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*

«Most of the modern theories (of the aetiology of tumors, sic) are merely fantastic inventions of a philosophic character, proposed by men who do not seem to have lived with tumors, nor even to have been particularly familiar with them. They always introduce many new terms and a new classification of tumors, with specially constructed Greek names, and the whole merely adds to the confusion and reminds one of the scholarship of the middle ages. We are quite ignorant of the real cause of tumor growth, and therefore all theories may be wrong» W.C. MacCallum, *A Textbook of Pathology.* WB Saunders Co., Philadelphia, 1924, p. 1115.

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 became isolated or fixed while they still possessed great energy. These cells would normally become differentiated in the
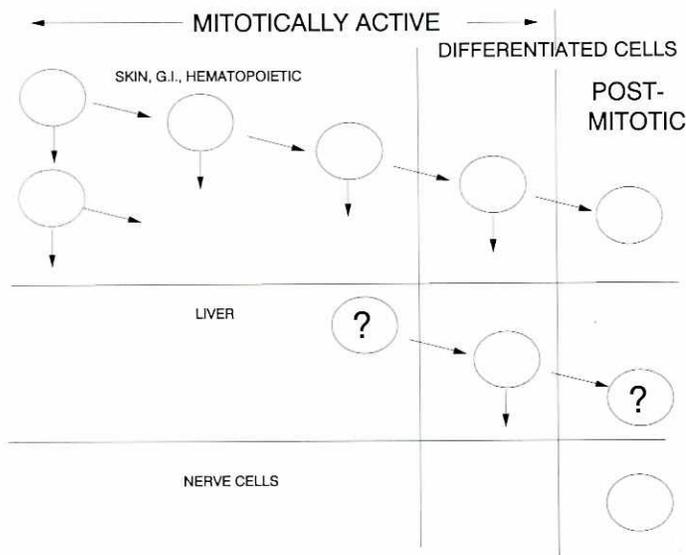
Fig. 1. Model of cell renewal and carcinogenesis. *The stem cell is depicted on the left with progressive differentiation to terminally differentiated cells at the right (post-mitotic). Expression of the malignant phenotype could occur at any stage of differentiation of mitotically active cells. Initiation may take place at the level of the stem cell, but 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 periportal 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).*

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 chemi-

Abbreviations used in this paper: AAF, N-2-acetlyaminofluorene; AFP, alphafetoprotein; ANIT, alpha-naphylisothiocyanate; CCl$_4$, carbon tetrachloride; CD-AAF, choline deficient diet containing AAF; DAB, diaminoazobenzene; DDPM, 4,4'-diaminodiphenylmethane; DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen.

cals (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)*.

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

TABLE 1

A SHORT SELF-SERVING HISTORY OF OVAL CELLS AND ALPHAFETOPROTEIN (AFP)

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1944	Opie describes small cells in the liver after exposure of rats to butter yellow.
1954	Price *et al.* note similar cells after DAB exposure.
1956	Farber coins the term «oval cells» for the small cells.
1961	Popper concludes that oval cells arise from bile duct cells.
1964	Grisham and Porta conclude that oval cells may differentiate into bile duct cells or mesenchymal cells.
1964	Ruben, using autoradiography concludes that oval cells can differentiate into hepatocytes.
1975-76	Dempo, Onoe, Fujita, Onda, *et al.* localize alphafetoprotein to oval cells early during hepatocarcinogenesis.
1977-78	Bannikov, Sell, Kuhlmann, *et al.* report AFP is not in foci or nodules.
1978	Shinozuka, *et al.* report massive proliferation of oval cells in rats fed carcinogens in choline deficient diets.
1980	Sell and Leffert propose oval cells as precursor cells for hepatocellular carcinomas and culture oval cells *in vitro*.
1984	Sell and Salman identify proliferating periportal cells early after carcinogen exposure with later ductal cell proliferation and suggest that this may be the stem cell for hepatocellular carcinoma.
1985	Yaswen *et al.* note increased expression of proto-oncogenes (c-*myc* and c-K-*ras*) in oval cells when compared to normal liver.
1985	Hixson and Allison use monoclonal antibodies to identify oval cells and immature liver cells.
1989	Dunsford 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.
1990	Sell proposes that liver stem cell may be either periportal cell or transition duct cell.

I. DEDIFFERENTIATION OF HEPATOCYTE

II. STEM CELL MATURATION ARREST
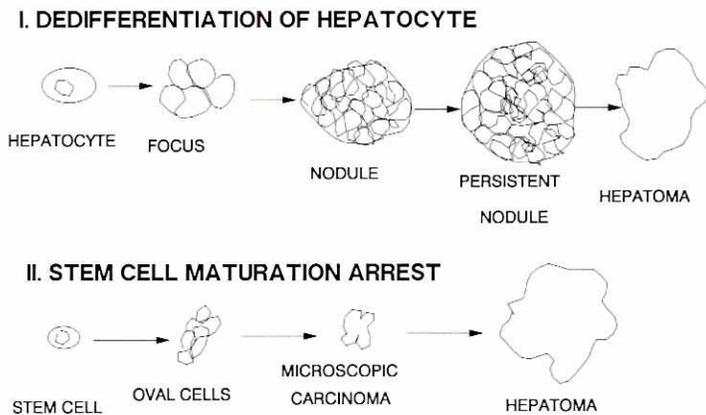
Fig. 2. Possible cellular pathways to liver cancer. *Two cellular lineages to liver cancer are presented:* **(I)** *dedifferentiation of mature hepatocytes and* **(II)** *aberrant differentiation of stem cells. The classic interpretation of the effect of chemical carcinogens in producing so-called premalignant foci and nodules implies dedifferentiation. The induction of proliferation of oval cells by chemical hepatocarcinogens is more consistent with derivation from stem cell maturation arrest (blocked ontogeny). Oval cells may differentiate into duct-like cells or transitional hepatocytes, which, in turn, may remold into normal ducts or hepatocytes or may progress to intermediate lesions such as nodules or atypical hyperplasia before becoming cancer.*

cancer kept the concept of a stem cell origin of cancer alive. In particular, the characteristics of cancers, such as teratomas, teratocarcinomas, neuroblastomas, Wilms tumors, etc., which produce a variety of undifferentiated as well as fully differentiated cells, are more consistent with a stem cell origin (Virchow, 1863; Wilms, 1896; Wright, 1910). Teratocarcinomas contain structures resembling pre-somite embryos (embryoid bodies) intermixed with virtually every recognizable fetal and adult tissue (Pierce and Dixon, 1959; O'Hare, 1978). Pierce et al. (1960) showed that teratocarcinomas (embryonal carcinomas) not only contained malignant pre-somatic cells, but also included benign somatic tissue. Neuroblastomas also have embryonal characteristics. Neuroblastomas of infants (<2 years) tend to be poorly differentiated and show spontaneous regression with documented regularity, whereas neuroblastomas of older children (>2 years) tend to have more differentiated cells, but usually cause death even after intense chemotherapy (D'Angio et al., 1971). It is believed that neuroblastoma is a congenital neoplasm of the sympathetic nervous system which arises in fetal neural crest cells and that primitive neuroblastomas may be controlled by differentiation induced by the environment of the infant (Cushing and Wolbach, 1927), but that neuroblastomas arising in older children are not controllable in this manner.

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 testicles 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), Papaioannou 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 trophectoderm 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.

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-acetlyaminofluorene (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; Schulte-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; Schulte-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 tars to the ear did not result in tumors unless the site of application was scarred 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 hepatectomy, liver injury induced by CCl_4 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).

TABLE 2

OVAL CELL PROLIFERATION AND ALPHAFETOPROTEIN PRODUCTION IN SELECTED MODELS OF HEPATOCARCINOGENESIS IN RATS

| Carcinogen | Preneoplastic Morphologic Change | | Serum AFP Levels | | |
	Early	Late	Early	Late	Hepatomas
Cyclic AAF	Increasing OC, foci	nodules, OC, ducts	++	+	+++/0
DEN	Little change	OC, microcarcinomas	0	++	++++
CD-AAF	Massive OC	foci, nodules	+++	++	+++
DEN-AAF-PH*	Massive OC, foci	nodules	+++	++	+++/+
WY-14643	Hepatocyte Mitosis	microcarcinomas	+	0	0

AAF, *N*-2-fluorenylacetamide; OC, oval cells; DEN, diethylnitrosamine; CD, choline deficiency; DEN-diethylnitrosamine; PH, partial hepatectomy;*, Solt-Farber model; WY, Wyeth. (Modified from Sell et al., 1987).

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; Tchipysheva et al., 1977; Kuhlmann, 1978; Sell, 1978; Jalanko and Ruoslahti, 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 that appear 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 «Solt-Farber» model of inducing HCC. This regimen was designed to produce rapid development of foci, nodules and cancer (Solt 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 Solt-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 periportal 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

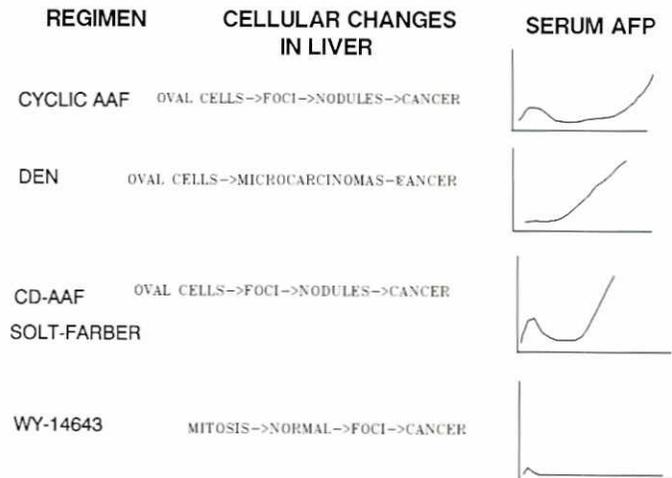| REGIMEN	CELLULAR CHANGES IN LIVER	SERUM AFP
CYCLIC AAF	OVAL CELLS→FOCI→NODULES→CANCER	
DEN	OVAL CELLS→MICROCARCINOMAS→CANCER	
CD-AAF SOLT-FARBER	OVAL CELLS→FOCI→NODULES→CANCER	
WY-14643	MITOSIS→NORMAL→FOCI→CANCER	

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 AAF 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 Solt-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.*

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,
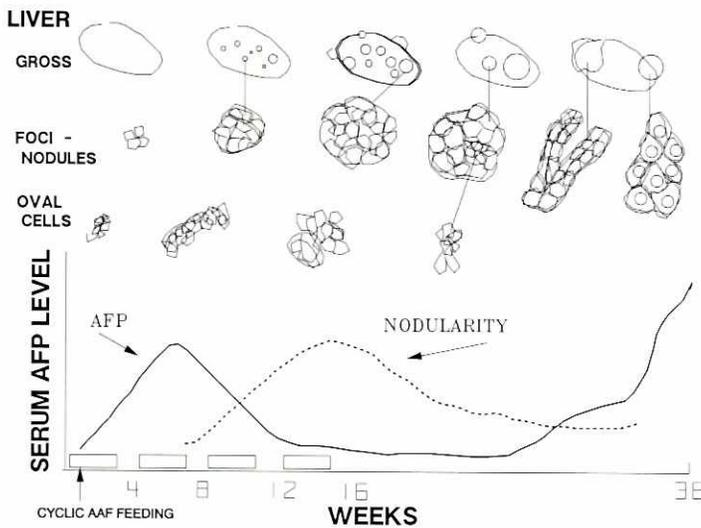
LIVER

GROSS

FOCI - NODULES

OVAL CELLS

SERUM AFP LEVEL

AFP

NODULARITY

CYCLIC AAF FEEDING   **WEEKS**

4   8   12   16   36

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.*

1964); the transition duct cell (Sell, 1991) or an ill-defined periportal cell (Sell and Salman, 1984). The relationships of these cell types is illustrated in Fig. 5. Earlier autoradiographic studies implicated terminal bile duct cells as the precursors of oval cells (Popper et al., 1957; Grisham and Porta, 1964). However, identification of the cell type by autoradiographic labeling of the cells proliferating early after carcinogen exposure depends upon the time after carcinogen exposure that labeling is done (Sell et al., 1981). The conclusion that oval cells come from duct cells was based on autoradiographic studies done several weeks after carcinogen exposure (Grisham and Porta, 1964). In contrast, during the first three days after CD-AAF, the majority proliferating cell population is not ductal, but periportal (Sell et al., 1981). After three days labeling increased in the duct cell population. By electron microscopy these early labeled cells were identified as small non-discript periportal cells, and later duct cells — in particular, transition duct cells (Sell and Salman, 1984; Sell, 1990). Later, when oval cell proliferation is at a peak, many of the oval cells infiltrating between the liver cords are transition duct cells (Novikoff et al., 1991). It is possible that both the transition duct cell and a periportal liver-determined stem cell have the potential to proliferate and differentiate into duct or liver

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 periportal 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 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 restitutive 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

regeneration of the liver (the first ? in Fig. 1). After partial hepatectomy or chemical injury (CCl_4, 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 (Sell, 1980), and adjacent to zones of liver injury after CCl_4 injury in the mouse (Abelev, 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 CCl_4, 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 (Demetris, 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 (Sell, 1980; Hixson and Allison, 1985; Germain *et al.*, 1988a; Shiojiri *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.*, 1986a) 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 periportal 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
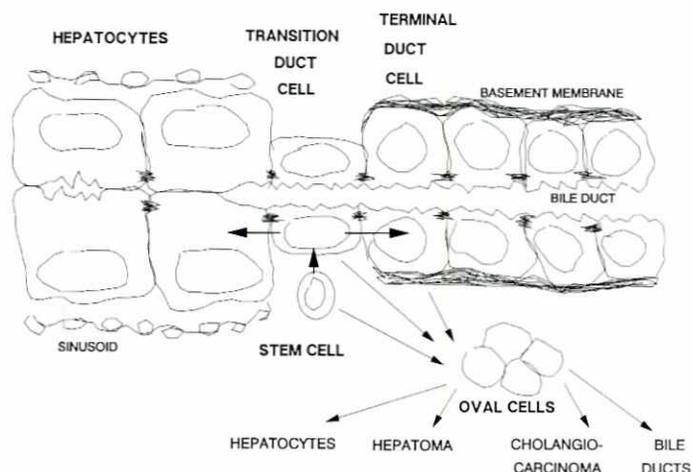
Fig. 5. Possible differentiation pathways during chemical hepatocarcinogenesis. *This figure depicts the architecture of the junction between hepatocytes and bile ducts including the three cell types that have been implicated as giving rise to hepatocellular carcinomas: the terminal duct cells, transition duct cells and the putative periportal liver stem cell. Transition duct cells have tight junctions with hepatocytes on one side and bile duct cells on the other. Many oval cells seen after exposure to hepatocarcinogens have extensive plasma membrane appositions between hepatocytes and bile duct-like cells, including desmosome structures, tight junctions, and microvilli interdigitations (Novikoff et al., 1991). Similar to other epithelial organs, the cell that is stimulated to proliferate depends upon the degree of the proliferative stimulus. Normally, the putative stem cell or transition duct cell may differentiate into either hepatocytes or duct cells, depending upon which direction the daughter cell takes (thick arrows). It is postulated that HCC arise from either the putative periportal stem cell or from the transition duct cells, or their progeny (thin arrows), which may resemble duct-like cells or transitional hepatocytes.*

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 10^{11} 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.

INTESTINE
(2-5 DAYS)

SKIN
(12-30 DAYS)

LIVER ACINUS
(1 YEAR)

DIRECTION OF DIFFERENTIATION

ONE WAY

CRYPT   BASAL LAYER   PORTAL ZONE

Fig. 6. Comparison of cell renewal in intestine, skin and liver proliferons.
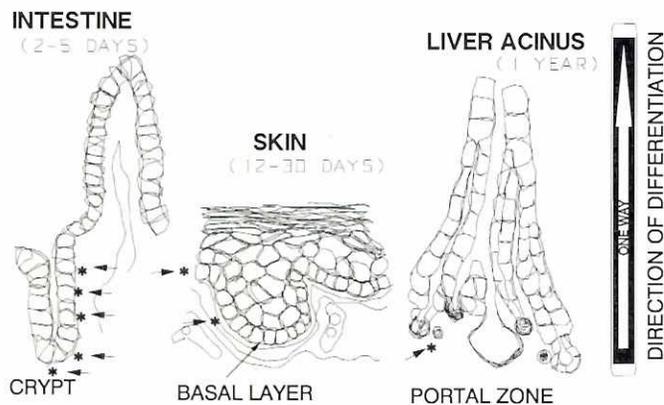*Normal cell turnover in the G.I. tract, skin and liver appears to proceed
similarly, but at greatly different rates. The small arrows and asterisks
denote the location of proliferating stem cells. Proliferating cells may be
demonstrated in the crypt of the G.I. tract up to the opening of the crypt,
limited to the basal layer of the skin and very rarely in the liver. Toxic or
destructive events may increase the proliferation rate in these organs so
that proliferating cells may be seen in higher layers in the skin and in the
hepatic cords. Induction of proliferation of hepatic stem cells requires
either massive loss of hepatocytes or inhibition of hepatocyte proliferation
by a necrotic dose of a genotoxic carcinogen.*

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 consid-
ering 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 prolifera-
tion (non-genotoxic), allowing «internal» or «spontaneous» muta-
tions 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 pro-
liferation 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
lasting 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 differ-
entiation 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
(Junqueira *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 (Peraino *et al.*, 1977; Solt and Farber, 1977). For example, in
unpublished experiments of Becker (Becker, F. personal commu-
nication), administration of phenobarbital 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 (hepatocholangiocarcinomas, 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_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[α]pyrene; whereas direct hyperplasia induced by liver mitogens, such as lead nitrate or nafenopin does not induce foci.

Why should agents that remove liver cells serve as «promoters» whereas non-necrotic mitogens do not? The answer could be that compensatory hyperplasia following liver cell death stimulates new hepatocyte formation by stem cells, whereas hyperplasia resulting in increasing the number of liver cells is returned to normal by apoptosis that does not increase proliferation of the determined liver stem cell population.

Another finding that is difficult to explain is that, while hundreds of foci and nodules are formed, only one or two, hepatocelluar carcinomas eventually develop (Teebor and Becker, 1971), fulfilling the Apostolic law: «Many are called, but few are chosen». If there are so many initiated cells, why are there not more primary HCC induced by carcinogens? It may be that development of foci is not an accurate measure of the ultimate effect of an initiator. It has been proposed that foci are produced as an «adaptive» response to the carcinogen because the cells in the foci are resistant to lethal effects of the carcinogen (Teebor, 1975; Farber, 1984; Sell and Dunsford, 1989). It is not surprising, since foci and nodules have increased levels of detoxifying enzymes, that foci may be enhanced and enlarged by chemicals that require metabolism, such as phenobarbital, and yet not by chemicals that stimulate mitosis directly. Therefore, persistent nodules may provide a supportive environment for proliferation of differentiating stem cells that give rise to cancers, yet not contribute directly to the cell lineage of the cancer (Sell and Dunsford, 1989).

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 Ellwein, 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

TABLE 3

EFFECT OF EXPRESSION OF HBV PROTEINS IN TRANSGENIC MICE

Trans-gene	Promoter	Effect	References
PSX	Albumin	Necrosis, Hepatoma	Chisari et al., 1985,1986,1987, 1989
PSX	Metallothioneine	Necrosis, Hepatoma	Chisari, et al., 1985,1986,1987,1989
PSX	HBV PrS	No Nec. or Foci, Hepatoma	Dragani, et al., 1989
HBx	HBx Trans. Enhanc.	Foci, No Nec., Hepatoma	Kim et al., 1991
HBx	α-1-antitrypsin	Focal Necrosis, No Hepatoma	Lee et al., 1990

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.

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