The role of determined stem-cells in the cellular lineage of hepatocellular carcinoma

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ABSTRACT The concept that hepatocellular cancer (HCC) arises by dedifferentiation of mature hepatocytes is challenged by more recent interpretations indicating that HCC arises from arrested maturation of determined stem cells. Either hypothesis is supported by the cellular changes that occur in the rodent liver following different hepatocarcinogenic regimens. The formation of foci and nodules from altered hepatocytes supports dedifferentiation; the proliferation of small oval cells with the potential to differentiate into either biliary ducts or hepatocytes supports arrested maturation of determined stem cells. The stem cell model predicts that genotoxic chemicals induce in the determined stem cell mutations that may be expressed in its progeny. Promoting agents act by inducing cell proliferation and increasing the chances for the additional mutations needed for expression of the malignant phenotype in proliferating progeny of the stem cell. Events which increase hepatocyte proliferation, such as cirrhosis or hepatitis B infection, may allow the number of critical mutations to occur in the absence of exposure to an external carcinogen. Exposure to hepatocarcinogens, such as aflatoxin, or integration of virus DNA, such as in hepatitis B infection, may produce transforming mutations. However, these mutations are most likely not sufficient to cause cancer, but are potentiated by increased endogenous mutations that occur when proliferation is increased, leading to very high incidence of HCC in areas in which there is endemic hepatitis B and aflatoxin contamination of the diet.

KEY WORDS: stem cells, hepatocellular carcinoma, carcinogenesis, virus, hepatitis

Introduction

Although we have advanced considerably in our knowledge and understanding of cancer during the 70 years since the state of understanding of the origin of cancer pronounced by MacCallum, many fundamental questions about cancer remain unanswered. One of the most basic questions regarding cancer is: what cells are transformed to become cancer. There are two possibilities: tumors arise from dedifferentiation of mature cells, or from aberrant differentiation of immature stem cells. Both of these hypotheses are supported by experimental and clinical data, and the same data are used by different investigators to support the hypothesis of their choice. Because of the marked differences in different kinds of cancer, it is just as reasonable to conclude that all theories of cancer are correct, as it is to say, ala MacCallum, that all theories are wrong.

Embryonal theory of cancer

The stem cell or embryonal origin of cancer may have been the first generally accepted theory of the etiology of cancer. Recamier in 1829 proposed that tumors arose from proliferation of embryonal cells that had persisted until adulthood. According to Oberling (1944) this idea attained enthusiastic support through the nineteenth century. Today, the embryonal rest theory of cancer has been attributed to Cohnheim (1875), who proposed that cancers arose from displacement of embryonic cells. At some stage of embryonic life cells become isolated or fixed while they still possessed great energy. These cells would normally become differentiated in the...
**Fig. 1. Model of cell renewal and carcinogenesis.** The stem cell is depicted on the left with progressive differentiation to terminally differentiated cells at the right (post-mitotic). Expression of the malignant phenotype could occur at any stage of differentiation of mitotically active cells. Initiation may take place at the level of the stem cell, or tumors may appear to arise from more differentiated cells when there have occurred additional mutations that permit expression of the malignant phenotype. The top lineage would hold for rapid-turnover cell populations, such as the G.I. tract, skin and hematopoietic tissue. The second lineage represents tissues which normally turn over slowly, such as the liver. The question marks for the liver indicate that previously, the ability of adult liver cells to proliferate and replace destroyed liver cells led investigators to conclude that there were neither stem cells nor terminally differentiated cells in the liver. However, more recent studies indicate not only that normal liver cell turnover involves perilobal stem cells, but also that mature liver cells in the central zone are terminally differentiated. The bottom lineage depicts nerve cells of the adult, which are terminally differentiated, do not proliferate and do not give rise to tumors. (Modified from Pierce et al., 1978).

### Stem cell tumors

The stem cell concept is clearly provocative today, given the ruling dogma that cancer is caused by some viral or chemical mutagenic effect, usually interpreted as causing transformation of a differentiated cell. However, the similarity of histologic appearance and growth characteristics between embryonic tissues and adult, but because of their isolation they could manifest embryonic capacity for continued growth in the adult. Rippert (1911) expanded the embryonic rest theory to include the possibility that cells expressing embryonic potential for growth could arise in the adult. Rotter (1921) proposed that primitive sex cells might lodge anywhere outside the ultimate sex glands during development and serve as the origin of tumors. He also thought that the growth of epithelial tumors depended upon primary changes in the underlying connective tissue which allowed invasion and expansive growth of epithelium. With the identification of cell-free filtrates (Rous, 1910) and chemicals (Yamagiwa and Ichikawa, 1918) that could induce cancer, attention shifted away from the embryonic theory to the idea that viruses or chemicals induced dedifferentiation of cells to produce tumors.

### Irritation and infection

Two other 19th century theories on the origin of cancer postulated that irritation or infection (parasites) produced changes in adult tissues leading to increased proliferation and cancer. It was proposed that irritation led to selection of cells that were better fitted to multiply and eventually grow autonomously (see Oberling, 1944, p. 22). This would be consistent with either a stem cell or dedifferentiation hypothesis. Hansemann, who introduced the term anaplasia, as well as others (see MacCallum, p. 1120), argued that a change took place in cells, under stress, which allowed them to proliferate rather than function (dedifferentiation).

### TABLE 1

<table>
<thead>
<tr>
<th>Year</th>
<th>Event or Study</th>
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<tbody>
<tr>
<td>1944</td>
<td>Opie describes small cells in the liver after exposure of rats to butter yellow.</td>
</tr>
<tr>
<td>1955</td>
<td>Price et al. note similar cells after DAB exposure.</td>
</tr>
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<td>1956</td>
<td>Farber coins the term «oval cells» for the small cells.</td>
</tr>
<tr>
<td>1961</td>
<td>Popper concludes that oval cells arise from bile duct cells.</td>
</tr>
<tr>
<td>1964</td>
<td>Grisham and Porta conclude that oval cells may differentiate into bile duct cells or mesenchymal cells.</td>
</tr>
<tr>
<td>1964</td>
<td>Ruben, using autoradiography concludes that oval cells can differentiate into hepatocytes.</td>
</tr>
<tr>
<td>1977-78</td>
<td>Bannikov, Sell, Kuhlmann, et al. report AFP is not in foci or nodules.</td>
</tr>
<tr>
<td>1980</td>
<td>Sell and Leffert propose oval cells as precursor cells for hepatocellular carcinomas and culture oval cells in vitro.</td>
</tr>
<tr>
<td>1984</td>
<td>Sell and Salient identify proliferating perilobal cells early after carcinogen exposure with lacto ductal cell proliferation and suggest that this may be the stem cell for hepatocellular carcinoma.</td>
</tr>
<tr>
<td>1985</td>
<td>Yaswen et al. note increased expression of proto-oncogenes (c-myc and c-K-ras) in oval cells when compared to normal liver.</td>
</tr>
<tr>
<td>1985</td>
<td>Huxson and Allison use monoclonal antibodies to identify oval cells and immature liver cells.</td>
</tr>
<tr>
<td>1989</td>
<td>Dunford and Sell, using monoclonal antibodies, conclude that oval cells represent proliferation and differentiation of a liver stem cell and that foci and nodules are adaptive changes and not precursors to hepatocellular cancer.</td>
</tr>
<tr>
<td>1990</td>
<td>Sell proposes that liver stem cell may be either perilobal cell or transition duct cell.</td>
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</table>

**Abbreviations used in this paper:** AAF, N2-acetylaminofluorene; AFP, alphafetoprotein; ANIT, alpha-naphylisothiocyanate; CCI4, carbon tetrachloride; CD-AFF, choline deficient diet containing AAF; DAB, diaminobenzene; DDFM, 4,4'-diaminodiphenylmethane; DEN, diethylnitosamine; HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen.
I. DEDIFFERENTIATION OF HEPATOCYTE

Transplantable teratocarcinomas

G. Barry Pierce, using transplantable teratocarcinomas of mice, provided irrefutable evidence for the stem cell origin of this form of cancer (see Pierce et al., 1978). Transplantable teratocarcinomas were first produced by Roy Stevens (1970) by injection of the cells from the genital ridge of F1 mice into the testes of parental mice. The normal genital ridge cells grew in the testes and gave rise to solid teratocarcinomas that have now been passed for over 200 generations in inbred parental mice. Pierce and Wallace (1971) demonstrated that proliferating malignant transplantable teratocarcinoma cells would differentiate into benign cells located in well differentiated keratin pearls within the tumor. Later Brinster (1974), Papaionannou et al. (1975) and Mintz and Illmensee (1975) transplanted the tumor producing «core» cells of malignant teratocarcinomas into normal blastocysts and obtained chimeric mice in which the malignant precursor cells had been induced to form normal differentiated adult cells. Labeled embryonal carcinoma cells were shown by Pierce et al. (1987) to localize preferentially in the mural trophoderm of the blastocyst, but also in the primitive endoderm and, rarely, in the inner cell mass. The carcinoma cells differentiated into tissue cells in accordance with their localization. Thus, at least for this form of cancer, the malignant potential exists in the stem cell and may be controlled by environmental factors present in differentiating tissue.

II. STEM CELL MATURATION ARREST

Hepatocarcinogenesis

The experimental induction of liver cancer in rodents has been one of the most extensively studied examples of how cancers arise. In the remainder of this paper, the question of which cells give rise to chemically induced hepatocellular cancers will be addressed.

Dedifferentiation and chemical hepatocarcinogenesis

Classically chemical hepatocarcinogenesis in rodents has been interpreted as showing dedifferentiation of mature cells to form cancer. As an example of this approach, rats are exposed to chemicals that induce liver cancer (carcinogens). The hepatocellular carcinoma (HCC) appears a considerable time (months or years) after carcinogen exposure and is preceded by a series of pathologic changes in the liver termed «pre-neoplastic» (Farber, 1974, 1984). Perhaps the best example of this model is the cyclic feeding of N-2-acetylaminofluorene (AAF) to rats as described by Teebor and Becker (1971). After four 2 weeks on/1 week off cycles of feeding AAF (a total of 12 weeks), rats will develop one or sometimes two hepatocellular carcinomas 24 weeks later. After each AAF feeding the livers of the rats contain a progression of lesions from small foci of basophilic hepatocytes to larger eosinophilic foci to nodules of increasing size that distort the liver. If the fourth cycle is not fed, all of the changes are reversible. After the fourth cycle most of the nodules will still disappear («remold»), but HCC will develop 24 weeks later from what appear to be «persistent nodules» that do not regress (Teebor, 1975). The progressive histologic and enzymatic changes in the foci and nodules preceding the appearance of HCC led a number of investigators to conclude that foci represent «initiated» hepatocytes that may be «selected» by further treatment to express the malignant phenotype (Peraino et al., 1973; Pitot and Sirica, 1980; Bannasch, 1984; Schulthe-Hermann, 1985; Sarma et al., 1986). In fact, the measurement of development of foci has become an accepted standard assay for determining the carcinogenic effect of a chemical in the rodent liver model (Scherer et al., 1972; Pugh and Goldfarb, 1978; Pitot et al., 1980; Campbell et al., 1986; Goldsworthy et al., 1986; Columbano et al., 1990; Schulthe-Hermann et al., 1990).

In regard to the origin of liver cancer, the question is: does...
Initiation of the carcinogenic process take place at the level of a mature differentiated cell that dedifferentiates or at the level of the stem cell or its immediate progeny, which produce tumors by failing to differentiate to terminally differentiated cells (maturation arrest), a form of "blocked ontogeny" (Potter, 1978). In order to present the relationship between chemical carcinogenesis and the stem cell theory of lineage, the concepts of initiation and promotion and blocked ontogeny will be now be presented.

Initiation and promotion

The concepts of initiation and promotion were originally applied to the skin by Berenblum (1941) and Rous and Kidd (1941). In the rabbit model studied by Rous (Rous and Kidd, 1941; Friedewald and Rous, 1944), single applications of carcinogenic tar to the ear did not result in tumors unless the site of application was scured using a cork borer. The application of the carcinogen (tar) became known as initiation; the scarring as promotion. One of the key observations in this process was that the promoting agent could be applied many months or years after the initiating event and tumors would still appear (Van Durren et al., 1975; Pitot, 1981). This implies that the initiating event is not only inheritable, but also takes place in a cell that persists in the skin for the lifetime of the animal.

The concept of initiation and promotion was then extended to chemical hepatocarcinogenesis (Peraino, 1981). Initiators are considered to be "genotoxic" and induce mutation in the initiated cell; promoters are agents which stimulate proliferation of the initiated cells. Mutagenic chemicals such as diethylnitrosamine (DEN), AAF, and diaminoazobenzene (DAB) are believed to be metabolized to chemically reactive species that bind to DNA (Miller, 1978), form adducts and produce mutations. Initiated cells are selected for by events that cause liver cell proliferation: restitutive proliferation, such as replacement of liver after partial heptectomy, liver injury induced by CCl₄ or galactosamine toxicity; or mitogens, which do not produce cell death, such as phenobarbital, nafenopin, lead nitrate, etc. (Peraino et al., 1975; Choie and Richter, 1978; Pitot and Sirica, 1980; Schulte-Hermann, 1985; Goldsworthy et al., 1986; Columbano et al., 1990; Columbano et al., 1991).

Blocked ontogeny

The concept of blocked ontogeny, advanced by Potter (1978), is a postulate of the stem cell model of carcinogenesis and cell renewal as visualized by Pierce et al. (1978), see (Fig. 1). In this scheme the undifferentiated stem cell is represented at the left of the figure. During organogenesis stem cells differentiate to produce tissue stem cells. Stem cells which are committed to form a certain tissue are called "determined". The determined stem cells are the cells that are available to proliferate to form a given organ or cell lineage. The determined stem cells give rise to progeny that begin to accumulate in their cytoplasm the molecules of specialized cell types. During normal cell renewal the determined stem cell divides to produce two "son" cells. One son cell remains as a stem cell; the other son cell expresses a more differentiated state. These differentiating cells are capable of additional rounds of proliferation, eventually giving rise to terminally differentiated cells. Note that in Pierce's model the existence of a determined liver stem cell is highlighted by a question mark, and the presence of a terminally differentiated liver cell is also highlighted by a question mark. In this article the nature of the determined stem cell for the liver will be presented (the first ? in Fig. 1), as well as evidence that there are terminally differentiated liver cells (the second ? in Fig. 1).

Is there a liver stem cell?

The question "Is there a liver stem cell?" was posed in a recent review by the author (Sell, 1990). The concept of the liver stem cell and its role in chemical hepatocarcinogenesis has developed from a number of studies of the cellular lineage of chemically induced hepatocellular cancer (for reviews see Sell et al., 1980, 1987; Lombardi, 1982; Sell and Leffert, 1982; Sell and Dunsford, 1989; Fausto, 1990; Marceau, 1990). There are two possible interpretations of the cellular lineage of cancer during chemical hepatocarcinogenesis (Fig. 2). During the process of development of hepatocellular carcinomas (HCC) induced by chemicals, tumors may arise by dedifferentiation of adult hepatocytes or by aberrant differentiation of stem cells. On the one hand, the sequence of foci to nodules to cancer implies dedifferentiation of mature hepatocytes (Peraino et al., 1973; Farber, 1974; Pitot and Sirica, 1980; Bannasch, 1984; Farber, 1984; Schulte-Hermann, 1985; Sarma et al., 1986). On the other hand, the production of small bile duct-like cells, that arise at the portal zone, proliferate extensively, and migrate between the hepatic cords to the central zone, is more consistent with stimulation of a stem cell (Opie, 1944; Price et al., 1952; Grisham and Porta, 1964; Sell et al., 1981). These small bile duct-like cells are called oval cells (Farber, 1956; Grisham and Porta, 1964; see Table 1). Oval cells contain liver cell markers such as albumin and alphafetoprotein (AFP) (Onoe et al., 1973; Dempo et al., 1975; Tchipsyhesheva et al., 1977; Kuhlmann, 1978; Sell, 1978; Jalanko and Ruoslahi, 1979) and epitopes identified by monoclonal antibodies (Germain et al., 1985; Hixson and Allison, 1985; Dunsford and Sell, 1989; Dunsford et al., 1989) that are also found in the HCCs. It appears much later. Oval cells may differentiate into normal duct cells or hepatocytes (Grisham and Porta, 1964; Sell and Leffert, 1982; Everts et al., 1987; Germain, 1988a,b), or migrate into persistent nodules where they may give rise to HCC (Everts et al., 1987; Dunsford et al., 1989).

Carcinogen-induced oval cell proliferation

The effects of five carcinogenic regimens that show different kinetics of oval cell production and serum AFP elevations are compared in Table 2 and Fig. 3. AFP is a serum protein found in high levels in fetal serum that becomes re-elevated in adults following liver proliferation or development of HCC (Kroes et al., 1972, 1975;
Abelev, 1978; Sell and Becker, 1978; Sell et al., 1980). Serum AFP levels may be used to detect either liver or oval cell proliferation after carcinogen exposure, necrotic liver injury, mitogen administration or partial hepatectomy, as well as proliferation of hepatocellular cancers. In recombinant inbred mice, the serum AFP level may be used to predict which mice have developed neoplasia of the liver, either spontaneously (Dunsford et al., 1991a) or after carcinogen exposure (Dunsford et al., 1991b), and select animals for morphologic evaluation.

Our first study used the cyclic AAF feeding regimen of Teebor and Becker (Sell, 1978). Although it was anticipated that AFP would be found in foci and nodules during the early elevation of serum AFP that was found, it became clear that serum AFP became elevated before nodules appeared and that the cells containing AFP were oval cells and not foci or nodules (Fig. 4) (Sell, 1978). From these observations it was tentatively concluded that HCC might not arise from foci and nodules, but from oval cells (Sell et al., 1980).

A study of the early cellular events with diethylnitrosamine (DEN) gave a different insight regarding the cellular lineage of HCC (Dunsford et al. 1989). This model was chosen because there was little recognizable early oval cell change and no early elevation of serum AFP, a marker associated with early oval cell proliferation during carcinogen exposure (Sell and Becker, 1978). In this model, HCC appear to arise from microscopic foci of atypical hyperplasia, rather than from either oval cells or preneoplastic nodules seen in other models. However, when monoclonal antibodies were applied to identify different cell populations, the early appearance of oval cells was readily detected (OV6+) and morphologic evidence of transformation of oval cells to larger cells bearing the HCC phenotype (T6) was seen (Dunsford et al., 1989), suggesting that oval cells may give rise to the cells which form microcarcinomas.

Oval cells were also identified as the AFP-containing cells in the "Salt-Farber" model of inducing HCC. This regimen was designed to produce rapid development of foci, nodules and cancer (Sell et al., 1977). Cells are initiated by a non-necrogenic injection of DEN, followed by a two-week feeding of AAF, with a partial hepatectomy performed after one week of AAF feeding. The AAF is given to inhibit proliferation of the non-initiated hepatocytes that would be stimulated by the partial hepatectomy, thus allowing the growth stimulus of the partial hepatectomy to act on the DEN-initiated cells. However, this regimen not only induces foci and nodule formation, but also there is massive early oval cell proliferation. Later cells with oval cell phenotypes may be seen within persisting nodules. This is interpreted to indicate that even when an HCC arises within a nodule, it may have originated from the oval cell lineage (Dunsford et al., 1989).

The fourth regimen, feeding a choline-deficient diet containing AAF (CD-AAF), was chosen to identify the earliest proliferating cells after hepatocarcinogen exposure. This regimen induces HCC rapidly compared to other regimens and produces massive early oval cell proliferation associated with elevated serum AFP concentrations, similar to that seen in the Salt-Farber model (Shinozuka et al., 1979; Sell et al., 1981). Using autoradiography, it was found that the first cells to proliferate were located in the perportal zone, next to the bile ducts (Sell et al., 1981). After 3 days labeling of bile duct cells was seen, and after one week many bile duct cells were labeled. The proliferating cells expanded across the hepatic acinus from the portal triad to the central vein within one month. Differentiation into both bile duct cells and small hepatocyte cells was apparent after 2-3 weeks. Thus in these four different models of chemical hepatocarcinogenesis, selected because of different cellular changes preceding liver cancer, evidence for the development of cancers from oval cells in the liver was obtained (Sell and Dunsford, 1989).

The fifth hepatocarcinogen, namely WY-14,642, is a peroxisome proliferating agent that acts as a non-genotoxic mitogen (Reddy et al., 1980; Cattley et al., 1988) and induces short-term proliferation of mature hepatocytes. This hepatocyte proliferation, like that induced by phenobarbital (Smuckler et al., 1976a), is associated with a small elevation of AFP within a few days after administration (Reddy et al., 1979), and is followed by a prolonged time (over 1 year) in which there appears to be very little change in the liver. With prolonged administration, some enzyme-altered foci appear (Rao et al., 1984). These foci are different from those found after genotoxic carcinogens and promotion in that they do not contain increased carcinogen detoxifying enzymes (Rao et al., 1984, 1986b). Later AFP-negative hepatocellular cancers appear (Reddy et al., 1979).

Oval cell proliferation has not been clearly defined in this model and the cellular events may be different than in the genotoxic models.

**Where is the liver stem cell?**

If there is a liver stem cell, where is this cell in the normal liver and what is its relationship to oval cells? Three stem cell candidates are: the terminal duct cell (Popper et al., 1957; Grisham and Porta, 1958).

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**Table 1.**

<table>
<thead>
<tr>
<th>REGIMEN</th>
<th>CELLULAR CHANGES IN LIVER</th>
<th>SERUM AFP</th>
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<tbody>
<tr>
<td>CYCLIC AAF</td>
<td>OVAL CELLS-&gt;FOCI-&gt;NODULES-&gt;CANCER</td>
<td>[Graph]</td>
</tr>
<tr>
<td>DEN</td>
<td>OVAL CELLS-&gt;MICROCARCINOMAS-&gt;CANCER</td>
<td>[Graph]</td>
</tr>
<tr>
<td>CD-AAF</td>
<td>OVAL CELLS-&gt;FOCI-&gt;NODULES-&gt;CANCER</td>
<td>[Graph]</td>
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<td>SOLT-FARBER</td>
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<td>[Graph]</td>
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<tr>
<td>WY-14643</td>
<td>MITOTIC-&gt;NORMAL-&gt;FOCI-&gt;CANCER</td>
<td>[Graph]</td>
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</table>

**Fig. 3.** Cellular changes in the liver and kinetics of serum AFP elevations in rats exposed to different carcinogenic regimens (see text and Table 2). In the cyclic-AAF, CD-AAF and Solt-Farber models, early AFP elevation is associated with oval cell proliferation and late AFP elevation with tumor development. The cellular changes and serum AFP elevation seen with the CD-AAF and Solt-Farber models are very similar, except that the massive oval cell proliferation and serum AFP elevation is delayed in the Salt-Farber model until after discontinuation of the AAF feeding. DEN produces very little oval cell proliferation early, but oval cells and transition cells between oval cells and atypical hepatocytes can be seen using monoclonal antibodies OV6 and T6 (Dunsford et al., 1989). WY-14,643, a non-genotoxic peroxisome proliferator induces liver cell proliferation (mitogenesis) and a small associated AFP elevation, but subsequent elevation of AFP has not been reported.
cells. Which cell predominates may depend on the degree of stimulation. The greater the stimulus, the more likely it is to involve the less differentiated perportal cell.

Oval cells are most likely responsible for the change in the state of ploidy from largely tetraploid to diploid following carcinogen exposure. Isolation of the oval cells from livers of carcinogen-treated rats by density gradient centrifugation is possible because the oval cells are much smaller (and denser) than parenchymal hepatocytes of nodular cells (Sell and Leffert, 1982; see review by Sirica et al., 1990). Following hepatocarcinogen exposure diploid cells appear in a population of cells that was previously largely tetraploid (Styles et al., 1985). Since nodular cells are mostly diploid (Schwarze et al., 1984), it has been suggested that hepatocytes undergo a reduction in ploidy when stimulated to proliferate following carcinogen exposure. However, most of the diploid cells found are about half the size of hepatocytes (Schwarze et al., 1986), and the diploid cell population corresponds to those cells containing AFP (Scott et al., 1989). Thus, it appears that diploid oval cells give rise to diploid nodular cells that give rise to nondiploid tumors that are transplantable (Seater et al., 1989).

**Bile duct proliferation**

Agents that selectively stimulate bile duct proliferation do not induce oval cell proliferation and do not lead to hepatocellular carcinomas (Ruben, 1964). Induction of bile duct hyperplasia by non-hepatocarcinogens such as bile duct ligation, 4,4'-diaminodiphenylmethane (DDPM) or alpha-naphylisothiocyanate (ANIT), does not induce alphafetoprotein- or albumin-containing duct cells (Sell, 1981; Dunsford et al., 1985; Sirica et al., 1990). There is one published report that bile duct cells containing AFP were seen after ANIT-induced bile duct proliferation (Richards et al., 1982), but we were unable to repeat this and did not find either elevated serum AFP or AFP-containing cells after ANIT (Dunsford et al., 1985). Elmore and Sirica (1991) have demonstrated that ductular cells may be stimulated to proliferate and differentiate into cells expressing mucin (intestinal metaplasia) or cells resembling hepatocytes histologically, but not cells expressing hepatocyte proteins. This proliferation is most likely of bile duct-determined stem cells at a stage of differentiation past that of the common stem cell for hepatocytes and duct cells, perhaps the terminal duct cell. On the other hand, the oval cells that appear after carcinogen exposure share bile duct and hepatocyte phenotypes (Sell et al., 1981; Sell, 1983; Dunsford et al., 1985). There appear to be two distinct types of bile duct proliferation: one is non-malignant proliferation of ducts, the other proliferation of duct-like cells associated with hepatocarcinogen exposure (McLean and Rees, 1958). The difference may depend on the stage of differentiation of the proliferating cell. If the proliferation occurs at the level of the determined bile duct cells, it may be unable to dedifferentiate and thus form only bile ducts. If the stage of the proliferating cell is at the transition duct cell or oval cell, both ducts and hepatocytes could be formed.

**The liver stem cell and restorative proliferation**

One of the properties of determined stem cells is to restore amputated or damaged tissue. For example, stem cells for the limb of the amphibian are able to regenerate a normal limb after its amputation, but cannot form other organs (Stocum, 1984). The presence of a liver stem cell has been questioned in the past because it did not appear that a liver stem cell was required for

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**Fig. 4.** Morphologic changes and serum AFP levels during cyclic feeding of AAF. After four 2 weeks on/1 week off cycles of feeding AAF (a total of 12 weeks), rats will develop one or sometimes two hepatocellular carcinomas 24 weeks later. After each AAF feeding the livers of the rats contain a progression of lesions from small foci of basophilic hepatocytes to larger eosinophilic foci to nodules of increasing size that distort the liver. If the fourth cycle is not fed, all of the changes are reversible. However, after the fourth cycle most of the nodules will still disappear ("remold"), but HCC will develop 24 weeks later from what appear to be persistent nodules that do not regress (Teebor, 1975). The progressive histologic and enzymatic changes in the foci and nodules preceding the appearance of HCC led a number of investigators to conclude that foci represent initiated hepatocytes that may be selected by further treatment to express the malignant phenotype. However, there is also early proliferation of oval cells which differentiate into normal liver and bile duct cells, and migrate into nodules and transform into hepatocellular carcinomas. Elevations of serum AFP are associated closely with oval cell proliferation and tumor development, but not with nodule formation.
regeneration of the liver (the first? in Fig. 1). After partial hepatectomy or chemical injury (CCl4, galactosamine), the liver deficit is replaced by proliferating hepatocytes that are derived from adult hepatocytes (Higgins and Anderson, 1931; Steiner et al., 1966; Grisham, 1969; Bucher and Malt, 1971; Rabes et al., 1976). For example, following 2/3 partial hepatectomy of the rat, each hepatocyte in the remaining lobes divides once or twice within a 48 hour period, without involvement of a stem cell (Steiner et al., 1966; Fabrikant, 1968; Grisham, 1969; Rabes et al., 1976). In fact, AFP can be identified in large dividing hepatocytes after partial hepatectomy in the rat (Seil, 1980), and adjacent to zones of liver injury after CCl4 injury in the mouse (Abele, 1978). AFP elevations may also occur within a few hours of administration of phenobarbital (Smuckler et al., 1976a) before liver cell proliferation as well as both before and after liver cell proliferation following toxic injury to the liver (Engelhardt et al., 1976; Smuckler et al., 1976b). The temporal sequence of the appearance of AFP in the serum and cellular regeneration after toxic injury suggests that most AFP production is an expression of the altered phenotype seen during the "step-down" phase in liver regeneration (Smuckler et al., 1976b), but immunocytochemical localization indicates that liver cells preparing for mitosis may also produce AFP (Engelhardt et al., 1976).

However, with more severe liver injury a liver stem cell may be called upon to restore the liver. For example, after severe injury with CCl4, Engelhardt et al. (1984) found some small oval-like cells contained AFP, a marker for proliferation, and Petropoulos et al. (1985) reported finding mRNA for AFP in small non-parenchymal cells. After galactosamine injury, Tournier et al. (1988) and Lemire et al. (1991) found large amounts of AFP and mRNA for AFP in proliferating oval cells. Oval-like cells may be seen in human livers that have been removed after transplantation when the donor liver has suffered too much damage to restore function (Demetrus, J., personal communication).

**Stem cells in the developing liver**

Determined stem cells are also the precursors for normal adult cells in developing organs (Pierce et al., 1978). In the liver of the developing rat fetus, cells have been identified that have the characteristics of the oval cells seen in the adult (Seil, 1980; Hixson and Allison, 1985; Germain et al., 1988a; Shigiri et al., 1991). At days 12 to 14 of development there are bipotential precursor epithelial cells that are capable of differentiation into hepatocytes or into biliary epithelial cells (Germain et al., 1988a). The ability to induce pancreatic duct cells to differentiate into liver cells (Takahasi and Pour, 1981; Reddy et al., 1984; Scarpelli, 1985) and for liver cells to differentiate into acini of cells containing zymogen granules (Rao et al., 1966a) indicates that there is a common stem cell for liver, biliary ducts and pancreas. Thus, there appears to be a determined stem cell in the fetus that gives rise to pancreas and liver, and that is still present in the adult rodent.

**The role of the stem cell in normal liver cell turnover**

There is now evidence that the cells that make up the hepatic cords are replaced by proliferating cells that originate in the portal area and migrate to the central zone, where they terminally differentiate and are removed by apoptosis (Zajicek et al., 1985, 1988; Arbor et al., 1988, 1991). It is proposed that the hepatocytes are formed at the perportal tract rim, where determined stem cells interact with ductal and stromal elements. The assembled unit then "streams" across the three acinus zones until reaching the terminal hepatic vein where it is eliminated. The liver unit replaces itself in a manner similar to, but much slower than, the layered epithelium of the skin or bladder and the gastrointestinal tract (Fig. 6). In the skin, stem cells located in the basal layer of the skin divide to produce daughter cells, one of which differentiates and migrates to the next layer and eventually terminally differentiates into a non-nucleated squamous cell (Potten, 1979; Wright and Allison, 1984; Marceau, 1990). A steady number of gastrointestinal lining cells is maintained by a balance of cells proliferating in the lower levels of the epithelium to those differentiating in the mid-level of the epithelium and exfoliated at the surface (Potten and Loeffler, 1990; Eastwood, 1991). A crypt of the small intestine contains 4-16 actual stem cells in steady state, but up to 30-40 potential stem cells that may be activated to divide following perturbations that stimulate proliferation. In man approximately 1011 epithelial cells are shed every day in the small intestine (Potten and Loeffler, 1990). It is estimated that the skin replaces itself every 15-30 days or at the rate of half a million cells per 30 seconds (Joan London, Television commercial), whereas the liver may take over a year (Steiner et al., 1966). The number of potential stem cells in the liver that may be activated has not been estimated; oval cells most likely represent progeny of activated stem cells.
Cell proliferation (turnover) and cancer incidence

The incidence of cancer in different organs is closely related to the rate of cell turnover (Cairns, 1978). The incidence of cancer of the skin and gastrointestinal tract is much higher than that of the liver in the western world. However, in areas of the world where hepatitis B is endemic and infected individuals have a marked increased turnover of liver cells related to liver damage, HCC is the most common cancer (Szmuness, 1978; Beasley, 1982).

Chemical hepatocarcinogenesis, proliferation and liver stem cells

Cohen and Ellwein (1991) have recently emphasized the role of cell proliferation in the induction of cancer by chemicals. In considering the principle that two or more mutations must take place before the malignant phenotype is expressed (Knudson, 1971; Vogelstein et al., 1988), the role of chemicals in inducing HCC may be either to induce a mutation (genotoxic) or to stimulate proliferation (non-genotoxic), allowing internal or spontaneous mutations to take place. In this explanation initiators act to induce alterations in DNA and promoters stimulate proliferation of the initiated cells, increasing the likelihood for additional mutations to take place (Cohen and Ellwein, 1991). Both promoters and so-called endogenous carcinogens (Pitot, 1991) stimulate cell proliferation and decrease the time that it takes to produce cancers. Endogenous carcinogens are essentially hormones that stimulate cell proliferation.

The role of liver cell injury, death and regeneration in hepatocarcinogenesis, without genotoxic chemical exposure, has now been demonstrated in three models of hepatocarcinogenesis (Lombardi et al., 1991). These include choline deficient diet (Copeland and Salmon, 1946; Salmon and Copeland, 1954), the LEC mutant of the Long Evans rat strain (Masuda et al., 1988) and transgenic hepatitis B mice (Chisari et al., 1989, see below). In these animals there is no known genotoxic event; liver cancer arises in a background of increased liver cell proliferation secondary to liver cell death.

The critical questions can now be addressed: On what liver cells do initiators act, stem cells or differentiated hepatocytes, and which cells eventually produce cancers? There are three points that need to be addressed: 1) metabolic activation of carcinogens, 2) the last effect of initiation, and 3) what is promotion?

Metabolic activation of carcinogens

Since most genotoxic chemicals need to be activated to metabolites that bind to DNA (Miller, 1978), one could conclude that genotoxic chemicals must act on differentiated hepatocytes that contain the metabolic enzymes (e.g. the p450 system) needed to activate the carcinogen. However, it is possible that liver stem cells also are able to metabolize carcinogens, or that such cells may take up metabolites from hepatocytes, particularly if the dose of the carcinogen results in cell death. Transitional duct cells have close cellular contacts with hepatocytes, which might allow transfer of metabolites to these cells (Kelly et al., 1984). Thus, metabolism of carcinogens by differentiated hepatocytes does not rule out other cells as potential precursors of HCC.

Lasting effect of initiation

One of the principles of initiation is that once a genotoxic event occurs, it will persist essentially for the lifetime of the animal (see above). In skin carcinogenesis, it must be the stem cell that is mutated. Mutations that occur in cells that have begun the differentiation process will not develop into cancer since differentiated cells are committed to terminal differentiation and eventual death (Potten and Loeffler, 1990; Cohen and Ellwein, 1991). This must be true for skin and gastrointestinal and other epithelial cancers as the rapid turnover of these cells would remove all cells that have started the differentiation process within 2-5 days for gastrointestinal epithelium, 15 to 30 days for skin and 50 days for pancreas (Jungeira et al., 1983). Although the cellular turnover in the liver is much slower (up to 1 year), it is likely that the same principle holds for liver cancer. It has been reported that the alterations induced by chemical hepatocarcinogens may also persist for long periods of time (Perlino et al., 1977; Solt and Farber, 1977). For example, in unpublished experiments of Becker (Becker, F. personal communication), administration of phenobarbitale as late as one year after injection of a non-necrotic dose of DEN (50 mg/kg), which by itself does not cause tumors, gave rise to primary HCC within six months. However, because of the long cellular turnover time in the liver, it is possible that a partially differentiated cell with the potential to proliferate might give rise to cancer following chemical initiation, thus fulfilling the maturation arrest postulate of the differentiation theory of cancer (Pierce et al., 1978, see Fig. 1).
Fig. 7. Postulated levels of expression of carcinogenic events during hepatocarcinogenesis. The stem cell model of hepatocarcinogenesis postulates that carcinogenic events occur in proliferating cells at some stage during differentiation resulting in expression of the malignant phenotype (blocked ontogeny). Since carcinogenesis most likely results from the accumulation of more than one mutation, it is likely that the first mutation (initiation) takes place at the level of the stem cell and that later mutations occur at the level of the transition duct cells or in aberrantly differentiating cells (atypical hyperplasia or cholangiofibrosis). Hepatoblastoma may represent tumors that arise because of multiple mutations at the stem cell level. Tumors with combined features of hepatocytes and bile ducts (hepatoblastoma, very rare) may arise from multiple mutations at a later stage of differentiation. Garden variety hepatocellular carcinomas arise from a later stage of arrested differentiation.

The hypothesis has been raised that the cells that ultimately give rise to cancer of the liver may be present at birth in the rodent liver as endogenous or spontaneously initiated cells (Moore et al., 1987; Lee et al., 1989). The idea that initiated cells may exist at birth is another way of stating the embryonal theory of cancer and appears to be an attempt to extend the concept of chemically mediated genotoxicity (initiation) to explain a biological phenomenon for which the concept of initiation does not apply.

What is promotion?

The evaluation of promotion has often used morphologic events that may not be directly related to the cellular lineages preceding cancer. In order to shorten the time period used to evaluate carcinogens, many laboratories have used the development of foci as an indication of initiation (Scherer et al., 1972; Pugh and Goldfarb, 1978; Goldsworthy et al., 1986; Schulte-Hermann et al., 1990). Columbano et al. (1990, 1991) have demonstrated that only compensatory proliferation such as that induced by CCl\textsubscript{4} necrosis or partial hepatectomy is able to produce increased foci after non-necrotic doses of initiators such as DEN, N-methyl-N-nitrosourea or benzo[a]pyrene; whereas direct hyperplasia induced by liver mitogens, such as lead nitrate or nafenopin does not induce foci.

TABLE 3

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Promoter</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSX</td>
<td>HBV PrS</td>
<td>No Nec. or Foci, Hepatoma</td>
<td>Dragani et al., 1989</td>
</tr>
<tr>
<td>HBx</td>
<td>HBx Trans. Enhanc.</td>
<td>Foci, No Nec., Hepatoma</td>
<td>Dragani et al., 1989</td>
</tr>
<tr>
<td>HBx</td>
<td>α-1-antitrypsin</td>
<td>Focal Necrosis, No Hepatoma</td>
<td>Kim et al., 1991</td>
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Hepatitis B and hepatocellular carcinoma

The association of a high incidence of HCC with hepatitis B infection is most likely related to the increased turnover of liver cells secondary to destruction of liver cells, leading to stimulated proliferation of stem cells and other cells early in the liver acinus differentiation proliferon (Mondelli et al., 1984). Similarly, increased restitutive proliferation in cirrhosis is also associated with increased incidence of HCC (Kew and Popper, 1984; Callea et al., 1991). This increased liver cell turnover increases the likelihood of "endogenous" carcinogenesis because of the increased chance for mutations (Cohen and Elwein, 1991). In transgenic mice expressing hepatitis B viral proteins, liver cancer has developed both with and without preceding liver cell damage (Chisari et al., 1986, 1989; Dragani et al., 1989; Dunsford et al., 1990; Lee et al., 1990; Kim et al., 1991; see Table 3). In addition, in transgenic mice that express hepatitis B surface antigen (HBsAg) and show progressive liver cell proliferation preceding cancer, exposure to chemical
carcinogens, such as DEN or aflatoxin, results in HCC at a younger age (Sell et al., 1991). Thus, chemical hepatocarcinogens may provide one mutational event necessary, but not sufficient, to cause malignant transformation. The increased proliferation of liver cells induced by cell injury or mitogens (promoters) allows accumulation of the other mutations required for expression of the malignant phenotype.

The stem cell model for liver carcinogenesis

The role of proliferating and differentiating liver determined stem cells in hepatocarcinogenesis and cholangiocarcinogenesis is depicted in Fig. 7. This hypothesis postulates that all liver tumors arise from transformation events that accumulate during the differentiation of hepatocytes or cholangiocytes from a common stem cell. The type of cancer seen will depend on the stage of maturation at which arrest of differentiation is manifested in the cancer cells.

References


Stem cells in hepatocellular carcinoma

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