Transplantability and Biological Behavior of Mouse Liver Tumors Induced by Ethylnitrosourea

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SUMMARY

A study has been made of the transplantability, morphology, and biological behavior of hepatomas induced by ethylnitrosourea in C57BL X C3H F₁ newborn and adult mice. Eight tumors induced in newborn and five in adult mice were transplanted to isogeneic hosts, and their behavior has been followed for a period of 1 year.

Grafts from the neonatally induced hepatomas grew in 75% of the recipients and were serially transplanted for 22 generations. Transplantation time was reduced from the original 8.4 weeks to 2 weeks. These transplants grew expansively, invaded locally, and metastasized to regional and distant lymph nodes and the lungs, killing the recipients within 6 weeks.

Hepatomas that originated in mice treated with ethylnitrosourea as adults grew in 30% of the recipients, but growth was slower. The original transplantation time of 18.0 weeks has been reduced to 8.1 weeks during the observed five transplant generations. No metastases were observed thus far in this series.

INTRODUCTION

Questions have been raised as to the true nature of liver tumors that develop in mice either spontaneously or following the administration of various established or suspected carcinogens. These lesions have been variously described as ranging from nonspecific regenerative growths to malignant neoplasias. It is apparent that various investigators were looking at entirely different morphological entities and thus were drawing divergent conclusions.

In order to solve this problem, a number of investigators have studied the transplantation capabilities of mouse liver tumors. Andervont and Dunn (3—5) described the morphological and transplantation characteristics of primary hepatic tumors that either developed spontaneously or were induced by protracted exposure to o-aminooazotoluene, 3,4,4,6-dibenzcarbazole, or carbon tetrachloride. Twelve months frequently were required for a transplanted tumor to grow to 10 mm in diameter. In several instances, the basic morphology of tumors changed during serial transplantation.

The other investigators studied transplantability of spontaneous hepatomas (2, 7, 8, 10—13), observing an incidence of successful transplants that was significantly higher in male than in female recipients (1, 3, 6, 9). The morphology of spontaneous liver lesions in mice was correlated with their ability to grow upon transplantation and was used as a criterion for the assessment of frank neoplasias (14). Most of these lesions could not be transplanted.

The objective of our studies was to investigate the transplantability, biological behavior, and morphological characteristics of mouse liver tumors induced in newborn and adult mice by a single dose of ENU.

MATERIALS AND METHODS

Animals. All of the animals used in this study were C57BL X C3H F₁ mice that had been raised in our laboratory. They were fed Purina laboratory chow and had continuous access to water. Liver tumors were induced in male host mice, 1, 4, or 42 days old, by a single i.p. administration of 60 or 120 μg ENU per g body weight. Tumor-bearing animals were killed when 80 to 90 weeks old. The isogeneic recipients were from 4 to 6 weeks old at the time of transplantation.

Original Transplantation. The tumor-bearing animals were killed by ether, and the liver was removed and washed thoroughly in sterile 0.9% NaCl solution to remove most of the blood. After being washed, the organ, which had practically always been replaced by several large tumors, was cut perpendicularly, and the transplant area, containing tumor tissue of a constant 10 to 15 mm in diameter, was selected. At this tissue size, there was no way of assessing whether such lesions originated from single or several closely spaced primary nodules which became confluent. Neoplastic growth appeared to the naked eye as a grayish white, solid mass which contrasted with the characteristic appearance of the surrounding normal liver tissue. After the selected specimen had been removed and a representative sample was taken for histological processing, the main tumor tissue was freed of any necrotic elements and minced finely with scissors. The minced tissue was washed twice and then suspended in the sterile 0.9%
NaCl solution (1:4, v/v) within a glass homogenizer. The cells were then dispersed by means of a single stroke of a Teflon pestle which had a serrated tip and a “homogenizer chamber” clearance of 0.004 to 0.006 inch. The cell viability has been then assessed by the exclusion trypan blue method. Only the highly viable (85 to 95%) samples were used. One-tenth ml of the supernatant cell suspension thus obtained was injected s.c. into the lateral thoracic wall region of 5 male and 5 female recipients by means of a tuberculin syringe with a 20-gauge needle. The recipients were lightly anesthetized with ether prior to transplantation.

**Observations and Serial Transplantations.** The recipient mice were examined twice a week for the presence of growth. The time interval between transplantation and the appearance of a palpable nodule was taken as the measure of the latent period. When the tumors reached 10 to 15 mm, the animals were killed, and the tumor mass was removed and retransplanted. The time interval between transplantations was taken as the measure of generation time. This paper reports upon the morphology and behavior of primary and serial transplants within 52 weeks after transplantation of the original tumors.

**RESULTS**

Thirteen ENU-induced primary liver tumors were transplanted into 130 isogeneic male and female mice; 8 of these tumors (61%) thus far have shown growth. The analysis of data regarding the incidence of positive transplantations, the latent period, and the transplantation time suggested 2 trends related to the induction history of the primary hepatomas. The variation in the biological behavior of transplantable tumors was found to be independent of the age of the animals at the time of exposure to carcinogen. Thus, Series 1 pertains to tumors originating from mice exposed to carcinogen as newborns (1 or 4 days of age), while tumors of Series 2 came from the animals that had received ENU at 42 days of age (Table 1).

Tumors from newborn animals (Series 1) grew in 75% of the recipients, with a latent period of 4.1 and 5.8 weeks and the 1st transplantation time of 8.4 and 10.7 weeks for males and females, respectively. Upon subsequent transplantations, sex difference disappeared, transplantation time was shortened to 2 weeks, and tumors metastasized broadly, killing the hosts within 6 weeks. The cut sections showed that the tumors had a relatively soft consistency, were grayish white, and seemed to infiltrate the adjacent soft tissues. By the end of the 4th posttransplant week, the tumors were infiltrating the overlying skin and underlying tissues. By the end of the 2nd week, both the lungs and regional lymph nodes already showed gross metastases. To date, these tumors have been followed for 22 generations.

The primary tumors originating from adult mice (Series 2) showed a significantly lower incidence of growth (30%; p < 0.001) and a longer 1st transplantation time (18.0 weeks). Because of their slower growth, they could be followed for only 5 generations within 12 months. No metastases were observed thus far in this series.

Microscopically, the original liver cell tumors were composed of groups of cells that showed considerable variation in size and staining properties (Fig. 1). They were arranged in trabeculae, cords, or compact masses, displacing the surrounding normal liver tissue from which they were distinguished by the lack of normal liver organization (Figs. 2 and 3). The cytoplasm of most of the neoplastic cells exhibited slight basophilia, and the normal distribution of intracellular glycogen was greatly altered and, in places, completely depleted (Fig. 3). Groups of neoplastic cells showing cytoplasmic vacuolization and intracytoplasmic inclusion bodies were evident in many areas of the growing tumors (Fig. 4). One primary tumor consisted of 2 cellular types, namely, epithelial (hepatocellular) and mesenchymal (sarcomatous). This tumor developed upon transplantation to either or both morphological entities, apparently depending upon cell types transplanted. Tumors induced in the adult mice were well differentiated.

Transplanted hepatocellular tumors were composed of polygonal or rounded cells with distinct cellular borders and basophilic cytoplasm, and with nuclei that showed moderate variation in size and staining properties. Mitotic figures were frequent. The tumor cells tended to form elementary trabeculae or to grow diffusely (Figs. 5 and 6). In certain areas, the tumor cells tended to line up over dense connective

**Table 1**

<table>
<thead>
<tr>
<th>Series</th>
<th>No. of donors</th>
<th>Total</th>
<th>Positive</th>
<th>No. that received transplant</th>
<th>No. in which transplants grew</th>
<th>Av. latent period (wk)</th>
<th>First transplant generation (wk)</th>
<th>Later transplant generations (wk)</th>
</tr>
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<tbody>
<tr>
<td>M</td>
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<td></td>
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<td>1</td>
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<td>6</td>
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<td>40</td>
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<td>8.4 ± 0.8</td>
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<td>2</td>
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<td>25</td>
<td>25</td>
<td>8 (32)</td>
<td>10.7 ± 1.2</td>
<td>18.0 ± 1.1</td>
<td>43</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 (75)</td>
<td>8 (32)</td>
<td>19.3 ± 6.0</td>
<td>19.3 ± 6.0</td>
<td>8.1 ± 0.7</td>
</tr>
</tbody>
</table>

*The primary host received a single i.p. injection of ENU at 1 or 4 days of age (Series 1) or at 42 days of age (Series 2).
*Primary tumors were used for transplantation.
*Time from inoculation of the original tumor to the detection of positive growth.
*Time interval between transplantations.
*Numbers in parentheses, percentage of transplants that grew.
*Mean ± S.E.
tissue. By the 10th transplant generation, the neoplastic cells were arranged in large groups surrounded by delicate bundles of supporting connective tissue. Although a diffuse growth pattern prevailed, areas showing a distinct trabecular arrangement were evident. In certain parts of the growing tumors, groups of isolated cells were freely detached and flowing into adjacent cavities (Fig. 7). Intracytoplasmic glycogen, in the form of fine granular material, was found in some of the neoplastic cells either at the primary site of transplantation or in the metastases (Figs. 8 and 11). However, the majority of tumor cells showed no evidence of glycogen. The earliest metastatic foci were identified in the perivascular and peribronchial lymphatic vessels in the lungs and in the peripheral lymphocolpus and hilar regions of lymph nodes (Figs. 9 and 10). Isolated tumor cells and/or tumor masses were frequently found in lymphatic glands far from the original sites of transplantation (Fig. 12).

DISCUSSION

This study has shown that mouse hepatomas induced by a single dose of ENU grew in great numbers upon transplantation into isogeneic mice. The most significant observation is the difference in the incidence, latent period, and transplantation time for the tumors induced in newborn as opposed to adult mice. The short latent period of the 1st transplant generation was impressive. The local invasion, formation of distant metastases, and killing capability of these tumors represent new information about the biological characteristics of mouse hepatomas. Also, although the transplantable hepatocellular tumors showed a rapid growth rate and metastasized widely, their basic morphology did not change throughout the subsequent passages, which is at variance with the observations of Andervont and Dunn (4, 5) and in agreement with studies on spontaneous hepatomas (13).

The basic question at the inception of this study was directed at finding the true nature of ENU-induced mouse liver tumors by assessing the transplantability of morphologically similar lesions. Thus, although the overall histological picture of the primary tumor may be helpful, it is obvious that it is the biological behavior that may provide the answer to the posed question. The morphological appearance, transplantability, and widespread metastases of the transplants, which caused the death of all of the recipients, gave all the essential parameters characteristic of the malignant growth in the case of the neonatally induced hepatomas. Similar malignant characteristics have been observed in studies in progress in which transplants originated from liver tumors that were induced transplacentally.

REFERENCES

Figs. 1 to 4. Morphology of primary hepatomas.

Fig. 1. Primary hepatoma 34EC1. Large tumor cells with abundant, slightly eosinophilic cytoplasm, large nuclei, and prominent nucleoli. These cells are intermingled with less-differentiated tumor cells that show considerable basophilia of their cytoplasm and a morphological resemblance to large monocytes. H & E, × 250.

Fig. 2. Primary hepatoma 21EC1. Tumor cells are arranged in wide cords. Note the variation in staining properties of both cytoplasm and nuclei. H & E, × 250.

Fig. 3. Primary hepatoma 34EC1. Growing tumor nodule has displaced the normal liver cells, which appear compressed and slightly elongated. Marked depletion of intracellular glycogen of the tumor cells is evident throughout. Compare the difference between this and the strongly positive reaction of the surrounding normal liver tissue. Periodic acid-Schiff, × 250.

Fig. 4. Primary hepatoma 7EC7. Vacuolization of tumor cell cytoplasm and intracytoplasmic "waxy bodies" are prominent. H & E, × 250.

Figs. 5 to 8. Morphology of transplanted hepatomas.

Fig. 5. Fourth generation of hepatoma transplant 21EC1,4. Rounded tumor cells show trabecular arrangement. Note mitotic figures at the bottom. H & E, × 80.

Fig. 6. Sixth generation of hepatoma transplant 21EC1,6. Large polygonal cells, suggesting liver origin to form elementary trabeculae. H & E, × 250.

Fig. 7. Eighth generation of hepatoma transplant 34EC1,8. Enlarged tumor cells appear rounded and detached. H & E, × 250.

Fig. 8. Third generation of hepatoma transplant 34EC1,3. Polygonal or rounded cells with abundant, slightly basophilic cytoplasm and large nuclei. Note the distinct cellular borders and the presence of intracellular glycogen. Periodic acid-Schiff, × 250.

Figs. 9 to 12. Metastases of transplanted hepatomas.

Fig. 9. Third generation of hepatoma transplant 34EC1,3. Lung metastasis. Invasion of a perivascular lymphatic channel is shown in the lower left. H & E, × 32.

Fig. 10. Second generation of hepatoma transplant 25EC4,2. Metastasis to an axillary lymph node. H & E, × 80.

Fig. 11. Fourth generation of hepatoma transplant 21EC1,4. Hilal region of a lumbar lymph node invaded by tumor. Most of the tumor cells contain glycogen. Periodic acid-Schiff × 200.

Fig. 12. Fifth generation of hepatoma transplant 21EC1,5. Tumor cells filling a lymphatic vessel in psoas muscle. H & E, × 80.
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