Hepatic progenitor cells, stem cells, and the biliary epithelium as transit compartment in models of liver injury

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Introduction

Numerous approaches have been made to study liver regeneration in human disease and also in various experimental conditions including hepatocarcinogenesis. The search for so-called stem cells being responsible for tissue repair has been a topic of most interest and debate. Hepatic progenitor cells being able to differentiate into mature hepatocytes and into cholangiocytes have long been postulated, but the location of hepatic stem cells still remained unclear. Because hepatic stem cells should be theoretically bipotent, they were expected to lie in the proximity to both hepatic and biliary compartments. This suggestion is supported by results of Theise et al. (1999) who studied normal livers and those with massive necrosis by immunostaining for cytokeratin 19 (CK19) and determined three-dimensional relationships by use of serial sections. From these studies it was concluded that *the actual interface of hepatic parenchyma and the biliary tree is not the limiting plate, but rather the zone of hepatocytes adjoining the canal of Hering, radiating from the portal tract.* Then, the canals of Hering are likely to be the source of ductular reactions in a variety of acute and chronic liver diseases and consist of facultative hepatic stem cells.

In order to study in detail progenitor cells in liver repair, suitable animal models are needed. From numerous experiments, and in particular during the period when immunohistological methods were refined and applied in our as well as in other laboratories, it was shown that alpha-1-fetoprotein is a useful biological marker to follow the restitutive response of the liver to various injuries including hepatocarcinogenesis.

Alpha-1-fetoprotein (AFP) is a normal constituent of fetal serum and amniotic fluid in many species. Under physiological conditions its synthesis occurs in the yolk sac, the gastrointestinal tract and the liver of embryos. After birth this protein disappears (Bergstrand and Czar, 1956; Gitlin and Boesman, 1967a, b; Gitlin et al., 1967), but it may reappear in substantial quantities in adults during the process of malignant transformation (Abelev et al., 1963; Laurence and Neville, 1972; Teilum et al., 1975) and also in non-malignant diseases of human liver, i.e., forms of regeneration associated with viral hepatitis, alcoholic cirrhosis and partial hepatectomy (Geffroy et al., 1970, Matray et al., 1972; Delpré and Gilat, 1978).

At the experimental level, conditions of normal and pathological AFP synthesis were extensively studied with rodents. Thus, partial hepatectomy, CCl_4 and galactosamine induced liver injury have shown temporary elevation of AFP (Sell et al., 1974a, b; Taketa et al., 1975; Sell et al., 1976; Kuhlmann, 1978; Kuhlmann, 1979; Kuhlmann and Wurster, 1980; Kuhlmann, 1981). Furthermore, the resurgence of AFP was subject of numerous studies on the different stages of experimental hepatocarcinogenesis. Its elevation was supposed as being a response to the carcinogenic diet and was correlated either with carcinogenicity of the chemical agent or with subsequent liver alterations including oval cell proliferation (Watabe,

1971; Kitagawa et al., 1972; Kroes et al., 1972, 1973, 1975; de Néchaud and Uriel, 1973). Since elevated serum AFP levels without oval cell proliferation during and after feeding of very small quantities of N-2-fluorenylacetamide were observed, one can expect metabolic effects on liver cells with subsequent selective derepression of AFP synthesis (Kroes et al., 1972; Becker and Sell, 1974). In any case and with respect to cellular fluctuations in the liver, AFP elevation will largely depend on effects such as cell death, mitotic activity and dynamics of cellular differentiation (Kuhlmann, 1978).

The normal liver

Cells in the hepatocyte lineage include hepatocytes and intra- and extrahepatic bile duct cells. During ontogeny, hepatocytes and bile duct cells originate from a common forgut endodermal precursor cell. At the junction of the bile ducts and the hepatic cords, there are found the terminal bile ductules, known as the canals of Hering. These structures connect the intrahepatic ducts with the hepatic cords. Intraportal bile ducts are restricted to the portal triad, whereas the terminal bile ductules extend across the limiting plate of hepatocytes (Grisham and Thorgeirsson, 1997).

The normal liver may be regarded as a non-growing proliferative tissue in which cell production and cell loss are balanced ("steady-state" tissue). In such a non-growing proliferative tissue production of new cells does not exceed cell loss. Growth control ensures that the cellular population is maintained at some defined level which can be assumed to be genetically determined and to allow adequate function. The mechanisms controlling such balance is not fully understood. Hence, in regenerative phase, an increase in the proliferative population (clonogenic cells) will be an essential requirement.

Under physiological conditions, hepatocytes are proliferatively quiescent and turnover very slowly. It has been estimated that the liver is replaced by normal tissue renewal about once a year (Steiner et al., 1966). Apart from normal turnover, parenchymal loss caused by partial hepatectomy or uncomplicated infection induces a rapid regeneration by hepatocyte self-replication; all hepatocytes are capable to undergo mitosis. The participation of putative stem cells has never been demonstrated. However, when liver damage is extensive and chronic, or in the case when hepatocyte proliferation is inhibited as for example by viral infection, putative liver stem cells may be activated to proliferate and to differentiate into hepatocytes (Alison et al., 2001). The intrahepatic biliary tree as well as haematopoietic stem cells may contribute to the renewal of hepatocytes.

Liver cells and the response to injury

Liver restitution after different injuries will involve the proliferation of cells at different levels in the liver lineage. It is hypothesized that, similar to other organ systems, lineage cells consist of stem cells (including shortterm stem cells and longterm stem cells), precursor cells, and mature cells (Sell, 2001).

Numerous experimental efforts have led to new concepts how the liver responds to injury. At least the following levels of cells seem to be implicated in the neoformation of hepatocytes and bile ducts: (a) mature hepatocytes; (b) bile duct cells; (c) ductular progenitor cells; (d) multipotent progenitor cells of presumed extrahepatic origin (Sell, 2002; Forbes et al., 2002; Fausto and Campbell, 2003; Sell, 2003). From the various models of liver injury including

hepatocarcinogenesis, it can be hypothesized that different insults evoke a response of different cells in the hepatic lineage, and that these cells will have the potential to differentiate into various cell types. Furthermore, such cells will serve as the cellular origin of hepatomas and hepatocellular carcinomas.

The proliferating cells in the liver will include:

- The mature, differentiated hepatocytes in all parts of hepatic plates.
- The original tissue-determined stem cells which are represented in the adult organ by cells of the canal of Hering.
- Multipotent stem cells derived from circulating bone marrow stem cells.

Recent studies support the view that two origins of tissue renewing stem cells will exist: bile ductules as endogenous stem cells being derived from the cells responsible for the embryonic development of the liver, and bone marrow cells as exogenous stem cells which appear after formation of the organ. The bone marrow derived exogenous stem cells are thougth to be few in number but of longterm proliferation capacity. Bile ductules as the endogenous liver progenitor cells are greater in number and of shortterm proliferation capacity. The latter are called in action under certain conditions, when the hepatocytes (which are greater in number but have more limited proliferation activity) are insufficient or unable to respond to injury.

Liver restitution after injury

Partial hepatectomy was for many years the most classic model for the study of liver regeneration. The loss of two thirds of the liver is replaced within two weeks by proliferation of the remaining hepatocytes (see above, The normal liver) without evidence of stem cells involved (Alison, 1986).

The question remained then for a long time, under what circumstances other cells in the hepatic lineage would be activated for restitutive response. From a variety of experimental models it became obvious that putative liver stem cells may be activated to proliferate only if liver damage is extensive or chronic or if proliferation of mature hepatocytes is inhibited by metabolic and infectious mechanisms. The most studied models included injuries by CCl_4, CCl_4 combined with N-2-acetylaminofluorene, furan, galactosamine, allyl alcohol and different chemical carcinogens. These models identified the following restitutive cells in the liver: (a) hepatocytes; (b) ductular stem cells; and (c) periductular stem cells; for references see Sell (2001), Fausto and Campbell (2003), Forbes et al. (2002).

Biliary epithelium as progenitor compartment

Much progress in the search for tissue renewing stem cells and progenitor cells in liver repair derived from studies with hepatotoxic chemicals, and especially the galactosamine induced experimental hepatitis proved to be useful. Galactosamine will produce dose dependent liver cell injury (panlobular); with low doses in the order of 200 mg/kg body weight biochemical disturbances are caused in the liver without necrosis, with higher doses liver cell necrosis is provoked (Keppler et al., 1968; Lesch et al., 1970). In contrast to CCl_4 injury, subsequent regeneration was characterized by extensive proliferation of bile ducts and ductular epithelial cells. Moreover, the intense immunostaining of AFP in these canalicular epithelial cells was a prominent feature (Kuhlmann and Wurster, 1980); normal quiescent bile ducts and normal canalicular epithelial cells failed to stain for AFP. Moreover, between day 3 and day 5 after

galactosamine injury, faint AFP immunoexpression could be observed within parts of the cytoplasm of few randomly distributed hepatocytes. In the majority of hepatocytes, however, AFP was not detected.

Results from our serological and immunohistological AFP studies after galactosamine injury are summarized in Table 1. It became evident that proliferating cells of the bile duct system developed to a level of cytodifferentiation encompassing concomitant reactivation of fetal genes. This process was marked by AFP synthesis and suggested a certain degree of retrodifferentiation (reversal of ontogeny) with potential stemness. During the phases of regeneration, marked mitotic activity of biliary epithelial cells was prominent. This in turn led to increasing numbers of cross-sections of ductules which might correspond to an increase in ductular structures, extensive arborization of expanding neoductules, or alternatively to prolongation of bile ductules. The new ductular structures are supposed to differentiate subsequently into hepatocytes and to represent liver progenitor cells.

Table 1. Detection of AFP after galactosamine injury, from Kuhlmann and Wurster, *Virchows Arch. A Pathol. Anat. Histol.* 387, 47-57, 1980).

Period of study	Serum AFP (μg/ml ± SD)	Hepatocytes	Canalicular epithelial cells
Day 1	< 0.1	– [a]	–
2	0.19 ± 0.04	–	(+)
3	0.54 ± 0.22	(+)	+
4	2.08 ± 0.67	(+)	+
5	1.53 ± 0.39	(+)	(+)
6	1.24 ± 0.48	–	(+)
10	0.16 ± 0.03	–	–
Normal control	< 0.1	–	–

[a] Intensity of immunocytochemical AFP reactions: – no; (+) faint; + strong

In conditions such as the galactosamine induced liver injury or in other experimental models when hepatocyte regeneration is impeded, the biliary system will act as a potential stem cell system. Proliferating cells of the biliary tree have been referred to as proliferating bile ductular cells, ductular structures or neoductules. In animals, the progeny of biliary stem cells are called ductular oval cells which are believed to be bipotential liver stem cells able to differentiate into hepatocytes and bile duct epithelia. Hence, oval cells will function as an amplification compartment for the regeneration of hepatocytes under appropriate conditions (Alison et al., 1997).

Fig. 1. Portal tract with increased cross-sections of bile ductules on day 3 after galactosamine injury. They are of various size. Bile duct cross-sections reach a maximum on days 3 and 4 after galactosamine injury. Afterwards they decrease, but remain still on a higher level than normal on day 10.

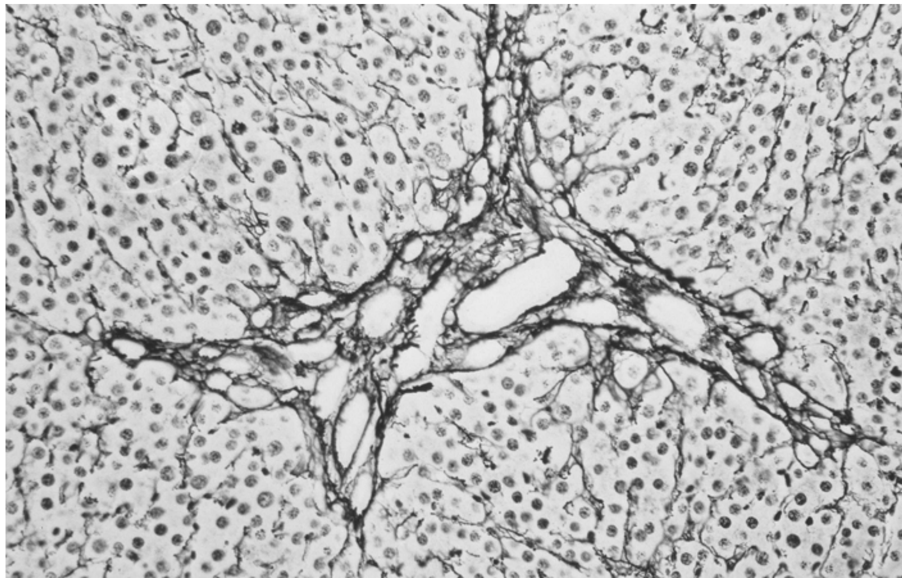
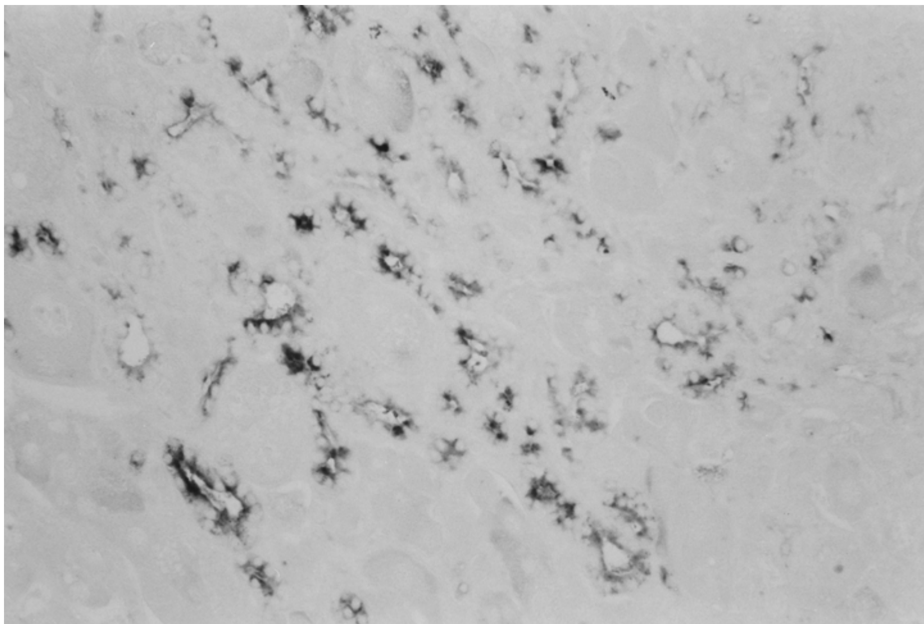


Fig. 2. AFP-positive bile ductular cells within portal tract and periportal area on day 3 after galactosamine injury.



Support to the above conclusion comes from a study of Theise et al. (1999). The location of hepatic stem cells, which are thought to be bipotent and important for the regeneration of hepatic parenchyma and biliary epithelium, is expected to lie in the proximity to the junction of both compartments. This tissue area was systematically studied by the above authors in order to establish the relationship between hepatic stem cells, canals of *Hering* and ductal plate remnants from fetal develoment. To this aim, three-dimensional reconstructions were made from serial sections of normal human liver and of liver with acetaminophene induced necrosis being immunostained for cytokeratin 19 (CK19); immunostainings for AFP, Hep Par1 ("hepatocyte paraffin 1" which is a mouse monoclonal antibody that reacts specifically with hepatocytes) and c-kit were also performed. In massive necrosis, clusters of CK19-positive cells were observed around single portal tracts which linked up to each other in complex arborizing structures and connected to the bile ducts. Furthermore, the development

of HepPar1 and AFP positive cells was observed which co-expressed CK19; in normal canals of Hering, no Hep-Par1 and no AFP could be detected. It is suggested that the preexisting canals of Hering undergo reactive proliferation and arborization. The failure of HepPar1 and AFP in normal canal of Hering indicated that this fetal phenotype will be only recapitulated during a regenerative process following injury. The authors concluded also that the small cells of the canals of Hering can regenerate hepatocytes in massive liver necrosis and, thus, represent facultative hepatic stem cells.

In our liver injury model (galactosamine), AFP could also be found in randomly distributed hepatocytes (Kuhlmann and Wurster, 1980). In a later study, AFP positive hepatocytes were shown by *in situ* hybridization to be localized in the vicinity of oval cells (Lemire et al., 1991). The fetal form of AFP was expressed by many oval cells, by duct cells and by occasional hepatocytes. Furthermore, oval cells expressed bile duct-type markers. The authors concluded by following the fate of epithelial cells (thymidine labelling experiments) that duct cells can generate both oval cells and small hepatocytes in response to galactosamine induced injury.

The relationship between ductular cells, proliferating oval cells and their differentiation into hepatocytes is supported by many experiments (Tournier et al., 1988; Dabeva et al., 1995; Alison et al., 1996; Yin et al., 2002a) including tissue culture methods (Yin et al., 2002b). From all these observations one can assume the important role of bile ducts in restitutive response and that oval cells may function as facultative liver stem cells. This is in agreement with the concept that proliferating cells in the liver will include the original tissue determined stem cells which are represented in the adult organ by cells of the canal of Hering (Sell, 2001).

All the above results do not mean exclusively that proliferation of bile duct structures is always associated with differentiation into hepatocytes. Under certain conditions, stimulation of bile duct proliferation turns out without differentiation into hepatocytes as seen after bile duct ligation or bile duct necrosis with defined chemicals (Sell, 2001).

Adult stem cells offer the possibility of regeneration, reconstruction and recovery of functionality after tissue loss. The reversibility of cell differentiation was successfully shown in amphibian regeneration where terminal differentiated cells generated new organs due to plasticity and reprogramming processes (Brockes and Kumar, 2002). Therapeutic cloning is another evidence for reversibility of the differentiation process: nuclear transfer of a differentiated somatic cell into an enucleated egg cell can give rise to stem cells which potentially develop into embryos (Anderson et al., 2001; Holden and Vogel, 2002; Wilmut et al., 1997).

Multipotent stem cells of extrahepatic origin

Cell differentiation is regulated by a hierarchy of totipotent, pluripotent (embryonic stem cells) and multipotent stem cells (committed stem cells). The latter are usually restricted to the lineage of a particular organ. A typical example of this group of stem cells is the hematopoietic stem cell which generates all of the cell types of the blood and immune system. Thereby, it is traditionally understood that organ specific stem cells will not differentiate into other tissues, and they are restricted to generate differentiated cells of the tissue in which they reside.

Generally, the biological properties of stem cells are their capacity to divide and renew themselves for long periods, and to be unspecialized until they give rise to specialized cell

types. A number of signals (inside and outside the cells) which trigger stem cell differentiation are needed: internal signals are controlled by the cell's genes which carry instructions for all the cellular structures and functions; external signals include chemokines and cytokines secreted by other cells as well as molecules in the microenvironment and physical contact with neighboring cells.

Adult (or somatic) stem cells will typically renew and differentiate into specialized cell types of the tissue in which they reside. Stem cells are small in numbers, they are thought to reside in a specific area of each tissue where they remain non-dividing for a long time until they are activated by injury or disease. Some recent experiments have shown the possibility that stem cells from one tissue will be able to generate cell types of a completely different tissue, a phenomenon described as *plasticity*.

The observation of "transdifferentiation" or so-called developmental plasticity of adult stem cells to give rise to cells of different organs is surprising and of increasing interest. Evidence of reprogrammed adult stem cells to differentiate into other cell types, e.g. the differentiation of multipotent adult progenitor cells from bone marrow into hepatocytes and other epithelial cells, is shown by a number of experiments which have caused numerous discussions (Alison et al., 2000; Anderson et al., 2001; Bjornson et al., 1999; Clarke et al., 2000; Holden and Vogel, 2002; Körbling et al., 2002; Krause et al., 2001; Lagasse et al., 2000; Petersen et al., 1999; Schwartz et al., 2002; Theise et al., 2000a, b).

Recent studies have shown a relationship between liver lineage cells and bone marrow inasmuch as hepatic oval cells and hematopoietic stem cells share CD34, Thy-1 and C-kit mRNA and protein (Fujio et al., 1994; Omori et al., 1997; Petersen et al., 1998). Indeed, a relationship between hematopoietic stem cells and liver could be deduced from studies in which tissue transplantion and liver injury were combined: for example, after transplantation of male bone marrow into lethally irradiated syngeneic females, the male Y-chromosome could be observed in the hepatocytes of the female recipient animal after liver injury; or in transplantation experiments with different donor/recipient expression of marker molecules where the marker molecules were detected in the recipient hepatocytes. Hence, an extrahepatic source for liver repopulation seems possible (Petersen et al., 1999; Theise et al., 2000a, b; Alison et al., 2000). The transdifferentiation experiments of bone marrow stem cells are in great favor for their plasticity to differentiate into other tissue types.

An alternative explanation for the ability of cells to generate progeny of another tissue type is the formation of hybrids by cell fusion. The possibility of spontaneous cell fusion to give rise to heterokaryons was recently proven (Terada et al., 2002; Ying et al., 2002; Wang et al., 2003). Bone marrow transplantations, transplantation experiments of bone marrow derived hepatocytes and cytogenetic analyses of karyotypes have shown doublings of chromosomes in recipients with patterns of tetraploid hybrids being indicative of fusion between donor and host cells. Then, hepatocytes derived from bone marrow will arise from cell fusion instead of differentation of haematopoietic stem cells. Such cells were able to divide, and the expression of previously silent genes became induced. If the bone marrow derived hepatocytes in animals with original liver defect were fused cells, then they would be expected to expand to a cell population which could repair the genetic defect.

Adult stem cells offer the possibility for regeneration, reconstruction and recovery of tissue functions. Several questions are subject of further research, f.e. (a) which humoral signals and transcriptions factors are necessary for recruitment and transdifferentiation of adult stem cells; (b) how to enhance the process of transdifferentiation; and (c) what about transdifferentiation as phenomenon of cell fusion? Functionality of stem cell plasticity must still open to be demonstrated. Indeed, transdifferentiated cells must be stable and represent a

quantitatively relevant population; this population must significantly contribute to normal functionality of the regenerated tissue type.

The so-called “nondescript” periductular cells which are reported to occur in response to certain injuries can be likely candidates for these bone marrow derived liver stem cells. The multipotent hematopoietic stem cells, even if they occur only in small numbers in the liver, will have a long proliferation potential. They may enter the liver through the portal vasculature, and are expected to locate near to the bile ducts in the portal triad.

There is indeed agreement that the liver will usually regenerate from populations confined to the liver, and the major liver stem cell is an epithelial cell that is a liver resident. Nonetheless, bone marrow derived stem cells are thought to serve as source for the regeneration process of injured liver tissue. At least, bone marrow derived cells may replace endothelial cells, fibroblasts and monocytes and provide factors required for the healing process (Grompe, 2003; Dabeva and Shafritz, 2003; Thorgeirsson and Grisham, 2003). Especially monocytes are known to produce large quantities of growth factors such as VEGF (vascular endothelial growth factor), bFGF (basic FGF/FGF-2; fibroblast growth factors) and MCP-1 (monocyte chemoattractant protein-1). All these observations will have a great importance for a general view of stem cell biology.

A number of questions about adult stem cells include:

- How many kinds of adult stem cells exist ?
- What are the sources of adult stem cells, f.e. are they “leftover” embryonic stem cells or do they arise by some other way ?
- Is the phenomenon of plasticity a fact or only an experimental artifact ?
- What are the signals that regulate proliferation and differentiation, and which factors are responsible for relocation of regenerated cells to the sites of tissue injury ?
- Is it possible to enhance proliferation of adult stem cells to produce sufficient tissue for transplantaion ?

Chemical hepatocarcinogenesis

Hepatocarcinogenesis can be regarded as a complex and special issue from all the types of hepatic injuries. Due to the inherent properties of carcinognic substances, alterations occur at molecular and cellular levels leading to dose dependent acute and chronic injuries with mutagenic and malignant changes included.

Chemical hepatocarcinogens were used for decades in the search of the early and late steps in tumor development and to trace the involved hepatic lineage cells. The regulation of drug metabolizing enzymes within hepatocytes was shown to be an extremely important factor in the initiation of chemical-induced carcinogenesis, and, similar to other chemicals, hepatocarcinogens are activated in different zones of the liver. This difference as well as inhibitory effects lead to different patterns of cellular proliferation.

Models of chemical hepatocarcinogenesis are associated with the sequential appearance of phenotypically altered cell populations, which can be characterized by changes in the expression of different marker enzymes. There is evidence that at least some of these enzyme altered foci are precursor lesions, which are causally related to malignant transformation (Emmelot and Scherer, 1980; Friedrich-Freksa et al., 1969; Goldfarb and Pugh, 1981; Buchmann et al., 1985; Aterman, 1992). These preneoplastic lesions will have a growth

advantage over normal cells (Rabes et al., 1982) and are indicators of distinct stages of carcinogenesis. From these animal models with apparently no signs of massive toxic liver injury it is concluded that precursor lesions originated from adult hepatocytes. Nevertheless, the heterogeneity of markers observed between individual foci and the differences in their growth rates point to different cellular origins as well as to variations of malignant potency of these preneoplastic lesions.

Many carcinogens are associated (in a dose dependent manner) with marked proliferation of so-called oval cells. The latter are readily identified by immunostaining for AFP (Teebor and Becker, 1971; Kuhlmann, 1978; Sell et al., 1981; Sell and Dunsford, 1989; Sell and Becker, 1978; Sell et al., 1987; Alison et al., 1993). Especially, the many serological and immunohistological studies with AFP as marker enabled much progress in understanding the processes in the development of hepatomas. When AFP appeared very early in the course of N-nitrosomorpholine (NNM) hepatocarcinogenesis, this was due to its toxic effect (mainly at high dose NNM feeding) and the paralleled proliferation of bile ductular cells, long before preneoplastic nodules were formed. At the cellular level, this oncofetal protein was localized in oval-shaped cells and in canalicular epithelial structures (Kuhlmann, 1978). The oval-shaped cells proliferated rapidly within the zones of necrotic hepatocytes and were found among non-proliferating hepatocytes ([^{3}H]thymidine autoradiography). During subsequent regeneration (when NNM was no longer applied), oval cells disappeared and foci of small basophilic hepatocytes with nodular appearance were seen. It has been suggested that oval cells play an important role in the histogenesis of liver carcinomas.

In hepatocarcinogenesis with high-dose NNM feeding as well as in other experimental models being associated with substantial loss of hepatocytes, proliferating canalicular epithelial cells or oval cells are the progeny of potential stem cell system of biliary origin (reversal of ontogeny, see above chapter for galactosamine injury). Hence, oval cells will function as an ampli-fication compartment for the regeneration of hepatocytes under appropriate conditions (Alison et al., 1997). It must be further emphasized that ductular oval cells represent a target population for carcinogens causing liver carcinomas.

Table 2. Experimental hepatocarcinogenesis, from Kuhlmann, *Int. J. Cancer* 21, 368-380, 1978. Animals: 6-, 12- and 20-week-old male inbred rats of strain BD X were used. * Not significant

BD X Rats	Group I 6 weeks	Group II 6 weeks	Group III 12 weeks	Group IV 12 weeks, Group V 20 weeks
Induction phase				
NNM treatment (per kg per day)	6 mg/12 weeks	20 mg/6 weeks	6 mg/12 weeks	20 mg/6 weeks
DNA replication	—*	Oval-shaped cells Hyperplastic areas	—	Oval-shaped cells Hyperplastic areas
Proliferative period (oval-shaped cells)	—	Days 21-56	—	Days 21-56
Intracellular AFP	0	Oval-shaped cells	0	Oval-shaped cells
μg AFP/ml serum	0	1.62±0.47	0	1.41±0.55
Hepatoma stage				
Induction time	200 days	90 days	200 days	90 days
DNA replication	Neoplastic hepatocytes	Neoplastic hepatocytes	Neoplastic hepatocytes	Neoplastic hepatocytes
Intracellular AFP	Neoplastic hepatocytes	Neoplastic hepatocytes	Neoplastic hepatocytes	Neoplastic hepatocytes
μg AFP/ml serum	<0.1 to >4,000	<0.1 to >4,000	<0.1 to >4,000	<0.1 to >4,000

In the late 1970s, we combined double tracer techniques, i.e. AFP immunohistology and [^{3}H]thymidine autoradiography, to study cellular fluctuations during toxic injury, premalignant changes and stages of hepatoma development in experimental models of hepatocarcinogenesis with N-nitrosomorpholine (Kuhlmann, 1978). The main points from these experiments are summarized:

- Hepatocellular carcinomas were induced by low doses as well as by high doses of N-nitrosomorpholine (NNM).

- Necrosis of hepatocytes was only seen at high dose NNM feeding, and regeneration occurred by prominent proliferation of oval cells forming canalicular epithelial structures.
- Elevated serum AFP concentrations occurred only with the high NNM doses in the early phase of carcinogenesis, and this protein was localized in the cytoplasm of proliferating oval-shaped cells (bile ductular cells).
- No elevation of AFP and no proliferation of oval-shaped cells could be detetcted at low dose NNM feeding.
- [^{3}H]thymidine labelling in pulse-chase experiments revealed the oval-shaped cells to be actively engaged in DNA synthesis, and their development into hepatocytes was demonstrated.
- At the final carcinoma stage (when the carcinogen has been already withdrawn for a long time), liver carcinoma cells proliferated and could stain for AFP.
- Both AFP staining and non-AFP staining carcinomas were found side-by-side within a given tissue section.
- The wide range of serum AFP levels and also the wide range within the AFP increasing rates in individual animals indicated the heterogenous character of induced carcinomas with respect to AFP immunoexpression.

AFP is an oncofetal protein typically expressed by fetal and regenerative hepatocytes. It might serve as a modulator or modifier of various cell growth regulatory pathways during embryonic and fetal development, and a suppressor protein may normally limit AFP expression (Mizejewski, 1995). In hepatocellular carcinomas, AFP expression was considered to be the direct consequence of retrodifferentiation during the neoplastic change of hepatocytes in which stages are reached where synthesis of AFP takes place (Uriel, 1969; Uriel, 1976). Thus, various degrees of retrodifferentiation will generate neoplastic hepatocytes of clonal origin leading to either AFP positive or AFP negative carcinomas.

Lemire et al. (1991) have shown that oval cells may function as facultative liver stem cells and tumor progenitors in liver carcinogenesis. These cells are detected in early carcinogenesis as well as in response to liver injury by galactosamine. Oval cells form duct structures outside the portal spaces and express bile duct-type markers. Furthermore, the fetal form of AFP (mRNA in situ hybridization studies) has been shown in oval cells, some bile duct cells, and in occasional hepatocytes.

2-Acetylaminofluorene (AAF) administration in combination with partial hepatectomy or the ligation of the common bile duct are useful models to study growth patterns of subsequent biliary cell proliferation (Paku et al., 1991). In both experiments, intense biliary cell proliferation was observed. Morphological data at early time points demonstrated that AAF induced oval cells were preferentially generated by proliferation of the terminal biliary ductules. Comparable to our observations, the oval cells formed ductular structures representing an extension of the canals of Hering, strongly indicating that liver stem cells are located in the biliary system. The population of oval cells can be regarded as the potential candidates to constitute the primary hepatic stem cell niche. The stem cell potential of the larger biliary ducts, however, cannot be excluded. After bile duct ligation, biliary cell proliferation appeared to be different from the oval cell proliferation in the AAF/partial hepatectomy model; proliferating biliary cells did not show signs of differentiation into other cell types. The increased intraductal pressure is thought to be the primary stimulus. Furthermore, different expressions of drug metabolizing enzymes in different segments of the biliary system (comparable to hepatocytes in different zones of the liver) will provide additional explanations for differences in subsequent cellular responses to the chemical regimens.

Even if the authentic liver progenitor cell may be difficult to identify, a key for the existence of progenitor cells is the appearance of so-called oval cells in different liver injury models, and their differentiation into either hepatocytes or cholangiocytes. Some problems involved in current discussions on stem cells are discussed by Potten and Loeffler (1990). They suggest that stemness is not a property but a spectrum of capabilities from which to choose.

Similar to the response to different injuries, the reaction of the liver to different chemical hepatocarcinogens involves different levels of cells. The following possibilities of the origin of hepatocellular carcinoma are suggested (for review see Sell, 2001):

- Hepatocyte: the original mature hepatocytes.
- Bile ducts: the original mature bile duct cells.
- Ductular stem cells: the tissue-determined stem cells of the adult liver, described as ductular precursor cells and represented by the terminal bile ductules (canals of Hering).
- Extra-hepatic stem cells: the multipotent stem cells derived from circulating bone marrow and localized in the liver as periductular stem cells.

Fig. 3. Detection of AFP in liver during early stages of hepatocarcinogenesis with high dose NNM feeding; liver from day 28, note necrosis of hepatocytes and regeneration by prominent proliferation of AFP positive oval-shaped cells forming bile duct epithelial structures.

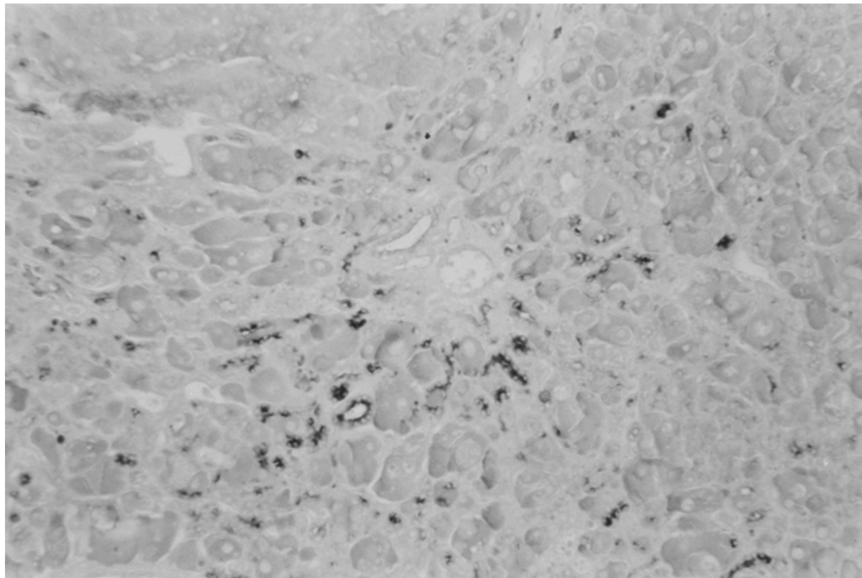
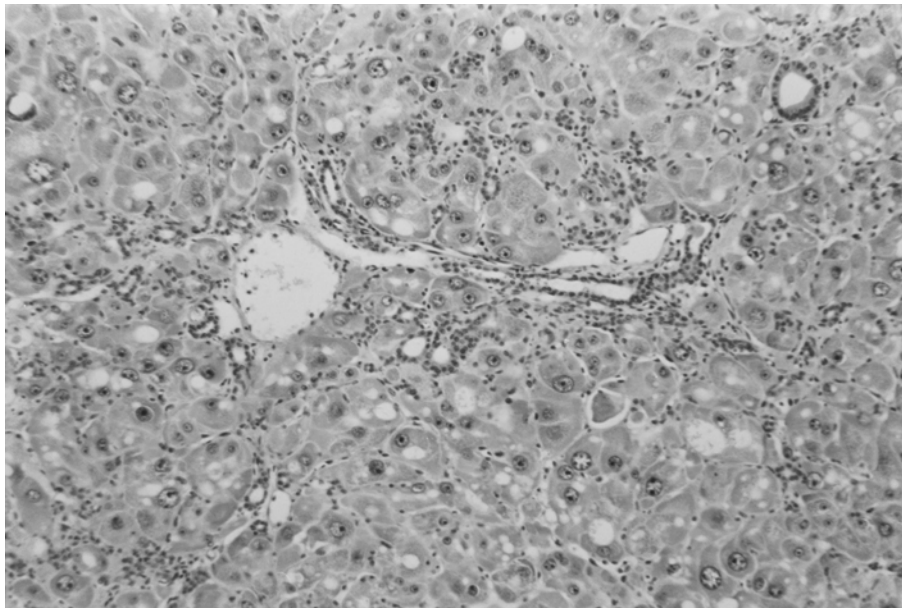


Fig. 4. Liver from day 35 at high dose NNM feeding. (a) HE stained preparation; (b) same liver, serial section stained for AFP, note localization of AFP in bile ductular structures.



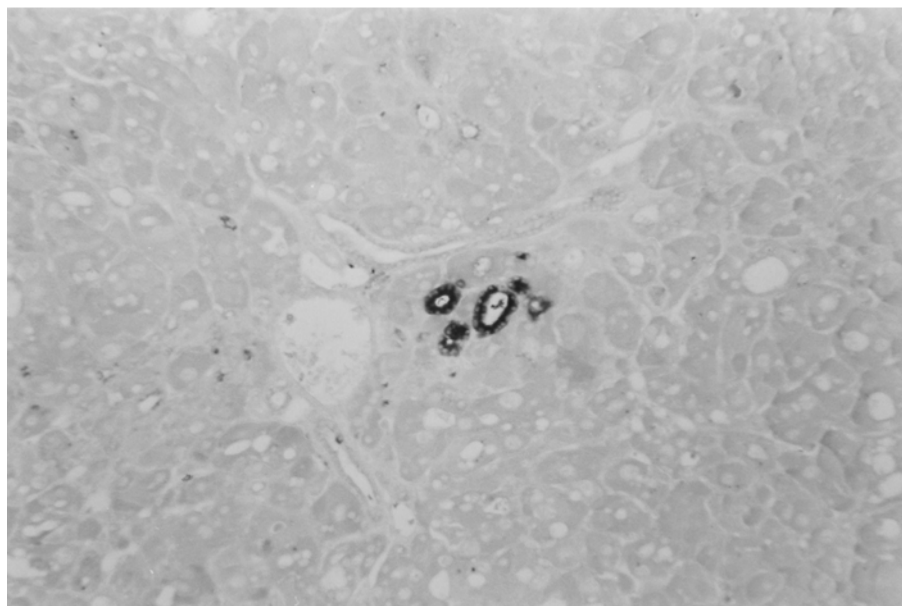
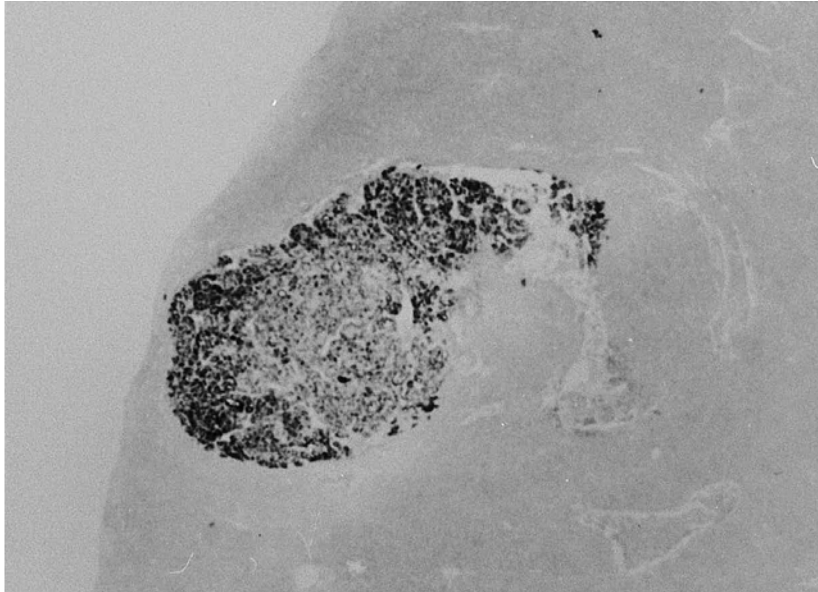
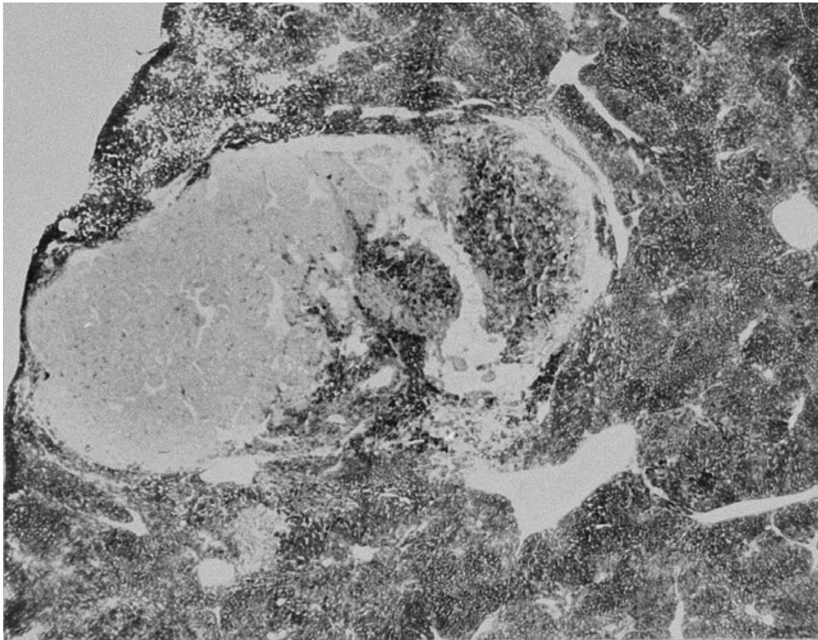


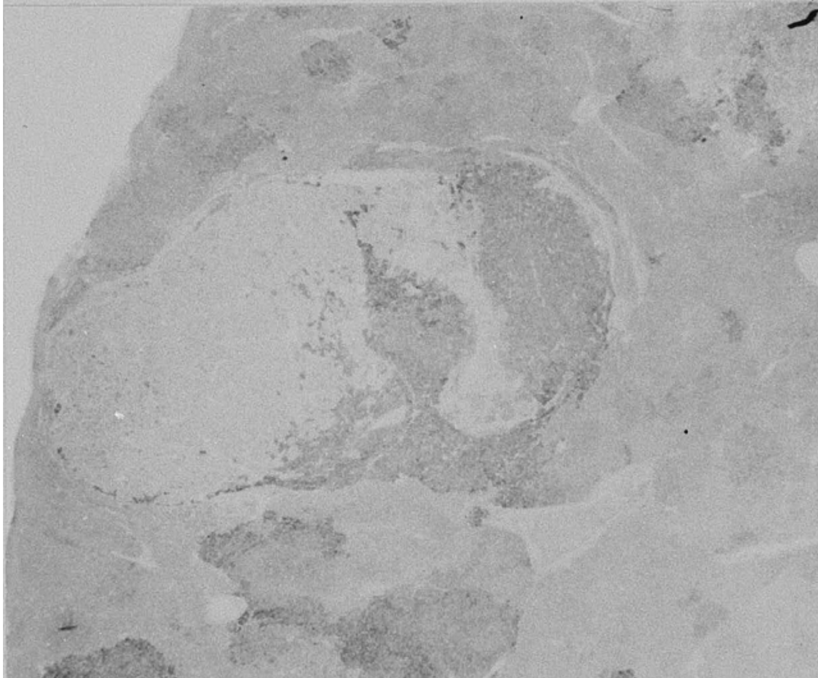
Fig. 5. Localization of AFP at the carcinoma stage. (a) Low magnification view of AFP-positive carcinoma. (b) Serial section from the same liver area was reacted by PAS; normal hepatocytes are strongly stained, whereas the AFP positive carcinoma cells remained PAS-negative. (c) Serial section from the same liver area was immunostained for liver microsomal epoxide hydrolase (EH). This enzyme is known to become increased by many xenobiotics. Hepatocarcinogens such as NNM can lead to a focally elevated EH which may persist in benign hepatomas. In contrast, elevated EH protein is no longer observed in malignant hepatocarcinomas as for example in AFP positive hepatocarcinomas which are basophilic, PAS negative and have characteristic malignant morphology (Kuhlmann et al. *Biochem. Biophys. Res. Comm.* 98, 417-423, 1981).



(a) AFP

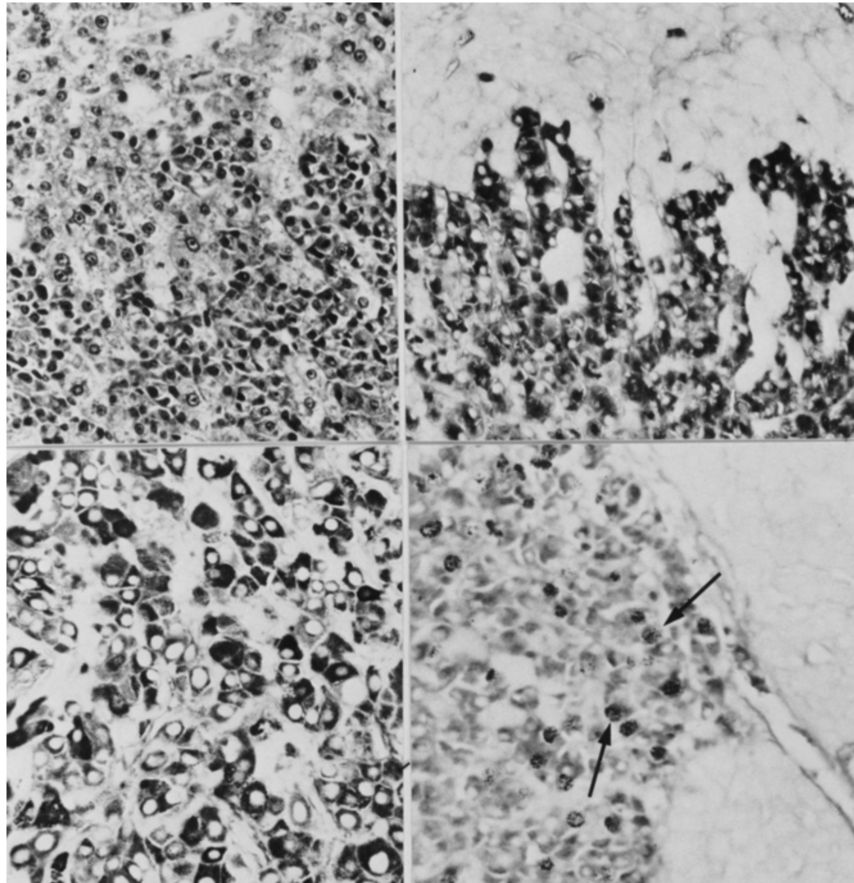


(b) PAS



(c) EH

Fig. 6. Localization of AFP at the carcinoma stage, from Kuhlmann, *Int. J. Cancer 21, 368-380*, 1978. (a) Border of a carcinoma stained by HE. (b) Serial section from same carcinoma region and reacted for AFP; neoplastic hepatocytes penetrate normal liver, and AFP reaction is only in the carcinoma cells. (c) Another view from an AFP-staining carcinoma. (d) Thymidine incorporation in carcinoma cells with AFP immunoexpression (←); adjacent normal liver is not labelled.



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