<th>Late hepatocellular carcinomas</th>
<th>All hepatocellular carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wk 44</td>
<td>Wk 68</td>
<td>Wk 44</td>
<td>Wk 68</td>
<td>Wk 44</td>
<td>Wk 68</td>
</tr>
<tr>
<td>1.25</td>
<td>6</td>
<td>0</td>
<td>20</td>
<td>5</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>2.5</td>
<td>12</td>
<td>30</td>
<td>20</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>5.0</td>
<td>12</td>
<td>40</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Irrespective of dose</td>
<td>30</td>
<td>70</td>
<td>2</td>
<td>51</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Irrespective of time</td>
<td>72</td>
<td>91</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Column 2. Adenomas were predominantly composed of basophilic cells in addition to cells that stored excessive amounts of glycogen (Fig. 2a) and fat (Fig. 1b), respectively. The basophilic cells often formed a ring around the periphery of the adenomas. This cell population frequently showed an increase in the number of mitotic figures. SYN and PHO were highly active in the cells storing glycogen in excess but were only moderately active or even inactive in the basophilic cells poor in, or free of, this polysaccharide (Fig. 1c). The ADC was generally reduced in glycogen storage portions of adenomas (Fig. 1d) but regularly elevated in fat-storing areas. In some adenomas, ADC was uniformly elevated despite a conspicuous glycogen deposition. G6Pase (Fig. 1e), ATPase, and ACPase were increased in some but were decreased in other areas of adenomas. ALKPase was increased around the capillaries but showed a normal or decreased activity in the adenoma cells (Fig. 1f). The γGT could not be detected in any of the hepatocellular adenomas. The activity of G6PDH and GAPDH was strongly increased in all adenomas (Fig. 1, g and h), exceeding that observed in the intermediate cell foci. With the exception of decreased SYN and PHO, no significant differences in the histochemical patterns were found between the ring of basophilic cells and the rest of adenomas.

The appearance of hepatocellular carcinomas was accompanied by additional histochemical changes (Fig. 2, a–h): fat, glycogen (Fig. 2a), as well as the activities of SYN, PHO (Fig. 2b), ATPase, and in many cases also that of G6PDH (Fig. 2f), were strongly reduced in comparison with adenomas. In contrast, ADC (Fig. 2c), ACPase (Fig. 2e), and GAPDH (Fig. 2h) showed an increase in their activities. G6Pase (Fig. 2e) and ALKPase (Fig. 2g) were more elevated in the early carcinomas than in the surrounding adenomas. Similarly to the preceding lesions, γGT was lacking. The fully developed hepatocellular carcinomas showed essentially the same histochemical pattern as the early carcinomas, although most of the changes were more manifest (Fig. 3, a–h).

### DISCUSSION

Sequential histochemical changes occurring during hepatocarcinogenesis from preneoplastic lesions to the hepatocellular adenomas were studied extensively to date in rats (10, 11). In mice, however, histochemical studies were of more limited scope. The present study was directed to fill this void by investigating a large number of enzymes, particularly those of alternative pathways of carbohydrate metabolism.

The histochemical pattern of preneoplastic hepatic lesions induced in the infant mouse model by DEN has been described previously (3). In the present investigation, the spectrum of enzymes studied in the early lesions was complemented by demonstration of the activities of ADC and ALKPase, which are involved in signal transduction and transport processes of
Fig. 1. Serial sections through a hepatocellular adenoma. Remnant liver parenchyma is visible at the left margin of the micrographs. Bar, 100 μm. 

- a, glycogen: reduced deposition in large areas of the adenoma; b, neutral lipids: pronounced storage in some portions of the adenoma (arrows). Inset, higher magnification. Note intensely stained lipid droplets in contrast to nuclei of the tumor cells. 
- c, glycogen synthase: decreased activity in large areas of the adenoma. 
- d, adenylate cyclase: increased enzyme activity in the majority of adenoma cells. 
- e, glucose 6-phosphatase: activity is decreased in some but slightly increased in other parts of the adenoma. 
- f, alkaline phosphatase: weak activity in adenoma cells but strong in capillaries (arrows). Inset, higher magnification. Note, enzyme activity is confined to the cell membrane of adenoma cells. 
- g, glucose-6-phosphate dehydrogenase: strongly increased in adenoma. 
- h, glyceraldehyde-3-phosphate dehydrogenase: strongly increased in adenoma.
Fig. 2. Serial sections through an area showing transition from adenoma (upper, lower, and left margin) to carcinoma. Bar, 100 μm. a, glycogen: gradient leading from marked storage in the adenoma to almost complete reduction in the center of the carcinoma. b, glycogen phosphorylase: decrease activity in subpopulations of the adenoma and in all cells of the carcinoma. c, adenylate cyclase: strongly increased enzyme activity in carcinoma in contrast to the surrounding adenoma tissue. d, glucose-6-phosphatase: variable activity in adenoma but strong increase in the majority of carcinoma cells. e, acid phosphatase: activity of the enzyme is increased at the transition zone between adenoma and carcinoma and in the central part of the carcinoma (right upper corner). f, glucose-6-phosphate dehydrogenase: variable activity in adenoma but decreased in carcinoma. g, alkaline phosphatase: low activity in adenoma but strong increase in carcinoma. h, glyceraldehyde-3-phosphate dehydrogenase: increased activity in adenoma but even higher in carcinoma.
Fig. 3. Serial sections through a frank hepatocellular carcinoma. Bar, 100 μm. a, glycogen: strongly reduced in majority of tumor cells. b, glycogen phosphorylase: decrease of enzyme activity throughout the tumor. c, adenylate cyclase: activity is found in plasma membrane as well as in cytoplasm of carcinoma cells. d, glucose-6-phosphatase: all tumor cells show a strong increase in activity. e, acid phosphatase: strongly increased enzyme activity in all tumor cells. f, glucose-6-phosphate dehydrogenase: strongly increased activity in some tumor areas but decrease in most of the carcinomas cells. g, alkaline phosphatase: strong increase in activity throughout the carcinoma. h, glyceraldehyde-3-phosphate dehydrogenase: enzyme activity is strongly increased.
the plasma membrane. Whereas ADC activity was reduced, that of ALKPase remained unchanged in preneoplastic lesions.

The adenomas shared a number of features with the preneoplastic foci. Thus, in addition to the pronounced basophilia, which has been known for some time, the adenomas were particularly characterized by strongly increased activities of G6PDH as detected in a number of tumor types, including hepatocellular tumors of the rat, by biochemical (12) and histochemical methods (13). In contrast to early lesions, the murine adenomas usually stored fat and especially glycogen in excess in large tumor areas with SYN and PHO being active at the plasma membrane. Whereas ADC activity was reduced, that stimulated under normal conditions is dependent on the relative oxidative pathway, which provides precursors for ribonucleo-pentose phosphate pathway. There is, however, also the non-oxidative, which has been known for some time, the adenomas were merely reflects the proliferative activity of the respective tumor. Our studies were focused in the oxidative part of the pentose phosphate pathway. There is, however, also the non-oxidative pathway, which provides precursors for ribonucleotide synthesis and glycolysis without production of NADPH. Whether both parts are acting or either of them is preferentially stimulated under normal conditions is dependent on the relative requirements for NADPH and ribose-5-phosphate (14). Thus, in the fast-growing hepatocellular carcinomas with low G6PDH activity, most of the ribonucleotide precursors might be supplied by an elevated nonoxidative pentose phosphate pathway and glycolysis as indicated by the increased GAPDH activity observed in the present investigation. Another feature of developing carcinoma cells was the striking increase in ALKPase, an enzyme known to catalyze the hydrolysis of various phosphate esters, in liver, especially proteins phosphorylated at tyrosine residues (15). The elevation of ALKPase was correlated with an increased ADC. The ability of cyclic AMP to induce alkaline phosphatase has been described by Firestone and Heath in mouse L-cells (16). However, an isoenzyme shift of ALKPase leading to a protein of higher catalytic activity cannot be precluded (6, 7, 17).

The sequential cellular changes observed in the experimental model used have been shown to be consistent with earlier studies in the same infant mouse model (3), which is highly sensitive to the hepatocarcinogenic DEN (4). Following treatment of the adult mice with the same carcinogen (DEN), even after its repeated applications, the frequency of preneoplastic and neoplastic lesions was significantly lower (2). Tinctorial foci similar to those observed in the infant mice were also induced in rat livers by a single dose of aflatoxin B1 (18) and were referred to as “tigroid cell foci.” However, although both types of foci share similar morphology and lack of γGT, they are different with respect to other parameters. Thus, G6Pase is regularly reduced in mouse foci but frequently unchanged in rat tigroid cell foci. On the other hand, γ-GT-positive hepatocellular foci were encountered after feeding mice safrone (19). Glycogen storage foci being precursor lesions of hepatocellular carcinomas in the rat can also be found in mouse liver after administration of ethylnitrosourea (20), DEN, and dieldrin (21). Therefore, several factors such as species, strain, age at treatment, sex, experimental protocol, and the type of carcinogen may influence the kinetics of emerging lesions and some aspects of their phenotypic expression (22). Common characteristics, however, predominate across the lesions.

The present article characterized for the first time the sequential change in the histochemical pattern taking place from small preneoplastic hepatocellular foci to adenomas and carcinomas triggered by single carcinogenic treatment of infant mice. In many respects, the observed histochemical changes during hepatocarcinogenesis in the infant mouse model are comparable with those seen during hepatocarcinogenesis in the rat. Despite some conspicuous differences in the histochemical pattern that are not fully understood as yet, a shift from glycogen metabolism towards the pentose phosphate pathway and particularly to glycolysis seems to be a common denominator of hepatocarcinogenesis. These metabolic changes are apparently closely related to the neoplastic conversion of hepatocytes (10, 13).

ACKNOWLEDGMENTS

We thank Erika Filsinger for excellent histochemical work, Gabriele Becker and Erwin Zang for printing the photomicrographs, Dr. D. Mayer for critically reading the manuscript, and Brigitte Pétillon and Myrtle Tuzar for typing the manuscript.

REFERENCES


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