Characterization of Primary Hepatocellular Carcinomas and Initial Transplant Generation

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SUMMARY

Transplantable rat hepatomas (THC) have been utilized in numerous experiments which were aimed at delineating crucial similarities or differences between normal and malignant tissues. These tumors demonstrate a broad diversity of relatively stable phenotypic patterns. The present study examined the relatedness of phenotypic pattern of several THC to the primary hepatomas from which they arose. Four modalities were examined: morphology, karyotype, plasma protein production, and \( \alpha \)-fetoprotein synthesis. These characteristics of THC bore a strong similarity to those of the primary hepatocellular carcinoma. However, the most striking result of transplantation was the presentation of a much more homogeneous phenotypic pattern in THC than was seen in the original primary hepatocellular carcinoma.

A second finding of this study was the inverse relationship between karyotype and function. Thus, aneuploid, rapidly growing tumors demonstrated intense production of normal plasma proteins and \( \alpha \)-fetoprotein while near-diploid tumors demonstrated little or none.

INTRODUCTION

THC\(^1\) have been used in numerous studies to delineate differences between normal and malignant cells. The availability of a number of tumors with varying growth rates and phenotypic patterns and the ease of obtaining large amounts of tissue have enhanced the popularity of these tumors as experimental materials (16, 22). In addition, the characteristics of these tumors are relatively stable over a number of transplant generations in most instances. However, we may question the relevance of data derived from studies of THC to the principles of basic tumor biology since the relationship of the characteristics of these tumors to those of primary malignancy is not understood. It is the difference between the primary malignant cells and normal cells that

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\(^2\) The abbreviations used are: TCH, transplantable hepatocellular carcinoma; PHC, primary hepatocellular carcinoma; FAA, N-2-fluorenylacetamide; \( \alpha \)IF, \( \alpha \)-fetoprotein.

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orcin staining technique, and the method for determining chromosomal composition have also been published (4).

Fragments of tumor tissue were placed in short-term culture and the resultant plasma protein production was studied by immunoelectrophoresis autoradiography utilizing rabbit anti-rat plasma antisera. A similar analysis performed upon a separate set of PHC has been reported along with the details of the methods (6). This method permits the identification of 15 normal plasma proteins synthesized \textit{in vivo} by approximately 75 mg of tissue.

It was possible to detect synthesis of \(\alpha1F\) as well. Immunoelectrophoresis-autoradiography was used as described previously (6) except that amniotic fluid was used as “carrier” and an antiserum to amniotic fluid was used to develop the immunoelectrophoretic patterns. The combination chosen showed 1 major precipitin line. The identity of this line with \(\alpha1F\) was confirmed using a monospecific antiserum.

The rats were exsanguinated via the inferior vena cava under light ether anesthesia. The blood was allowed to clot at 4° and then was centrifuged. The resultant serum was frozen at \(-20°\) until examined. The samples were coded and the concentration of \(\alpha1F\) was determined by radioimmunoassay (24). Their origins were not revealed until the results were recorded.

For \textit{in vivo} transfer tumor fragments were further minced with iris scissors in 0.9% NaCl solution at approximately 10°. Of the resultant cell supernatant, 0.02 ml (approximately \(10^6\) cells and small fragments) was injected s.c. at 2 sites via a 20-gauge needle. The resultant THCs were analyzed (as the primaries had been) at an average diameter of 3 \(\pm\) 1 cm. All procedures for the examination of these were identical to those for PHC.

To avoid confusion in karyotyping between tumor cells and the inflammatory cells of the host, the recipient rats were usually female. In the case of diploid tumors, tumors were carried in male rats as well. However, no functional or chromosomal differences were detected that were related to the sex of the host-rat.

RESULTS

PHC

General

Six tumors were used for this study (Table 1). All of the PHC examined were detected between Days 285 and 390 of the experiment (173 to 278 days after the cessation of the diet). Two tumors, 252 and 253, were detected by random laparotomy and were used approximately 90 days later. During this 90-day period, only slight growth was observed. No consistent relationship was noted between any of the characteristics measured in this study and the time of appearance of the PHC.

Karyotype

Three of the tumors were primarily aneuploid and 3 were predominantly diploid. Thus, Hepatomas 251, 241, and 243 demonstrated aneuploid patterns similar to those reported earlier with a broad spread of chromosome numbers per cell and occasional chromosome markers (4) but lacked the bimodal presentation. In the tumors reported here, even though the spread was considerable, dominant modes were noted most frequently in the subtetraploid area, and diploid cells were rare or absent.

Hepatomas 252, 253, and 242 were predominantly diploid tumors with small numbers of tetraploid cells. A minor number of cells in each, including PHC 253, were aneuploid. The presence of aneuploid cells in PHC 253 was interpreted as further evidence of its (entrance) into a truly malignant form despite its morphological similarity to hepatic nodules. A previous study of a large number of hepatic nodules failed to demonstrate a single aneuploid cell (4).

Morphology

Gross. All of the tumors except PHC 253 were rounded, penetrated through the substance of the lobe in which they originated, and protruded from its surface. Although several were adherent to or invaded neighboring structures and were invasive and destroyed adjacent liver parenchyma, none had metastasized. They were invariably yellow-white with central areas of hemorrhage and necrosis.

The exception, PHC 253, measured 2.5 cm in diameter; it was similar to liver in appearance and protruded from the surface of the left lobe. No necrosis could be detected grossly or microscopically. It resembled in many respects hepatic nodules (8, 23, 26).

Light Microscopy. Three basic histological patterns were seen in these tumors. Tumors 251, 241, and 243 were poorly differentiated hepatomas identical to those previously reported (4). They were composed of sheets or nodular masses of large, anaplastic cells with large nuclei of variable size and irregular shape and with poor cell margins (Fig. 2).

Tumors 252 and 242 could be considered, respectively, very-well-differentiated and well-differentiated tumors. They displayed cords of cells with intervening vascular spaces that appeared to be lined by endothelial or flattened cell elements. Most of the nuclei were regular and resembled those of normal hepatocytes. Areas of Tumor 242 contained more solid accumulations of similar cells (Fig. 1) (9, 11, 15, 21).

Tumor 253 appeared similar in many respects to previously described hepatic nodules (9). The cells were large and eosinophilic with some slight nuclear variability. However, in at least 1 area there was invasion of the adjacent parenchyma, a finding not seen previously in our hepatic nodules (26). In addition, in widely separated areas within the tumor there were foci of intense mitotic activity. This level of cell division far exceeded that seen in our previous experience with numerous hepatic nodules (5). The combination of these 2 findings led us to believe that this tumor represented a hepatic nodule with early malignant activity. The karyotypic findings described above support this contention.
Electron Microscopy. The relationship between the light microscopic and electron microscopic appearance was much less clearly defined than we would have expected from previous reports (10). The poorly differentiated Tumors 251, 241, and 243 conformed to prior descriptions. They displayed a minor increase in smooth endoplasmic reticulum; absence of stainable glycogen; greatly reduced granular endoplasmic reticulum which was vesicular, tortuous, poorly granulated, and estimated at one-tenth that of normal hepatocytes; increased free ribosomes; and many mitochondrial abnormalities. Golgi complex was rarely identified. Bile canaliculi were not well developed (Fig. 3).

The majority of the cells of the histologically well-differentiated Tumors 252 and 242 were identical to those of the poorly differentiated tumors. Some cells demonstrated surfaces more nearly normal in structure, occasional glycogen aggregates, and rare stacks of granular endoplasmic reticulum; in the main, however, these cells were also poorly differentiated. Tumor 253 was composed of 2 populations of cells, some of which appeared identical to those of the other tumors and others that were similar to those described in hepatic nodules (13).

Protein Synthesis. Tumor 251 demonstrated synthesis of plasma proteins in quantitative excess of normal liver. In addition to the 5 species of protein seen in incubates of normal livers, the proteins designated βF-globulin and βE-globulin were also detected. Both Tumors 241 and 242 produced low levels of albumin and normal βG-globulin. All other tumors were totally inactive in in vitro plasma protein synthesis.

αIF. The availability of only a single determinant of circulating αIF in the presence of PHC makes it difficult to evaluate the significance of borderline elevations. The αIF levels associated with PHC 251 and 241 were clearly elevated while that of PHC 242 was not. The elevation of αIF associated with Tumors 243, 252, and 253 could not be evaluated as to its significance.

In vitro analysis for αIF production by PHC correlated with the results determined upon circulating levels. Strongly positive patterns were noted for PHC 251 and 241 with a weak reaction for PHC 243. All others were negative by this method.

THC

THC 251a, c

Two of the THC’s (Table 2) that resulted from planting PHC 251 were designated as a and c and, as early as the 2nd transplantation, were clearly distinguishable by karyotype and protein-synthetic activity. They have averaged from 3 to 4 weeks between transplantations.

Karyotype. In its earliest transplant generations, 251a had a modal chromosome number of approximately 105, and this mode predominated through the 10th generation. Tumor line 251c had a modal chromosome number of approximately 70 which decreased gradually during transplantation.

Morphology. Both lines have remained morphologically poorly differentiated. Each has assumed a distinctive pattern of growth that permits comparison. Tumor line 251a grows predominantly as small clusters or large “trabeculae” with a distinctive fibrovascular stroma and focal necrosis. Tumor line 251c grows as large masses of cells and without stromal or obvious vascular development. The nuclei of tumor line 251a were very large and irregular while those of tumor line 251c were smaller and tended towards regularity. These differences were sufficiently distinctive to be invariably correlated with karyotype (Fig. 4).

Plasma Proteins. Both lines demonstrated vigorous plasma protein synthesis throughout the 1st 10 generations. However, tumor line 251c persistently produced more total protein and a greater number of protein species than tumor line 251a or normal liver.

αIF. Tumor line 251c produced enormous serum levels of αIF throughout all 10 generations tested. After an initially high value for line 251a in the 2nd generation, the

### Table 1

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Time of appearance (days)</th>
<th>Histology</th>
<th>Karyotype</th>
<th>Plasma proteins</th>
<th>Circulating αIF (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spread*</td>
<td>% cells at mode</td>
<td></td>
</tr>
<tr>
<td>251</td>
<td>285</td>
<td>Sheets, cords, pleomorphic cells</td>
<td>39-146</td>
<td>70-80</td>
<td>56</td>
</tr>
<tr>
<td>241</td>
<td>304</td>
<td>Sheets, cords, pleomorphic cells</td>
<td>38-78</td>
<td>70-80</td>
<td>80</td>
</tr>
<tr>
<td>243</td>
<td>363</td>
<td>Sheets, cords, liver-like</td>
<td>50-90</td>
<td>80-90</td>
<td>50</td>
</tr>
<tr>
<td>252</td>
<td>355</td>
<td>Liver-like</td>
<td>37-80</td>
<td>42</td>
<td>72</td>
</tr>
<tr>
<td>253</td>
<td>355</td>
<td>Hepatic-nodule + invasion Liver-like, + solid</td>
<td>30-83</td>
<td>42</td>
<td>60</td>
</tr>
<tr>
<td>242</td>
<td>323</td>
<td>Liver-like, + solid</td>
<td>41-88</td>
<td>42</td>
<td>55</td>
</tr>
</tbody>
</table>

*Chromosome number in 25 to 50 metaphase cells; counted consecutively.
†Numerator, quantity, 0 to 4 +; denominator, no. of protein species; control livers, 2+/5.
‡In vitro determination of tumor synthesis.
§Control 0.016 ± 0.007.
serum level dropped to that of control rats. From the 4th through the 7th generations, line 251a demonstrated elevated circulating levels of α1F which were statistically significant, but relatively low when compared with line 251c. From the 8th generation through the 10th, the α1F progressively increased. Despite this increase, at the 10th generation, line 251c continued to exceed α1F levels of line 251a by at least 1 order of magnitude. α1F synthesis in vitro was present in all transplant generations of line 251c tested. Synthesis was not observed in generations 2 and 4 of line 251a but was observed again in generation 10.

THC 252 and 242

Transplantable hepatomas 252 and 242 (Table 3) have been transplanted 4 times. Their average growth period between transplantations is approximately 7 weeks, which is twice as long as that of the aneuploid tumors. At 8 months, a growing tumor has been identified in 2 recipients of THC 253; these are, however, too small for analysis as yet.

Karyotype. The karyotype of both lines has remained predominantly diploid. However, at the 4th generation, a strong tendency toward an additional aneuploid mode was detected in Hepatoma 242 while Hepatoma 252 remained more dominantly diploid. Despite this divergence in karyotype, both tumors remained similar in morphology and function.

Morphology. An increasing tendency towards growth in solid masses has been noted in both lines with resultant decreases in trabecular structure. Nuclear pleomorphism also increased, but the predominant cell remained relatively hepatocyte-like with a small regular nucleus. In line 242, numerous clusters of cells with giant, mononuclear nuclei were present from the 1st transplant generation. These were never noted to divide (Fig. 5).

Plasma Protein. These tumors continued to produce little or no plasma protein.

α1F. The production of α1F remained at a relatively low level from the 1st transplant generation. This was somewhat elevated when compared to normal rats, but statistical significance was not achieved. α1F synthesis was not detected in vitro in any generation of THC 252 or 242.

DISCUSSION

The findings of this study have added to our understanding of the nature of the PHC's that result from chemical

Table 2

<table>
<thead>
<tr>
<th>Characteristics of Aneuploid THC's</th>
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<tbody>
<tr>
<td><strong>Karyotype</strong></td>
</tr>
<tr>
<td>----------------------------------</td>
</tr>
<tr>
<td>Tumor</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>PHC 251</td>
</tr>
<tr>
<td>251-2a</td>
</tr>
<tr>
<td>251-3a</td>
</tr>
<tr>
<td>251-4a</td>
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<tr>
<td>251-10a</td>
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<tr>
<td>251-3c</td>
</tr>
<tr>
<td>251-4c</td>
</tr>
<tr>
<td>251-10c</td>
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</tbody>
</table>

* Chromosome number in 25 to 50 metaphase cells; counted consecutively.
* Numerator, quantity, 0 to 4+; denominator, no. of protein species; control livers, 2+/5.
* In vitro determination of tumor synthesis. Two tumors.
* Control 0.016 ± 0.007. Average of 2 to 4 bloods.
* Weeks from planting until harvesting.

Table 3

<table>
<thead>
<tr>
<th>Characteristics of &quot;diploid&quot; THC's</th>
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<tbody>
<tr>
<td><strong>Karyotype</strong></td>
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<tr>
<td>----------------------------------</td>
</tr>
<tr>
<td>Tumor</td>
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<tr>
<td>-------</td>
</tr>
<tr>
<td>PHC 242</td>
</tr>
<tr>
<td>242-2</td>
</tr>
<tr>
<td>242-3</td>
</tr>
<tr>
<td>242-4</td>
</tr>
</tbody>
</table>

* Chromosome number in 25 to 50 metaphase cells; counted consecutively.
* Numerator, quantity, 0 to 4+; denominator, no. of protein species. Control livers, 2+/5.
* In vitro determination of tumor synthesis. Two tumors.
* Control 0.016 ± 0.007. Average of 2 to 4 bloods.
* Weeks from planting until harvesting.
carcinogenesis and their progression to THC’s. It is clear that 2 THC’s, after repeated transplantations, bear a resem-
blandance to the PHC from which they arose but may vary in
-crucial characteristics. Their capacity for plasma prote-
-in and $\alpha$F synthesis as well as their degree of morpho-
-logical differentiation may remain similar or may gradually
-modify and their chromosome composition may demon-
-strate significant alteration. Diploid PHC’s have demon-
-strated a tendency to maintain this state through the limited
-number of transplants studied. Aneuploid tumors remained
-aneuploid, but line 251 and one other not reported have
given rise to 2 series of differing THC, which are charac-
-terized by strongly modal but widely differing aneuploid
-karyotypes. The histological pattern of each of these tumors
-has adapted consistent and recognizably differing pat-
-terns. However, they both remain undifferentiated. It is not
-within the scope of this paper to review the multitude of ex-
-plans that have been offered for chromosome drift in
-tumor lines (14, 17, 27). Either of the 2 major possibilities,
-that the deviating cells originated de novo or that they ori-
-ginated in the primary tumor, are equally likely from these
-results. It is possible that the tumor responded to pressures
-exerted by the host by selection of an optimal growth pat-
ttern and selected functional characteristics or even more
-likely that these lines represent overgrowth of one of several
-“wild types” of tumor cells (20). The progressive develop-
-ment of these THC’s will be of great interest in view of ap-
-parently continuous but less dramatic “molding” of tumor
type as is seen in the progressive reduction of modal chro-
-mosome number in line 251-10c.

A 2nd major result from these studies is the demonstra-
-tion of a remarkable dichotomy between karyotype and
-selected hepatocyte functions. Prior studies have suggested a
gross correlation between ploidy, histological apparance,
growth rate, and selected enzymatic parameters (19, 28-30).
However, if plasma protein synthesis is accepted as a highly
-characteristic function of normal hepatocytes, then the cor-
-relation between this activity and chromosomal pattern is
-the reverse of that expected. No diploid PHC or THC
-studied demonstrated significant plasma protein synthesis.
This included 253 which greatly resembled an hepatic nodule as well as those diploid THC’s that have drifted to a
-somewhat less diploid state. This functional failure is espe-
cially odd in view of the high degree of histological differ-
-entiation seen in these primary diploid tumors. However,
-the finding that they were very poorly structured at the
electron microscopic level and that their granular endo-
-plasmic reticulum was severely altered was in agreement
-with previously reported series (6, 12) and demonstrated the
-absence of a correlation between these methods of morpho-
-logical mensuration.

The histological and electron microscopic picture of the
-aneuploid lines demonstrated an almost total loss of differ-
-entiation. Their production of large amounts of plasma pro-
teins is even more difficult to understand both from karyo-
type-function and histological structure-function correlates.
Justification of this testing technique as a true measure of
-synthesis and secretion has been offered previously (6). In
-addition, production of $\alpha$F by these tumors in vitro cor-
-related well with serum levels and constituted further proof

that these tumors were functioning in vivo. We can suggest,
therefore, that these tumors also contribute plasma proteins
to the circulating pool in vivo.

Although an occasional diploid tumor was found to be
-producing minimal amounts of $\alpha$F, intense production was
demonstrated only in association with aneuploid lines. In
-view of the “contamination” of diploid tumors with num-
-bers of aneuploid cells, we plan to follow the production of
-this protein by such tumors if they eventually drift towards
-aneuploidy. Whether the production of $\alpha$F represents a
-recapitulation of fetal function as part of the malignant
-state or is a broader phenomenon is as yet unclear. The ab-
-sence of significant $\alpha$F production by the diploid tumors is
-in support of data presented elsewhere (25). These results
-suggest that $\alpha$F production is not an inherent quality of
-malignant transformation.

At present, we cannot explain either the functional sup-
pression of diploid tumors or the functional release of the
-aneuploid tumors. Previous investigators have suggested
-that the phenotypic differences between hepatomas may
-result from the “freezing” effect of carcinogen exposure
-upon the hepatocytes (18). Thus the functional state of the
tumor reflects that of the hepatocytes at some crucial point
during its exposure to carcinogens. Becker (3) has
-further suggested that carcinogens may induce certain
-functional alterations in the target cells and that the inter-
-relationships of these alterations or demands with the mi-
totic thrust of the altered cells might result in carcinogenic
-alteration.

It is also possible that the broad variation in tumor types
-seen in this series may result from the effects of carcinogen
-upon target cells of differing type. Thus, differences be-
tween tumor lines such as 252, 242-diploid, nonfunctional
-versus 251-aneuploid, functional would represent selec-
tion of hepatocytes of differing functional emphasis, e.g.,
-hepatocytes primarily devoted to plasma protein synthesis
-and cell division (pericentral hepatocytes) versus those
devoted to other functions (pericentral hepatocytes) (1, 2, 7).

ACKNOWLEDGMENTS

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Mitotic Activity during N-2-Fluorenylaceticamide Hepatocarcinogene-
Primary Hepatomas and Initial Transplants


Fig. 1. Histological section of PHC 242 demonstrating the striking similarity of this malignant tumor to normal liver. Other areas, which represented less than 25% of the tumor mass, demonstrated more solid arrangements of similar, hepatocyte-like cells. H & E, x 250.

Fig. 2. Histological section of PHC 251 demonstrating sheets of tumor cells. These cells have indistinct margins and variably sized nuclei and bone a minimal similarity to hepatocytes. Many areas of necrosis were noted and only in rare areas were trabecular structures noted. H & E, x 150.

Fig. 3. Electron photomicrograph of PHC 251, lead stained following glutaraldehyde and osmium fixation. Final magnification, x 20,000. This represents a cell typical of the aneuploid tumors. Little or no glycogen was visible. Mitochondria were variably reduced in number and usually rounded. There was a striking diminution of granular endoplasmic reticulum in all cells and Golgi complexes were rarely identified. Less than 5% of the cells of the well-differentiated PHC 242 demonstrated a more well-differentiated appearance.

Fig. 4. Histological sections of 251–10a and 10c stained by our modification of the Wilder silver stain to demonstrate nuclei and reticulum; in the 10th generation of the 2 lines derived from the PHC 251. x 250. In a, THC 251–10a demonstrated a distinctive stromal pattern with prominent thin-walled vessels. Mitotic activity was intense. The nuclei were large and irregular and often demonstrated a “folded” appearance. In b, THC 251–10c demonstrated very little stroma and poor vascularity. Mitotic activity was vigorous although less than that of 251–10a. The nuclei were smaller, rounded, or elliptical and quite regular.

Fig. 5. THC 242–4. From the 2nd generation through the 4th, there was a progressive increase in solid growth of this diploid tumor until less than 10% of the tumor demonstrated trabecular structure. Necrosis was minimal even in larger tumors when compared with THC 251. A consistent finding was the presence of clusters of huge polyploid cells, in a background of cells that still possessed normal-appearing nuclei. H & E, x 150.
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