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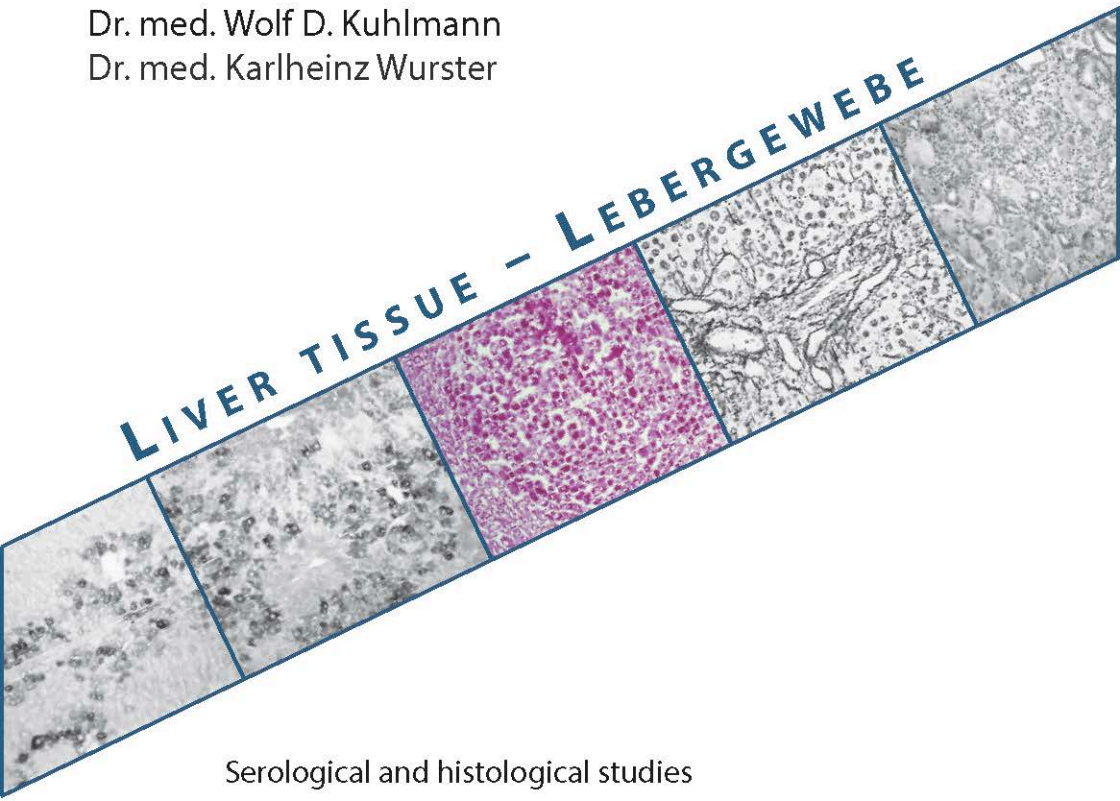

Serological and histological studies

Cellular fluctuations, alpha-fetoprotein expression, and the question of stem cells in experimental liver regeneration and hepatocarcinogenesis

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Preface

This review deals with processes of liver repair and the role of progenitor and stem-like cells in regeneration after hepatic injuries. The experimental models include cellular loss by surgical means as well as liver damage by hepatotoxins and hepatocarcinogens. The response of hepatic lineage cells and their potential of differentiation are studied by the expression of alpha-1-fetoprotein (AFP). AFP was selected as marker by virtue of its strong correlation with the activity of fetal gene expression in ontogeny, regeneration, and in oncodevelopmental situations.

For this purpose, we ought to solve general problems occurring in immunohistology such as biochemical, serological and histological methods including the reagents with appropriate sensitivity and specificity to analyse cell functions under normal and pathological conditions. Immunohistology serves to understand structure-function-relationships in experimental settings and, thus, making a bridge between biology and pathology. Our research work started in facilities of the Ludolf-Krehl-Klinik in Heidelberg, thereafter continued at the Institut de Recherches sur le Cancer in Villejuif and the Deutsches Krebsforschungszentrum in Heidelberg. Our projects have been supported by the Deutsche Forschungsgemeinschaft (DFG).

Stem-like properties of adult hepatocytes and stem-like properties of biliary epithelial cells became evident in the course of liver regeneration. In partial hepatectomy and after carbon tetrachloride injury, adult hepatocytes are the main source for liver restitution. In these cases of liver repair, stem cells with the ability of multilineage differentiation are not required. Increased serum AFP concentrations are associated with the proliferative activity of mature hepatocytes. However, when regenerative capacity of hepatocytes is blocked by defined injury conditions, liver repair follows another pathway which involves the proliferation of biliary epithelial cells by generating the so-called oval cells. Oval cells and their progenies are stem-like descendants from the canaliculi of HERING and from small interlobular bile ducts. These cells represent the tissue stem cells of the liver. During proliferation and maturation, oval cells reach levels with fetal gene activation leading to AFP synthesis as sign of reversal ontogeny.

Apart from regeneration of lost tissue, both oval cells and hepatocytes are susceptible populations for risk of malignant transformation. In this process, AFP resurgence displays characteristic of retro-differentiation. Pathways of cell differentiation towards malignancy, maturation stages and maturation arrest are often unpredictable features in cancer development. In this environment, AFP expression can be a sign of maturation arrest. It seems to be worthwhile to evaluate more facts and further characteristics by the use of molecular-genetical means, and thus, serving diagnostics and therapeutical approaches.

Koblenz, April 2023

W. D. Kuhlmann
K. Wurster

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Cellular fluctuations, alpha-fetoprotein expression, and the question of stem cells in experimental liver regeneration and hepatocarcinogenesis

WOLF D. KUHLMANN and KARLHEINZ WURSTER

Abstract

Hepatocytes and bile ductular cells possess stem-like potential in restoring liver tissue. This was derived from rat and mouse injury models such as (a) partial hepatectomy by surgery; (b) acute carbon tetrachloride intoxication; (c) injuries by D-galactosamine (GalN) or by N-nitrosomorpholine (NNM); (d) experimental hepatocarcinogenesis with NNM using different doses. Liver parenchyma was restored by adult hepatocytes after partial hepatectomy as well as after carbon tetrachloride intoxication. Concurrently, hepatocytes resumed synthesis of alpha-1-fetoprotein (AFP) which depended to a high degree on the animal species and strains used in that studies. In contrast to this way of regeneration, liver repair after heavy injuries by agents such as GalN and NNM was initiated by oval cell proliferation deriving from ductular epithelial cells of the canaliculi of HERING (canals of Hering, intrahepatic bile ductules) as sources of endogenous progenitors. Progenitor populations reach levels of differentiation with fetal gene reactivation and significant AFP expression. Proliferating oval cells operated as transit and amplification compartment during liver regeneration. Oval cell proliferation and concurrent AFP synthesis occurred also in the early stages of hepatocarcinogenesis when the carcinogen NNM was applied in toxic doses. The histologic features compared to those seen in GalN intoxication. NNM carcinogenicity was of concern for both oval cells and adult hepatocytes with risks of transformation and malignant development. At the cancer stage, carcinomas either synthesizing AFP or not can be found side by side in the same liver. AFP expression by cancer cells seems to be a special attribute to differentiation stages in carcinogenesis. Clonality, maturation arrest and retrodifferentiation feature cells with high autonomy.

Keywords

Alpha-fetoprotein, hepatocytes, oval cells, biliary epithelial cells, stem-like progenitor cells, liver regeneration, liver carcinoma

1 Introduction

The liver is an important organ with essential chemical functions for life. Cell production and cell loss are usually balanced, renewal of cells does not exceed cell loss. In restoring cell mass during physiological regeneration, adult hepatocytes switch from a quiescent state to a proliferative state. This capacity of normal adult hepatocytes can be blocked in certain situations, and other repair mechanisms become involved to replace lost liver mass [1, 2]. This leads to the all-moving question: are there stem cells in the adult liver for regeneration? In fact, the adult liver contains stem cells that can proliferate and differentiate

into different cell types. These cells are called hepatic stem cells or liver progenitor cells, they proliferate and differentiate into hepatocytes and bile duct epithelia, respectively.

Stem cell activation is an important mechanism for regeneration and is dependent on the severity of the damage. In some cases, liver regeneration may be impaired by progressive fibrosis, inflammation, or genetic factors, limiting the ability of cells to regenerate. Anyway, organ-specific stem cells are of importance in regenerative situations. Several studies have shown that stem-like cells can be found at the junction of the bile ducts and the hepatic cords. They reside in the terminal bile ductular system from which so-called oval cells derive [3, 4]. This cell population can act as progenitor cells and generate new lineages of liver epithelia.

In the search for stem-like liver cells it is reasonable to recall liver morphogenesis. In embryonic life, liver originates from the ventral foregut epithelium forming the hepatic diverticulum and the liver bud. In the early stages, growth factors and tissue interactions form endoderm and mesoderm, and they shape the process of liver development. During hepatogenesis, hepatic endoderm cells (hepatoblasts) differentiate and maturate gradually into functional hepatocytes and biliary epithelial cells. Other liver cells for example von Kupffer cells, stromal cells and blood vessels derive from the mesoderm. Many of the necessary genes and molecular pathways have been identified [5]. Endoderm patterning and pathways of hepatic maturation have an impact on liver regeneration and pathophysiology. In this context, differentiation markers are helpful to trace cellular fluctuation in repair situations. One of such marker molecules is alpha-1-fetoprotein (AFP).

Protein patterns vary in embryonic and postnatal life. They are signs of current gene activity in the developmental stages of histogenesis and organ differentiation. AFP has obtained some considerable relevance because of its association with cell differentiation in onco-developmental processes. Since its detection in embryonic life and in human diseases, this protein is studied in many experimental settings to unravel developmental pathways, regeneration, and carcinogenesis. Over the years, a lot of papers and opinions on liver physiology, liver diseases, regeneration and hepatocarcinogenesis were published [6-26]. Stem-like progenitor cells and their differentiation pathways are of special interest [27-32]. However, the properties of regenerative cells for tissue repair and their abilities such as differentiation, transdifferentiation, dedifferentiation and, most importantly, the possibility of reprogramming into certain cell types are not yet solved [33-36]. In this review we will show how alpha-1-fetoprotein (AFP) expression can mark cellular fluctuations in the liver under various pathophysiological conditions and highlight origin and fate of progenitor cells in the adult liver.

Historically, AFP became the first useful biomarker in liver research and diagnostics since G.I. ABELEV and YU TATARINOV described the occurrence of this protein in fetuses and in malignant liver tumors [37, 38]. Their observations confirmed the existence of specific fetal serum proteins that have been already postulated in previous reports [39, 40] and in reports regarding the relationship between embryonic and cancer tissues [41]. These observations have initiated new ideas in cancer research by linking carcinogenesis

and developmental biology [42], the era of oncodevelopmental aspects in tumor biology emerged.

AFP is a normal constituent of fetal serum and amniotic fluid of many species. Under physiological conditions its synthesis occurs in the yolk sac, the gastro-intestinal tract, and the liver of embryos in many species [43-45]. In late embryonic life, the liver is the main organ of AFP synthesis, then this protein disappears after birth [39, 44, 46]. AFP expression is strongly suppressed in adult life. However, AFP may reappear in substantial quantities during processes of liver repair and malignant transformation, and measurement of AFP has proved to be of value in the diagnosis of hepatocellular cancer and endodermal sinus tumors [33, 37, 38, 47, 48]. Elevated serum AFP levels also occur in non-malignant diseases of human liver, i.e., forms of regeneration associated with viral hepatitis, alcoholic liver cirrhosis and partial hepatectomy [49-51]. AFP expression has become a valuable marker in biomedical studies and in clinical medicine [33, 37, 47, 52].

In animal experiments, such as partial hepatectomy and ingestion of hepatotoxins (e.g., CCl_4 or galactosamine-HCl induced liver injury) or genotoxic carcinogens temporary elevation of AFP levels have been reported [53-66]. Interestingly, some studies have shown similarities between galactosamine-induced liver damage in the rat and acute human viral hepatitis [67, 68]. AFP elevation during the different stages of experimental hepatocarcinogenesis 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 [54, 57, 69-72]. 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 [71, 73].

In any case and with respect to cellular fluctuations in the liver, AFP elevation depends largely on effects such as cell death, mitotic activity, and dynamics of cellular differentiation [58, 74]. Furthermore, regeneration patterns differ from one another in their ability to cope with damage types. Some injuries impair or even block proliferation of adult hepatocytes while other injuries do not so. Possibly, stem-like cells become engaged to renew lost tissue [1]. It is expected that models of induced liver injury by surgical and pharmacological interventions and differences in traumatism allow the view on inherent distinct processes of regeneration.

The adult liver harbors progenitor cells with the ability to differentiate either into hepatocytes or into cholangiocytes comparable to hepatoblast differentiation in embryonic life [75, 76]. Then, hepatic progenitor cells should behave like bipotential stem cells and expected to lie in the proximity to both hepatic and biliary compartments. This suggestion is supported by results of THEISE et al. [4] who studied normal livers as well as those with massive necrosis by immunostaining for cytokeratin 19 (CK19). They determined three-dimensional relationships by use of serial sections. From those 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 [77], radiating from the

portal tract. 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.

Re-expression of AFP in adult life occurs when hepatocytes proliferate. Thereby, this protein is a dedicated marker in studies on cell differentiation, tissue repair and hepatocarcinogenesis. AFP proved already useful in earlier studies with serology and immunofluorescent labelling techniques [46, 57, 64, 65, 78-85]. In the search for details of AFP synthesis and cellular fluctuations, immunohistology with peroxidase methods proved very promising and more suitable to detect histological changes in more detail than conventional techniques with immunofluorescence [58, 86, 87]. It is straight to note that other models including transgenic animals are equally useful to enable insights into the inherent ability of hepatocytes or bile ductular cells for hepatic regeneration [17, 34, 88-96].

Some major discussions pertained to the possibility of canalicular epithelial cells in hepatic restitution of damaged liver tissue with pros and contras for the population of so-called oval cells as progenitor or stem-like cells originating from Hering's canaliculi, equivalent in meaning with the canals of Hering, the intrahepatic bile ductules [77]. The nomenclature for one and the same cell structure as putative progenitor cells is sometimes confusing. Popular formulations for the term "oval cells" are notations such as canalicular epithelial cells, bile ductular cells, biliary epithelial cells, bile ductules, ductular oval cells, neoductules and ductular progenitor cells. Finally, they all together address the same cell type.

In our studies, we followed cellular fluctuations in various injury situations with focus on putative progenitor cells. The experimental approach included several injury models and the use of AFP as differentiation marker. Re-expression of AFP in postnatal life seemed to be a useful rationale since this oncodevelopmental protein is known to be associated with differentiation stages and pathophysiological developments. The models included (a) partial hepatectomy by surgery; (b) acute carbon tetrachloride intoxication; (c) hepatotoxicity by D-galactosamine (GalN) or by toxic doses of N-nitrosomorpholine (NNM) which inhibit hepatocytes to regenerate; and (d) carcinogenesis based on the use of the genotoxic NNM in both low and high doses, respectively. The necessary immunological and histological methods were adapted for this approach.

2 Materials and Methods

2.1 Animals

Injury models served to study progenitor cells in liver repair and hepatocarcinogenesis. Rats and mice are the most widely used animals for such purpose. Twelve-week-old rats and mice of male sex were segregated for the experiments. The well characterized BD X rat inbred strain was chosen. This rat strain proved to be appropriate in long-term studies on carcinogenesis because of its low incidence of spontaneous tumors [97, 98]. BD X rats were originally obtained from Prof. Druckrey (Forschergruppe für Präventivmedizin, Max Planck Institut für Immunbiologie Freiburg, Germany) and later bred in facilities of the Institut für Versuchstierkunde at the German Cancer Research Center (DKFZ Heidelberg). BALB/cJ mice were from the The Jackson Laboratory (Bar

Harbor, USA), and C3H/He mice from the Institut für Versuchstierkunde (Hannover, Germany). The inbred mouse strains BALB/cJ and C3H/He were chosen because both strains show significant differences in synthesizing the onco-developmental marker AFP. Several mechanisms of AFP gene control are mainly responsible for this [99, 100].

Parts of the experimental work were carried out at the German Cancer Research Center (DKFZ, Heidelberg, 1975-2006) and supported by research grants from the German Research Foundation (DFG, Deutsche Forschungsgemeinschaft). Mice and rats were held in the animal care facility of the DKFZ and maintained in air-conditioned rooms on a 12-hour day night cycle with free access to food and water. Studies were in accordance with German rulings to protect animals and according to guidelines and welfare assurance [101-103]. Animal studies were done under veterinarian surveillance by the Institut für Versuchstierkunde (DKFZ). Experimental and control groups were processed in parallel, the studies are summarized in Table 1.

In the case of surgical intervention such as partial hepatectomy or at autopsy for liver preservation and at the end of experiments, animals were maintained under anesthesia e.g., IP injection of ketamine and xylazine combinations or sodium pentobarbital or inhalation anesthesia with isoflurane [101-103]. Exsanguination was done by cardiac puncture under anesthesia, then animals were sacrificed by cervical dislocation.

Table 1: Experimental models in studies on hepatic repair and chemical hepatocarcinogenesis

Animal models	**Treatment**	**Study period, controls**
Normal controls — BALB/cJ mice — C3H/He mice — BD X rats	No treatment animals kept on standard diet and tap water	12-week-old animals no treatment
Partial hepatectomy — BALB/cJ mice — C3H/He mice — BD X rats	70% resection controls: sham operation	1-7 days post-op 5 animals per day
CCl_4 intoxication — BALB/cJ mice — C3H/He mice — BD X rats	100 µl CCl_4/100 g body weight controls: oral ingestion of liquid paraffin	1-7 days following injury 5 animals per day
GalN injury — BD X rats	375 mg/kg body weight (a) first dose at 8:30 a.m. (b) second dose between 8.00 and 9.00 p.m. controls: no treatment	1-10 days following injury 5 animals per day
Carcinogenesis by NNM — BD X rats	Chemical carcinogenesis (a) low dose feeding group 6 mg NNM/kg/day for 12 weeks (b) high dose feeding group 20 mg NNM/kg/day for 6 weeks (c) latent period	Carcinoma development (a) all animals bled at weekly intervals for serum AFP and 2 rats studied by histology (b) all animals bled at weekly intervals for serum AFP and 2 animals studied by histology (c) all animals bled at monthly intervals for serum AFP and 2 rats studied by histology

Abbreviations: CCl_4, carbon tetrachloride; GalN, D-galactosamine; NNM, N-nitrosomorpholine.

2.1.1 Partial hepatectomy

Surgical resection of liver lobes was essentially as described [104-106]. The use of magnifying operative loupes was useful in the mouse model. Intense regeneration will follow partial hepatectomy (about 70% liver weight) and proceeds fast within 6 to 8 days after operation. Histology is shaped by mitotic activity of the remaining mature hepatocytes [107]. Animals were bled 1 to 7 days after injury, livers were dissected out for histology.

2.1.2 Carbon tetrachloride

Carbon tetrachloride is a classical hepatotoxin leading to necrosis and apoptosis mainly in centrilobular areas [108]. Toxicity depends on a cytochrome P450 dependent monooxygenase system of the hepatocytes and on mechanisms generating toxic products such as free radicals. The latter are responsible for a peroxidative decomposition of microsomal and membrane lipids leading to structural damage and cell death [109, 110]. CCl_4 injuries were caused in rats and mice by the application of 100 µL CCl_4/100 g body weight (1 mL of 10% CCl_4 in liquid paraffin/100 g body weight). A single dose was directly applied into the esophagus by means of a trocar and syringe [66]. Animals were bled and livers dissected out between 1 to 7 days following injury.

2.1.3 Galactosamine

Galactosamine-HCl (GalN) leads to liver alterations similar in appearance to human viral hepatitis [67, 68, 111]. Liver injury by GalN is caused by its interference with the cellular uridine pool accompanied by reduced RNA synthesis and blocking of transcription [112, 113]. Hepatocyte necrosis and inflammatory reactions occur while the intrinsic hepatocyte regeneration is impaired. Toxic effects are dose dependent, the structural injuries are suggested to be secondary to the metabolic lesion. GalN was applied by IP injections of GalN in 0.9% NaCl, 375 mg/kg body weight [60, 84]. Livers and blood were taken 1 to 10 days after injury.

2.1.4 N-Nitrosomorpholine

The genotoxic carcinogen N-nitrosomorpholine (NNM) was synthesized by Dr. R. Preussmann (DKFZ, Division of Toxicology, Heidelberg). NNM was given in different doses in drinking water: (a) 6 mg/kg/day for 12 weeks or (b) 20 mg/kg/day for 6 weeks; all treatments were stop experiments [114]. Carcinogen intake was controlled by daily measurements of water drunk. NNM is known to produce liver tumors. Different malignant phenotypes may develop in the same liver. While low NNM doses have no significant toxic effects, high NNM doses are clearly toxic [98]. Hepatocellular carcinomas developed with either of the used NNM feeding schedules. Animals were bled at defined intervals for AFP detection and histological analysis.

2.1.5 DNA synthesis

Tritiated thymidine (^{3}H thymidine) was purchased from NEN Radiochemicals (Boston, USA). Thymidine incorporation in vivo was studied by intraperitoneal injections of [^{3}H] thymidine (spec. act. 6.7 Ci/mmole) according to these schemes: (a) 60-min pulse labelling by injecting 500 µCi/rat; (b) pulse labelling over 24 h by three successive injections of 250 µCi each; (c) pulse labelling for 60 min by injection of 500 µCi/rat followed by daily injections of unlabelled thymidine for 7 days [58]. Livers were dissected out and treated as described in section *2.7 Tissue fixation and processing for microscopy* and in section *2.11 Autoradiography*.

2.1.6 Control animals

Animals were kept on standard diet and tap water ad libitum. They were either untreated or sham operated or given oral ingestions of liquid paraffin.

2.2 Chemical reagents

Biochemicals and standard laboratory reagents of highest available purity were obtained from Merck (Darmstadt) unless noted otherwise. Horseradish peroxidase (HRP, EC 1.11.1.7, approx. 1000 units/mg solid using ABTS) and glucose oxidase (GOD, EC 1.1.3.4, 100,000-250,000 units/g solid using peroxidase as the coupling enzyme) were from Boehringer (Mannheim) and from Sigma-Aldrich (München).

Gel filtration methods were applied for molecular size sieving. Separation of biological macromolecules according to their molecular weight is a major application. Different particle size grades and degrees of concentration or cross-linking enable various fractionation ranges for globular proteins. Furthermore, gel media are used for the preparation of immunosorbents by covalent coupling of antigens or antibodies onto the gel matrix. For these purposes, media from dextran, acrylamide, agarose, or combinations of all of them were used. Inert gel media were obtained from Sigma-Aldrich (München) e.g., Sephadex and Sepharose, products from Pharmacia Uppsala Sweden), then further processed according to laboratory needs. A selection of gels for the separation of high molecular weight molecules is shown in Table 2.

Table 2: Gel filtration media for sieving of high molecular weight proteins

Filtration medium	Gel matrix	Fractionation (MW) [a]
Sephadex G-200	Dextran	$5 \times 10^3 \ldots 6 \times 10^5$
Sephacryl S-200	Dextran/acryl.	$5 \times 10^3 \ldots 2.5 \times 10^5$
Sephacryl S-300	Dextran/acryl.	$1 \times 10^4 \ldots 1.5 \times 10^6$
Sephacryl S-400	Dextran/acryl.	$2 \times 10^4 \ldots 8 \times 10^6$
Sepharose 2B/CL-2B	Agarose	$7 \times 10^4 \ldots 40 \times 10^6$
Sepharose 4B/CL-4B	Agarose	$6 \times 10^4 \ldots 20 \times 10^6$
Sepharose 6B/CL-6B	Agarose	$1 \times 10^4 \ldots 4 \times 10^6$

[a] Molecular weight (Da), fractionation range

2.3 Biochemical and serological methods

Reagents for serology and immunohistology such as immune sera, purified antigens, antibodies, and antibody-enzyme conjugates were prepared using selected methods. Qualitative and quantitative controls followed standard methods (Table 3). These methods covered protein determinations with the Folin-phenol reagent [115] and the quantitation of enzyme activities [116]. Furthermore, standard methods for the qualitative and quantitative evaluation of antigens and antibodies are based on electrophoresis in polyacrylamide gels [117, 118], immunoelectrophoretic analysis in agar or agarose gels [119]. Also, various single and radial immunodiffusion techniques as well as electro-immunodiffusion methods in agarose gel were used [120-123]. For enhanced sensitivity of the gel diffusion techniques, an enzyme amplification method using GOD labelled antibodies has been adapted for quantitation [124].

Enzyme-linked immunosorbent assays (ELISA) and technical adaptations of the original method to microtiter plates served to measure very low quantities of AFP in serum [125-127]. Antigen measurements were carried out by an antibody sandwich method [125]. The solid phase coated with rabbit anti-AFP antibodies acted as capture antibodies for AFP binding from serum specimens. After washings, detector antibodies (peroxidase labelled sheep anti-AFP) followed with the next incubation step. The formed sandwich complexes were revealed with peroxidase substrate. The colored reaction product was measured in a photometer. Assays were performed with serum dilutions and in duplicate. The test profile included control animals and standards with known AFP concentrations. AFP concentrations were determined by a curve fitting programme of the spectrophotometer. The detection limit of serum AFP was in the order of 5 ng/mL [114].

Table 3: Standard methods for antigen and antibody evaluation

Method	Main use, application	References
Electrophoresis polyacrylamide	Fractionation and analysis of biomolecules	Shapiro AL et al. 1967 Weber K, Osborn M 1969
Double radial immunodiffusion	Immuno-serological properties of antigens and antibodies	Ouchterlony Ö 1949
Single linear immunodiffusion	Analysis of antigen-antibody reactions for quantification	Oudin J 1946 Oudin J 1949
Single radial immunodiffusion	Quantification of antigens and antibodies	Mancini G et al. 1965
Immuno-electrophoresis	Immuno-serological properties of antigens and antibodies	Grabar P, Williams CA 1953
Electro-immunodiffusion rocket electrophoresis	Immuno-serological properties, quantification of antigens	Laurell CB 1965 Laurell CB 1966 Clarke HGM, Freeman T 1968 Kuhlmann WD 1984
Enzyme-linked immunosorbent assay	Quantification of antigens and antibodies	Engvall E, Perlmann P 1971 Schuurs AH, van Weemen BK 1977 Kuhlmann WD, Peschke P 2006

2.4 Purification of AFP

AFP was purified from amniotic fluids (taken from 14 days old mouse and rat embryos, respectively) using preparative electrophoretic and immunosorbent methods. In a first step, amniotic fluid was submitted to gel electrophoresis in 5% acrylamide-0.8% agarose plates [128]. AFP molecules migrating in the α1-zone were washed out from the supporting matrix by homogenization of the gel matrix followed by centrifugation. Purity of AFP was determined by SDS polyacrylamide gel electrophoresis [129, 130] and by various gel diffusion techniques (section *2.3 Biochemical and serological methods*) making use of cross-reactivity between mouse and rat AFP. For controls, a rat AFP immune serum was obtained from Nordic Immunological Laboratories (Netherlands).

The purified AFP molecules were used to immunize rabbits to prepare specific anti-AFP immune sera. The latter were absorbed with glutaraldehyde cross-linked normal mouse serum proteins (rat serum proteins, respectively) with the aim of eliminate possibly contaminating antibodies and to render them monospecific. Immunosorbent columns were finally prepared from those monospecific immune sera [58, 86].

2.4.1. Scaling of AFP preparation

Immuno-affinity chromatography is presumed to be one of the most powerful separation techniques because of the high specificity of the antigen-antibody reaction. For example, antigens or antibodies can be coupled to agarose beads and used for selective isolation of antibodies or antigens, respectively. In the case of AFP purification, immunosorbents were prepared by covalent binding of γ-globulin fractions of anti-AFP immune sera (γ-globulin fractions fractionated beforehand by DEAE ion exchange chromatography) to Sepharose 4B matrix (Pharmacia, Sweden). First, cyanogen bromide is reacted with agarose hydroxyl groups to form imidocarbonates and cyanate esters [131, 132]. These active groups will form isourea linkage with amino groups of the ligands to be added in the second step (specific antibodies). In the third step, the immobilized ligand is used as an immunosorbent that will bind the corresponding antigen in a reversible manner from added amniotic fluid. Unreactive substances are washed off from the immunosorbent by adding phosphate buffered saline (PBS) followed by washing with phosphate buffered saline (PBS) and with 2 M NaCl in 0.1 M NaOH-glycine buffer pH 9. Bound AFP molecules are finally eluted from the immunosorbent by treatment with a desorption buffer pH 7.4 containing 3 M chaotropic ions [133].

Eluted AFP fractions were filtrated on immunosorbent columns prepared by coupling γ-globulin fractions from immune sera against normal mouse (normal rat serum proteins, respectively) to Sepharose 4B beads. These immunosorbent columns served to bind traces of contaminating normal plasma proteins to secure the purity of AFP. Purification processes were monitored by immunological means and by SDS polyacrylamide gel electrophoresis (see above).

2.5 Immune sera, antibodies

Antibodies are produced by immunization of animals. Typical species are rabbits. Also, sheep, goats, donkeys, or horses are immunized when large volumes of immune sera are needed. We made antibodies usually in rabbits and sheep.

Immune sera against the bulk of serum proteins were obtained by immunization of rabbits with normal adult mouse and rat serum proteins (whole serum, respectively). Primary injections were done with whole serum mixed up with complete Freund's adjuvant. Booster injections followed at monthly intervals with the same antigens emulsified in incomplete Freund's adjuvant. Injections were intramuscularly and subcutaneously at different sites [134, 135].

Immune sera against antigens such as rat and mouse IgG, rat and mouse AFP and enzymes (GOD) were prepared by immunization of rabbits with the respective antigens. Injections were done with pure antigen in saline and mixed with complete Freund's adjuvant. Booster injections (intramuscularly and subcutaneously) followed one month later with antigen dissolved in saline and mixed with incomplete Freund's adjuvant. Further booster injections followed at monthly intervals. The blood was tested for antibodies two weeks after the fifth injection.

Antibodies were also raised in sheep by immunization with rat AFP, mouse AFP and rabbit IgG, respectively. In each case, primary injections were done with purified antigen in saline and emulsified with Freund's adjuvant. Animals received subcutaneous injections at different sites (shoulder and hip region). Booster injections were repeated at monthly intervals for 6-8 months.

Prior to use, immune sera and purified antibodies were submitted to specificity testing because minor contaminants may produce significant amounts of undesired antibodies. Immunological specificity was controlled by standard methods (see section *2.3 Biochemical and serological methods*). Immuno-staining of tissue arrays with known antigen targets and known histological pattern were included as validation tools.

Immune sera raised against purified antigens were routinely absorbed with insoluble immunosorbents prepared by glutaraldehyde cross-linking of unrelated proteins (normal adult serum proteins). This measure secured the specificity of immune sera as much as possible. The effect was controlled by standard methods (section *2.3 Biochemical and serological methods*). There exist several techniques of antibody preparation from whole immune sera with varying degrees of purification (Table 4). The needed degree of purification, however, depends to a great extent on the type of experimentation. For routine work, we used antibodies being prepared either by DEAE ion exchange chromatography or by gel filtration (Sephadex G 200). Immunosorbent methods proved in all applications most suitable [132, 133, 136]. Because immunoaffinity chromatography is quite elaborate, this method was mainly preferred for the preparation of enzyme-antibody conjugates in immunohistology and in enzyme-immunoassays of the ELISA type to quantify serum AFP [124].

Table 4: Methods for antibody purification

Method	Application	Advantage	Annotation
Ammonium sulphate	Fractionation of immune sera by salt precipitation. Crude method to prepare γ-globulin fraction from whole serum	Not expensive step for prepurification of immunoglobulins, convenient for bulk preparations	Delimited purification, not recommended as single purification step
DEAE ion exchange	Chromatography for partial purification of γ-globulins/antibodies	Purification degree better than with ammonium sulphate precipitation	Higher claims of purity need additional steps, f. e. immunoaffinity chromatography
Caprylic acid Octanoic acid	Caprylic acid precipitation of immunoglobulins, easy but crude method to prepare γ-globulins and antibodies	Prepurification step and convenient for whole immune sera	Delimited purification, not recommended as single purification step, higher claims of purity need additional steps
Hydroxyapatite chromatogr.	Ion exchange and metal chromatography CHT) for preparation of immunoglobulins (Ig) and antibodies	Purification degree equivalent to DEAE chromatography	For higher claims of purity, additional measures are needed
Gel filtration Sephadex Sephacryl	Separation of proteins and antibodies according to molecular mass	Protein separation with high degree of purification	Limited capacity of gel columns, dilution effects to be considered
Protein A or Protein G binding	Protein A or G bound to insoluble matrix, affinity chromatography of immunoglobulins (Ig), species and isotype selectivity	High purification degree and high yield of Ig	Not suitable for all animal species and Ig isotypes
Immunosorbent affinity	Antigens or antibodies covalently bound to insoluble matrix and selective immunoaffinity chromatography	Top-level of purification, scaling possibilities	Elaborated method, sophisticated skills needed

2.6 Antibody-enzyme conjugates

From the described marker molecules, one can select a likely label for the study aim. In any case, enzymes and enzyme substrates must allow reliable visualization and sufficient sensitivity. Horseradish peroxidase (HRP), glucose oxidase (GOD), alkaline phosphatase (AP) or β-galactosidase (β-Gal) are of equal value for labelling purpose (Table 5). It should be minded that highly purified antibodies are preferred to our experience when using enzyme labelling techniques in histology [124, 137].

Table 5: Enzymes and enzyme substrates for immunohistology

Enzymes	Substrates [a]	Advantage	Annotation
Peroxidase (*horseradish*)	DAB (3,3'-diamino-benzidine *plus* H_2O_2 AEC (3-amino-9-ethyl-carbazole) *plus* H_2O_2 CN (4-chloro-1-naphthol) *plus* H_2O_2 p-Phenylenediamine dihydrochloride (Hanker-Yates)	Many enzyme substrates available Conjugation of antibodies with HRP easily done Dual or multilabelling with different antibody and substrate sets	Be aware of endogenous peroxidases Endogenous enzyme inhibition encouraged Protocols for enzyme inhibition available
Glucose oxidase (*Aspergillus niger*)	NBT (Nitro Blue Tetrazolium) *plus* Phenazine methosulfate INT (Iodophenyl-Nitrophenyl Tetrazolium) *plus* Phenazine methosulfate TNBT (Tetra Nitro Blue Tetrazolium) *plus* Phenazine methosulfate Enzyme amplification with two enzymes (coupled GOD-HRP method) Glucose *plus* DAB (3,3'-diamino-benzidine)	No endogenous enzyme activity in mammalian tissues Enzyme substrates for different colors available Dual or multilabelling with different antibody and substrate sets Enhanced sensitivity by enzyme coupling, amplification with co-immobilized HRP as secondary system enzyme	Sensitivity comparable to HRP Sensitivity enhanced by two-step enzyme amplification method Sensitivity enhanced by DAB-nickel method
Phosphatase (alk.) (*calf intestine*)	BCIP (Bromo-Chloro-Indolyl-Phosphate *plus* Nitro Blue Tetrazolium Naphthol AS-MX *plus* Fast Red TR Naphthol AS-MX *plus* Fast Blue BB Naphthol AS-BI *plus* New Fuchsin	Enzyme substrates for different colors available Sensitive coupled enzyme substrates available Double labelling with different antibody and substrate sets	Endogenous enzyme activities in many cells and tissues (species dependent) Endogenous enzyme inhibition encouraged
Beta-galactosidase (*E. coli*)	BCIG (Bromo-Chloro-Indolyl-Galactoside) *plus* ferri-/ferrocyanide	Different enzyme substrates available	Endogenous enzyme activities observed Endogenous enzyme inhibition suggested No interference with mammalian enzyme with marker enzyme at its optimal pH

[a] Enzyme substrates for immunohistology

Antibody-enzyme conjugation is done by covalent bonds between enzyme and antibody. Various conjugation reagents and methods are available. Most labelling strategies use methods that modify the amine, carboxyl, or thiol groups of proteins for stable cross-linking of the molecules. Conjugation procedures may be carried out in so-called one-stage, two-stage, or multiple stage reactions. Experience exists with hetero-bifunctional or homo-bifunctional reagents. Also, specific modification of protein molecules or the use of reagents with differing reactivity towards two protein molecules are useful (Table 6). For convenient bench work, ready-to-use reagent kits can be recommended.

Table 6: Techniques for conjugation of antibodies and enzymes

Technique	Reaction	Conjugation [a]	Advantage
Glutaraldehyde chemistry	H_2N groups	One- or two-step procedure	Easy operation
Carbohydrate chemistry	Aldehyde activated carbohydrates, H_2N groups	Two-step procedure [b]	Easy operation
Carbodiimide chemistry	COOH, H_2N groups	One-step procedure	Easy operation
Isocyanate chemistry	SH, H_2N groups	Two-step procedure	Easy operation
Maleimide-thiol and thiol-thiol chemistry	SH, H_2N groups	Thiolation	Easy operation

[a] Solubility, solvent and pH depend on the given reagent class

[b] Marker molecule (HRP) modified by fluorodinitrobenzene and carbohydrate portion oxidized by sodium periodate which gives HRP-aldehyde to form a Schiff base between aldehyde and amino group

We applied with good success glutaraldehyde as cross-linking reagent for antibody-enzyme conjugation. Glutaraldehyde reacts almost exclusively with ε-amino groups of proteins. Studies on the reaction of glutaraldehyde with proteins have been described earlier [138-140]. This covalent coupling molecule is preferably done in a two-step procedure [141]. The conjugation products prepared by this procedure have in gel filtration techniques an estimated molecular weight in the order of 200,000 Da.

In a first step, the enzyme marker (f. e. HRP) reacted with one of the two aldehyde groups while the second free aldehyde group remained free for the combination with an amino group of antibodies added in the second step. This conjugation type was possible because of the low proportion of lysine in HRP [142].

Unreacted glutaraldehyde was eliminated by gel filtration on a column of Sephadex G 25 fine. In the conjugation step, the "activated" enzyme fraction was mixed with purified antibody in 0.5 M carbonate buffer pH 9.5 for 24 hours at 4° C. Free (unreacted) aldehyde groups were blocked by dialysis against 0.1 M ethanolamine-HCl buffer pH 7.4.

Antibody-enzyme conjugates are purified from unconjugated molecules by gel filtration with Sephadex G 200 or with Sephacryl S-200 columns. Apart from gel filtration, antibody-HRP conjugates are readily purified by lectin affinity chromatography with Concanavalin A coupled to Sepharose 4B beads [124].

2.7 Tissue fixation and processing for microscopy

Various additive and coagulant solutions (aldehydes, solvents) and fixation times were tried to determine adequate liver fixation. Fixation of tissue blocks and slices of about 0.5 cm thickness in 96-99% ethanol-1% acetic acid mixture for 12-15 h at 0-4° C was most convenient for the detection of AFP in tissue sections from paraffin embedded specimen. Fixed tissue was dehydrated in absolute ethanol, cleared in benzene or benzene substitute

(chloroform, xylene) and embedded in paraffin (Paraplast®). Microtome sections with standard thickness of approx. 4 to 5 µm were collected on slides, deparaffinized in xylene and passed from absolute ethanol into 0.1 M phosphate-buffered saline pH 7.2 (PBS) prior to immunocytochemistry [86].

Alternatively, tissue blocks from fresh liver were prepared for cryostat sectioning and served for control purposes. Air-dried sections were fixed at 4° C for 5 min in PBS containing 6% formaldehyde and washed in PBS prior to incubation. Immuno-staining of AFP was essentially as described [143]. In addition, liver specimens were embedded in Epoxy resin. 1 to 1.5 µm thick tissue sections were mounted on glass slides. Prior to immuno-staining, the resin medium was partially removed by etching with sodium methoxide [144].

2.8 Immunocytochemistry

Methods for the detection of tissue antigens date back to the early work of AH COONS and co-workers [145-147]. The principles of direct and indirect antigen-labelling in cell preparations are still the same as those described for immunofluorescent staining [147, 148]. Numerous modifications have been published since then. Either direct or indirect assay principles may be chosen, and one can select from many detection and labelling formats. In any case, there is no defined standard method. The selection of a staining technique is based on the type of tissue to be stained. Usually, one must adapt the detection procedure to the diverse requirements. Thus, we needed extensive preliminary tests for our objectives [58, 86, 114, 149].

The procedure of AFP detection in tissue sections followed the antibody sandwich technique (Figure 1) where the primary antibodies (anti-AFP) derived from rabbits; the secondary antibodies were from sheep (anti-rabbit IgG being conjugated with HRP), and the formed enzyme-labelled antigen-antibody complex became finally detected by incubation of the tissue section in appropriate enzyme substrate.

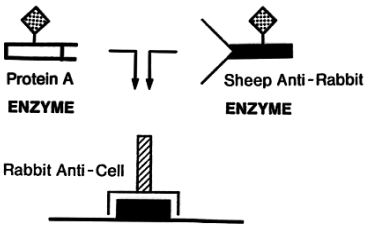


Figure 1: Indirect immuno-staining with enzyme-labelled sandwich antibodies or with labelled Protein A. In the first step, the cell preparation is incubated with unlabelled primary antibody (rabbit anti-cell antigen). In the second step, an enzyme labelled Sandwich (sheep anti-rabbit IgG) or enzyme labelled Protein A is added. After washing steps, an appropriate enzyme substrate is used to reveal the antigen-antibody complex.

Prior to immunohistological staining, endogenous peroxidases in tissue sections were blocked by treatment with 1 % hydrogen peroxide in PBS for 1 h at room temperature [86]. Slides were immuno-stained using an indirect method: (a) incubation was first with unlabelled anti-AFP antibodies (0.01, 0.1, 0.5 mg of anti-AFP antibodies for 20 min or alternatively with 0.005 to 0.01 mg of anti-AFP antibodies for 24 h at 4° C), then followed by (b) second incubation with HRP conjugated sheep anti-rabbit IgG antibodies (0.1 mg/ml) for 20 min at room temperature [114].

Non-reacting antibodies were removed by successive washings in PBS supplemented with 1 % bovine serum albumin and 0.5 M NaCl. Peroxidase activity was revealed by DAB (3,3'-diaminobenzidine-HCl), 0.5 mg/ml in 0.2 M Tris-HCl buffer pH 7.2 containing 0.01% hydrogen peroxide [150].

Apart from HRP as cytochemical marker, GOD labelling was a useful alternative to HRP as marker in AFP immunostaining. A two-step enzyme method was applied [151]. GOD and HRP molecules were co-immobilized in proximity by immunological bridging at the cellular sites of AFP in tissue sections. In this enzyme-amplification technique, H_2O_2 is generated by the reaction of GOD with D-Glucose which serves as substrate for HRP in the oxidation of chromogens such as DAB (Figures 2-3). The coupled enzyme substrate system delivers a colored and insoluble stain. Co-immobilization of both enzymes (GOD and HRP) and the coupled enzyme substrate system work efficiently in immunohistology. It is also advantageous that endogenous GOD activity is not present in mammalian tissue that might corrupt the cytochemical procedure. Finally, we can exploit the widely known usefulness of DAB as cytochemical reagent.

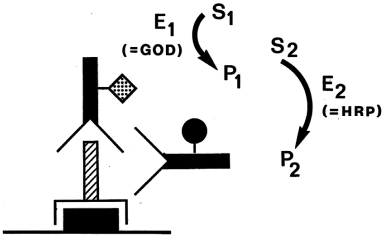


Figure 2: Enzyme amplification in immuno-staining by co-immobilization of GOD (E_1) and HRP (E_2) molecules. In the coupled enzyme reactions, E_1 catalyses substrate S_1 to give P_1 which in turn serves as substrate S_2 for HRP (E_2) to oxidize the added chromogen into the insoluble and colored product P_2 (for example DAB, diaminobenzidine).

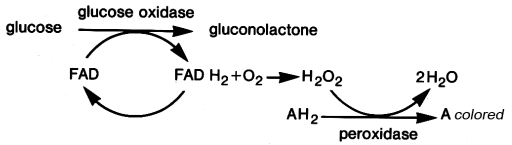


Figure 3: Two-step enzyme technique in the coupled GOD-HRP staining method with HRP as secondary system enzyme. GOD generates hydrogen peroxide by the oxidation of D-glucose. Then, hydrogen peroxide serves as substrate for HRP which oxidizes the chromogen AH_2 to yield an insoluble colored product ($A_{colored}$), described by Kuhlmann and Peschke [151].

Immuno-stained sections were optionally treated with 0.1% OsO4 in PBS for 1 min. In addition, tissue sections were counterstained with Mayer's hemalum for 2 min using a formula described previously [152]. For other histological procedures see *2.10 Histologic stains*.

2.9 Specificity controls

The blocking effect of endogenous peroxidases by hydrogen peroxide was checked out by incubation with peroxidase substrate alone. Further controls included tissue sections incubated in (a) normal rabbit IgG globulins; (b) rabbit anti-AFP antibodies absorbed by an immuno-adsorbent (AFP coupled to cyanogen activated Sepharose 4B); (c) rabbit anti-mouse and anti-rat IgG antibodies; (d) rabbit anti-glucose oxidase antibodies. Each step was followed by enzyme conjugates of sheep anti-rabbit IgG and enzyme substrate. Control incubations were used to detect unrelated staining such as non-specific binding of antibodies to tissue components and, also, to monitor uptake of serum proteins from extracellular spaces which might prevail in necrotic areas or from leakage of soluble substances into liver cells for various reasons [124].

2.10 Histologic stains

Tissue sections were conventionally stained by haematoxylin alone or by haematoxylin and eosin (HE), toluidine blue, methyl green, and by Gomori's silver impregnation. Glycogen accumulation was detected in tissue sections from overnight starved animals by the periodic acid-Schiff (PAS) method [152]. Semithin resin sections were stained with azure-methylene blue or with toluidine blue [153].

2.11 Autoradiography

The stripping film technique (Kodak AR 10 Glass Stripping Plates) was applied [154-156]. Microscopic preparations were exposed for 21 days at 4° C and developed with Kodak D 19 for 5 min [58]. After fixation, the preparations were optionally counterstained with haematoxylin and mounted in glycerol-gelatine. Labelled cells were taken to be those which had more than five developed silver grains above the nucleus.

3 Results

For serology and cell biological applications including immunohistology, pure and specific reagents in terms of immunology are needed. Purity of reagents, antigens and antibodies are important requirements for the specificity of experiments. Apart from the quality of reagents, immunohistological staining must be secured and monitored by adequate controls. For example, movement of molecules from extracellular spaces due to passive diffusion into tissue cells, displacements by damage and necrosis or other inaccuracy must be discerned from specific staining patterns. Methods and reagents were tested in parallel experiments and controlled using appropriate procedures.

3.1 Preparation of Reagents

Experimentation started with the purification of AFP molecules from mouse and rat amniotic fluids. The method of preparative electrophoresis in agarose-polyacrylamide gel as supporting matrix was chosen because classical purification methods such as solvent and salt precipitation or chromatography were not effective enough. AFP purification by preparative electrophoresis was a reliable procedure. Purity of AFP was confirmed by SDS polyacrylamide gel electrophoresis (Figure 4). Enough amounts of molecules could be obtained in each run for control purpose and for immunization of animals. AFP molecules and amniotic fluids yielded single precipitation reactions in both Ouchterlony's double diffusion tests and in immunoelectrophoresis (Figure 5). No reactivity was observed with normal adult plasma proteins.

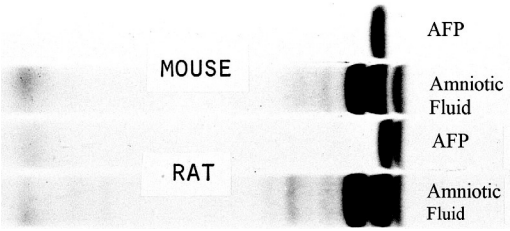


Figure 4: SDS-PAGE electrophoretic analysis of amniotic fluid from mouse and rat and the purified AFP molecules, respectively. After electrophoretic separation of the probes, the gels were stained with Coomassie Blue. Mouse AFP occurs as a single zone; rat AFP shows a characteristic double zone.

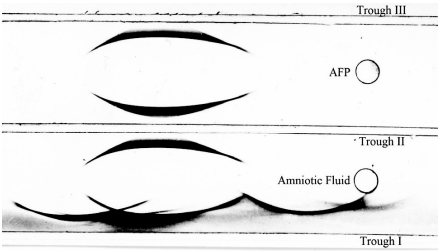


Figure 5: Immunoelectrophoretic analysis in agarose gel. First, electrophoretic separation of amniotic fluid and purified AFP. Then, troughs are cut in the gel and filled with immune sera. *Trough I*: rabbit anti-amniotic fluid serum. *Trough II*: rabbit anti-AFP serum. *Trough III*: rabbit anti-AFP serum. Amido black stain.

Preliminary experiments using different purification methods for the isolation of IgG class antibody molecules from immune sera have shown the superiority of immunoadsorption techniques over other preparation methods. The use of purified antibodies was most important in sensitive immunoassays such as immunohistology to avoid non-specific binding reactions.

Gel diffusion methods according to MANCINI and LAURELL were convenient for quantitative measurements of AFP in serum specimens. However, enzyme-immunoassays (ELISA) proved to be more reliable and were needed to measure very small amounts of molecules. This became evident in the course of liver regeneration, and depended also on the animal species under study.

The preparation of antibody-enzyme conjugates required attention to the formation of stable covalent linkage between the molecules and on the other hand, retaining antibody and enzyme activity, repectively. Glutaraldehyde proved as reliable linking reagent. The two-step conjugation method was favorable for controlled conjugation because the formation of complex structures by polymerization of the involved proteins was avoided. Finally, purification of antibody-enzyme conjugates was performed to eliminate nonconjugated molecules in two steps: (a) gel filtration using Sephadex G 200 in the first step, and (b) Concanavalin A affinity chromatography in the second step using the conjugation products obtained with peak I (void volume fraction) from Sephadex G 200 gel filtration. Thus, a high degree of purification was achieved (Fgure 6). The latter were most suitable for quantitative immuno-assays such as ELISA and for studies on cellular markers in immunohistology.

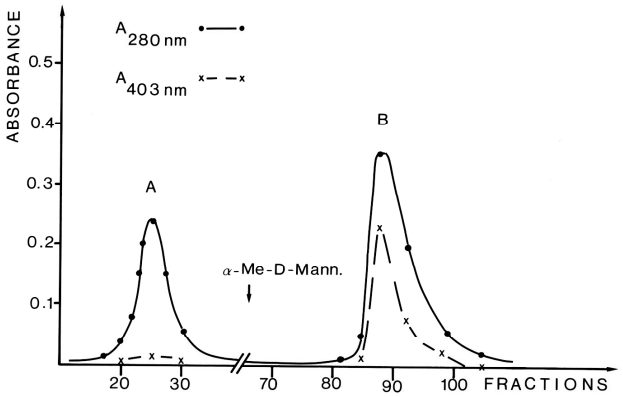


Figure 6: Purification of conjugates with gel filtration techniques. High degree of purification of HRP conjugates can be obtained with Concanavalin A (ConA) affinity chromatography. The column flowthrough (Peak A) contains unlabelled antibodies. Peroxidase conjugated molecules are eluted from the affinity gel matrix using 0.01 mol/L α-methyl-D-mannoside (Peak B).

3.2 Tissue processing for immuno-staining

All histological and immuno-staining methods used here were determined in preceding experiments. Even if certain concessions had to be made to morphological structures, the applied methods proved reasonably good for our studies. Tissue sections could be reliably immuno-stained for AFP. Furthermore, the use of conventional histological stains was possible. Endogenous enzyme activities were effectively blocked. Hydrogen peroxide used in the described manner did not damage tissue structure and had no adverse effect on the immunolocalization of AFP. A panel of control incubations including various immunological and cytochemical reagents secured immunohistological specificity.

3.3 Liver injury and recurrence of AFP

Substantial AFP synthesis was associated with liver regeneration after injury. The amount of AFP expression depended significantly on the injury type. Moreover, differences in AFP concentrations occurred within animal species and animal strains. All results are collected in Table 7.

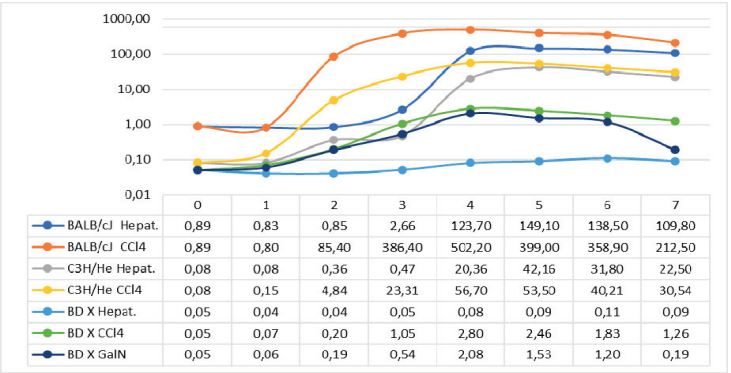
Table 7: AFP recurrence during liver regeneration and hepatocarcinogenesis

Animal models	AFP in serum [µg/mL] [a]	AFP immunohistology [b]
Normal controls	Baseline concentrations	Cellular detection of AFP:
— BALB/cJ mice	0.3-0.9	AFP in hepatocytes
— C3H/He mice	0.05-0.1	AFP not detected
— BD X rats	< 0.1	AFP not detected
Partial hepatectomy	Peak values at days 3 to 4	Cellular detection of AFP:
— BALB/cJ mice	120-200	AFP in hepatocytes
— C3H/He mice	20-45	AFP in hepatocytes
— BD X rats	< 0.1-0.8	AFP in hepatocytes (few/scanty)
CCl_4 intoxication	Peak values at days 3 to 4	Cellular detection of AFP:
— BALB/cJ mice	300-700	AFP in hepatocytes
— C3H/He mice	40-110	AFP in hepatocytes
— BD X rats	0.1-3.6	AFP in hepatocytes (few/scanty)
GalN injury	Peak values at days 3 to 5	Cellular detection of AFP:
— BD X rats	1.4-2.8	AFP in oval cells, ductular cells, canalicular epithelial cells, hepatocytes (few/scanty)
Carcinogenesis by NNM	Precancerous stage	Cellular detection of AFP:
— BD X rats	(a) low dose NNM feeding < 0.1 µg AFP/mL serum	(a) AFP not detected in early stages
	(b) high dose NNM feeding 1.1-2.3 µg AFP/mL serum	(b) AFP detected in oval cells, ductular cells, canalicular epithelial cells
	Carcinoma stage < 1.0 to > 100 µg AFP/mL serum	AFP detected in hepatocellular carcinoma cells

[a] Range of AFP concentrations in multiple samples

[b] Cellular detection of AFP by immunohistology

Liver regeneration was a signing feature in all experiments. Particularly, the great difference in AFP regulation within species and strains is highlighted. AFP data from different time intervals of regeneration are depicted in Figure 7. They illustrate the great variety of AFP concentrations in mice and rats after partial hepatectomy, CCl_4 or GalN intoxication, respectively.



	0	1	2	3	4	5	6	7
BALB/cJ Hepat.	0,89	0,83	0,85	2,66	123,70	149,10	138,50	109,80
BALB/cJ CCl4	0,89	0,80	85,40	386,40	502,20	399,00	358,90	212,50
C3H/He Hepat.	0,08	0,08	0,36	0,47	20,36	42,16	31,80	22,50
C3H/He CCl4	0,08	0,15	4,84	23,31	56,70	53,50	40,21	30,54
BD X Hepat.	0,05	0,04	0,04	0,05	0,08	0,09	0,11	0,09
BD X CCl4	0,05	0,07	0,20	1,05	2,80	2,46	1,83	1,26
BD X GalN	0,05	0,06	0,19	0,54	2,08	1,53	1,20	0,19

Figure 7: Serum AFP levels in mice and rats following partial hepatectomy, and CCl_4 or GalN injury, respectively. Each point in the graph represents the mean of 5 animals per day and study group, the relevant data are tabulated below the graph. *Abscissa*: untreated animals on day 0 and during the regeneration period from day 1 to day 7. *Ordinate*: AFP µg/mL serum.

3.3.1 Partial hepatectomy

The microscopic pattern resembled closely in all studied species. Repair started by proliferation of mature hepatocytes. While only few mitoses were found at 24 hours after hepatectomy, mitoses became then frequent and peaked the third day. This was about 24 hours earlier when serum AFP reached its highest level. Thereafter, mitotic activity declined rapidly.

In both mouse strains, AFP increase was observed from day 2 after partial hepatectomy. A straight increase occurred and levels reached a maximum between day 4 and day 5 (Figure 7). Then, serum AFP decreased again. AFP levels were about 5 to 10 times higher in BALB/cJ mice than in the C3H/He strain. By contrast, only weak increases of serum AFP were found in rats with partial hepatectomy. AFP elevation persisted for 2-3 days, then AFP decreased and reached normal adult values.

AFP immuno-staining was detected in mouse hepatocytes through days 2 to 7, and preferably localized in portal and periportal liver areas; few hepatocytes stained weakly for AFP in centrolobular and intermediate zones. AFP immuno-expression was all the time stronger in BALB/cJ than in C3H/He mice (Figure 8). In regenerating rat liver, cellular AFP immuno-staining was hardly detectable. Some few hepatocytes exhibited faint reactivities.

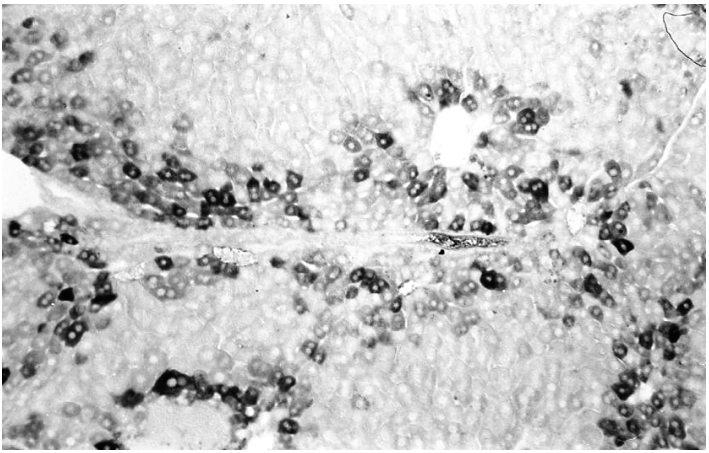


Figure 8: Balb/cJ mouse, day 3 after partial hepatectomy. Paraffin section with AFP immuno-staining. Strong AFP immuno-expression occurs in hepatocytes of portal and periportal areas. Immuno-staining 160 x.

3.3.2 Carbon tetrachloride intoxication

All species and strains displayed similar histological patterns. Oedema appeared within 24 h after CCl_4 ingestion followed by focal lesions such as ballooning of hepatocytes, necrosis, apoptosis, and an inflammatory response. Cellular infiltrates emerged on the second day (Figure 9). Necrosis and cell infiltrates persisted for up to four days. The boundary zones between viable and necrotic areas showed many dividing hepatocytes with high mitotic activity between day 2 and day 6 after injury. Finally, liver lobules became completely recovered.

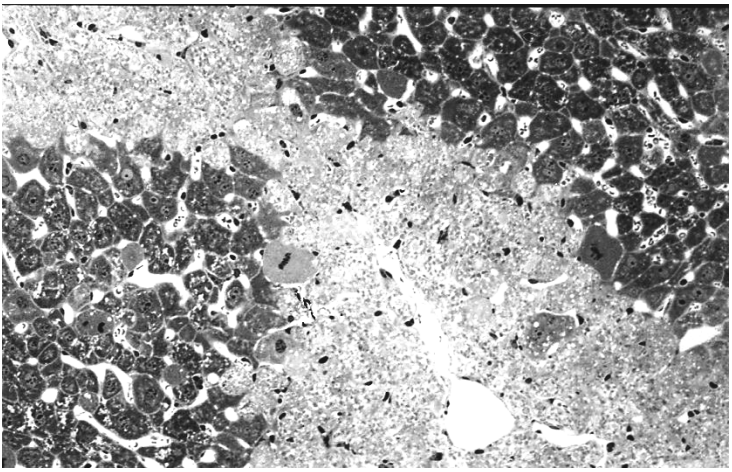


Figure 9: C3H/He mouse, CCl_4 injury. Semithin Epon section from regenerating liver. Toluidine blue 250 x.

Throughout all days, AFP increase was much higher in mice than in rats (Figure 7). Also, serum AFP levels were remarkably higher after CCl_4 intoxication than after partial hepatectomy. Highest concentrations were found on day 4. Then, AFP levels decreased. The slopes of AFP curves paralleled in both mouse strains, but AFP concentrations were about 10-fold lower in C3H/He than in BALB/cJ mice. In the rat, serum AFP levels were always much lower than in mice.

AFP immuno-staining was detected in hepatocytes of the portal and periportal zones. The strongest reactions occurred in hepatocytes of the zones adjacent to necrosis. AFP immuno-expression in BALB/cJ mice could be observed as early as on day one after CCl_4 injury and reached a maximum between days 3 and 4 (Figure 10). In C3H/He mice, AFP was hardly detected before day 2. During the following regeneration days, the pattern of AFP immuno-staining was comparable to that of BALB/cJ mice. However, staining intensities were always weaker in C3H/He than in BALB/cJ mice.

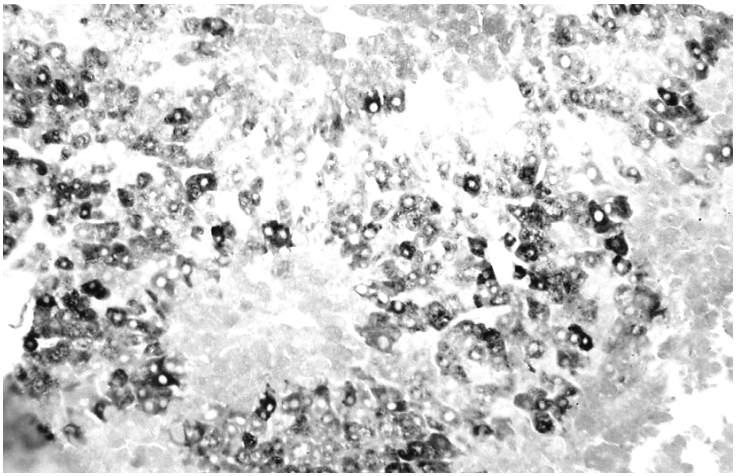


Figure 10: BALB/cJ mouse liver, 3 days after carbon tetrachloride injury. AFP immuno-expression reached a maximum between days 3 and 4 in hepatocytes of the intermediate zones adjacent to necrosis. Semithin Epon section. Immuno-staining 160 x.

Rat liver repair compared with that in mice. Serum AFP increased also. AFP-positive hepatocytes became detected on days 3 and 4. The number of AFP-positive hepatocytes as well as their staining intensities, however, were much lower than in mice. The staining pattern reflected well the low serum AFP levels. AFP-positive hepatocytes appeared as single stained cells scattered near to the necrotic zone and in periportal areas.

3.3.3 Galactosamine injury

Regeneration after injury is quite different from those observed in partial hepatectomy and CCl_4 intoxication. Histological patterns of severely injured liver with subsequent stages of regeneration are summarized in Table 8.

Table 8: Histologic observations in rat liver after GalN injury

Histologic changes	Day 1	Day 2	Day 3	Day 4	Day 5	Day 10
— Necroses	+++ [a]	++	++	+	(+)	–
— Councilman bodies	+++	++	++	+	(+)	–
— Inflammation	+++	++	++	++	(+) / +	–
— Canalicular proliferation	–	++	+++	+++	++	++
— Mitosis canalicular epithelial cells	–	++	+++	+	–	–
— Mitosis liver cells	–	+	++	+++	+	–
— Kupffer cells	++	+++	+++	+++	++	–
— Oedema	++	++	+	+	(+)	–
— Fibrosis	–	–	–	+	+	+
Cellular infiltrates						
— Inflammation						
portal	+	++	++	++	+	–
parenchymal	+++	++	++	++	(+)	–
— Histiocytes						
portal	++	++	+++	++	+	–
parenchymal	++	++	+++	++	(+)	–
— Granulocytes neutr.						
portal	++	+	+	–	–	–
parenchymal	++	+	+	(+)	–	–
— Granulocytes eos.						
portal	(+)	++	+	+	–	–
parenchymal	(+)	++	+	(+)	–	–
— Lymphocytes						
portal	–	(+)	+	+	(+)	–
parenchymal	+	+	+	+	(+)	–

[a] Intensity of histological changes: – no; (+) few; + significant; ++ strong; +++ very strong

Clusters of necrotic hepatocytes appeared within 24 hours; extensive necroses occurred especially near the portal tracts. At this time, cellular infiltrates prevailed the areas of necrosis. Histiocytes and neutrophilic as well as eosinophilic granulocytes dominated; lymphocytes were seen in small numbers. The formation of infiltrates changed rapidly, but histiocytes remained as dominant cells throughout inflammation. The histological changes induced by GalN injury compared well with those observed with high doses of the hepatotropic and genotoxic carcinogen NNM.

Beginning on day 2, necrotic areas decreased. Then, bile ductular cells (oval cells) and canalicular proliferations were observed (Figure 11). Parenchymal and bile ductular cells showed enhanced mitotic activity and peaked on day 3 and day 4. Afterwards, mitotic activity decreased rapidly. Histological sections exhibited now significant increases of bile ductular cross-sections along the borders of portal tracts with a maximum on day 3 and day 4 (Figure 12). During the following days some decrease occurred, but canaliculi were still increased over controls on day 10.

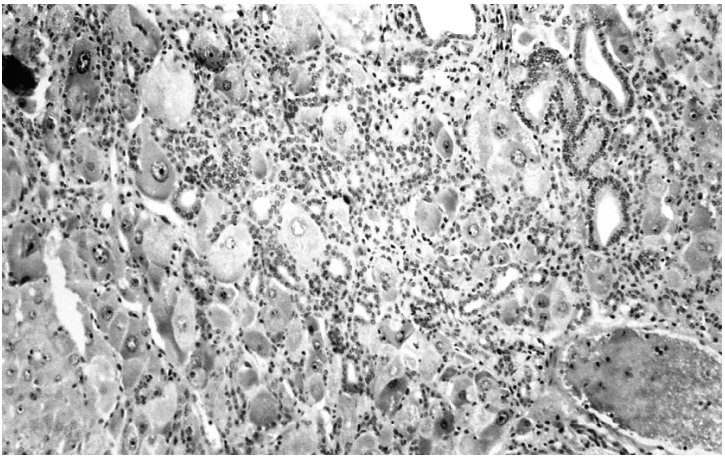


Figure 11: Rat liver tissue on day 3 after GalN injury with necrosis, inflammatory infiltrates, ductular proliferations and ballooning hepatocytes. HE 160 x.

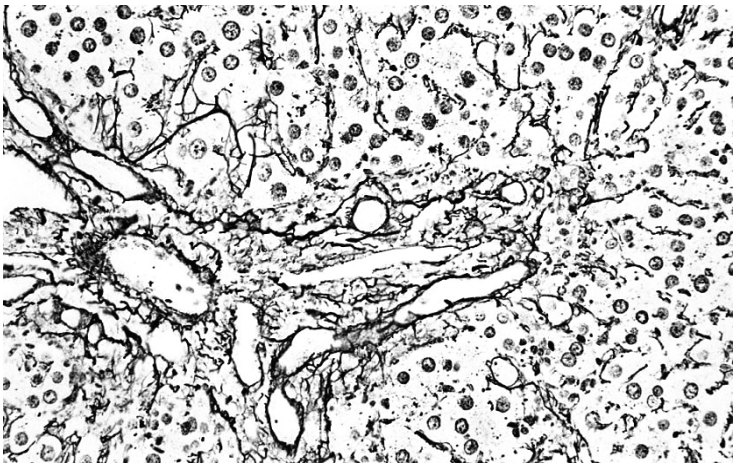


Figure 12: Rat liver on day 3 after GalN injury. Paraffin section, silver impregnation underlines the portal and periportal ductular proliferation. Gomori 250 x.

Serum AFP concentrations were slightly increased on day 2. Highest AFP levels occurred between day 3 and 5. Then, a rapid decline followed. When increased serum AFP was measured for the first time, cellular AFP staining became detectable in large numbers of small cells (oval cells) and in the cytoplasm (mostly in apical parts) of epithelial cells which formed bile-duct-like structures and rosettes (Figure 13). Preformed bile ducts in

typical portal spaces did not stain for AFP. Apart from AFP-positive oval cells, typical hepatocytes with AFP immuno-staining were found at random on day 3 and day 4. AFP staining was quite faint within cytoplasmic parts of hepatocytes. On day 5, AFP-positive canaliculi and bile ducts were significantly reduced. Between day 6 and day 10, liver structure returned to normal and proliferation of ductular epithelial cells ceased, meanwhile cellular AFP staining vanished. Most interestingly, proliferation of canalicular epithelial cells and AFP expression resembled those seen in toxic injury by high doses of NNM in the early stages of hepatocarcinogenesis.

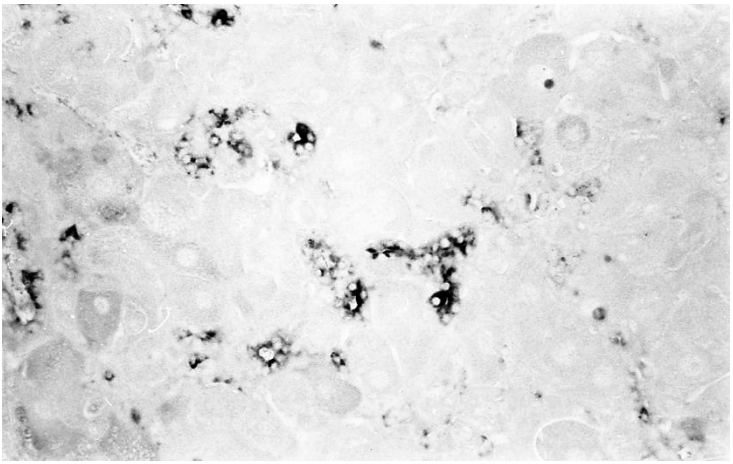


Figure 13: Rat liver on day 3 after GalN injury. Paraffin section with AFP immuno-staining. AFP-positive canalicular epithelial cells within portal tracts and in periportal areas. Immuno-staining 250 x.

3.3.4 Early stages of NNM hepatocarcinogenesis

Histotoxic patterns in rat liver depended largely on the applied NNM doses. With low NNM doses, necrotic effects and cellular proliferations were absent. Apart from that, increased serum AFP was not measured during the early stages of hepatocarcinogenesis. In addition, no cellular AFP could be observed by immunohistology.

In contrast to low NNM doses, severe necroses developed with high dose NNM feeding. Hepatocytes lost glycogen within the first two weeks. Thereby, the glycogen loss could reach into the portal fields. Oedema and necroses occurred in large numbers. Megalocytic and eosinophilic appearance was prevailing. Inflammatory infiltrates consisted mainly of histiocytes. Within few weeks, small oval-shaped cells proliferated and populated portal and periportal areas. They formed cell clusters and tubular structures. [^{3}H] Thymidine labelling experiments revealed significant incorporation of this precursor molecule in proliferating oval cells as sign of DNA replication (Figures 14-16).

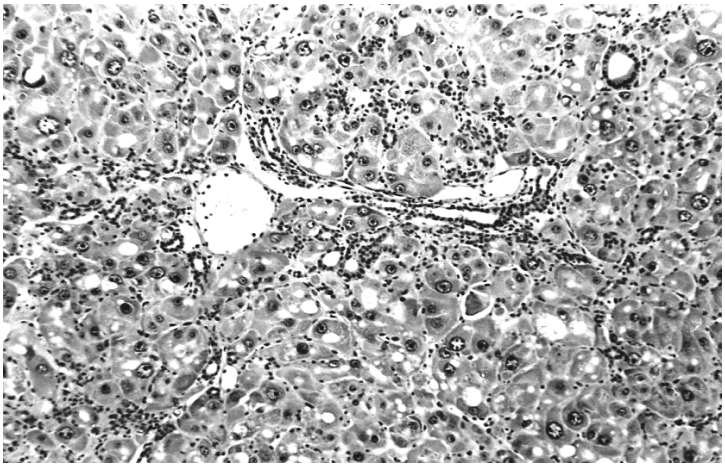


Figure 14: Rat liver tissue at high dose NNM feeding (day 35). Portal and periportal ductular proliferations with inflammatory infiltrates. HE 160 x.

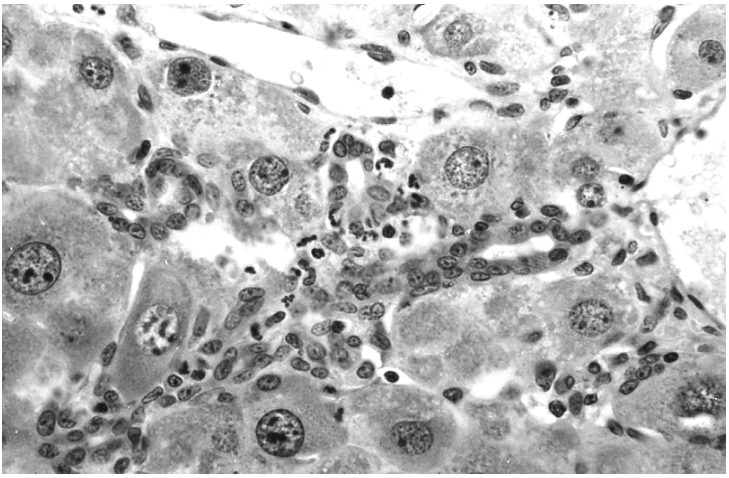


Figure 15: Rat liver from day 28-35 at high dose NNM feeding. Higher magnification view of biliary epithelial cells (oval cells) forming tubular structures. HE 540 x.

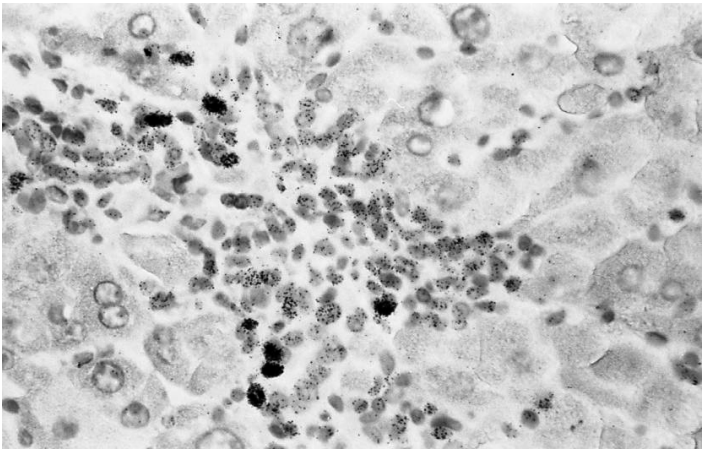
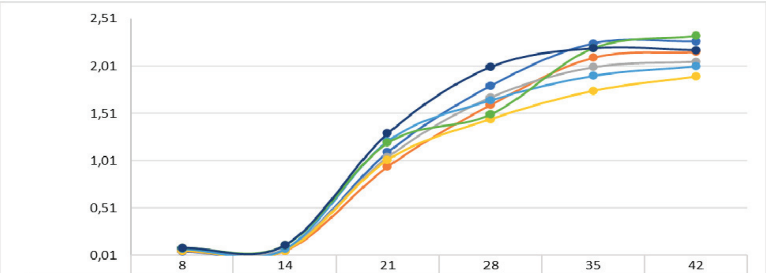


Figure 16: Liver from day 35 during high dose NNM feeding, pulse labelling with [^{3}H] thymidine. Thymidine incorporation in oval-shaped cells within areas of ductular reaction. Autoradiography and hematoxylin 540 x.

From day 35 proliferating cells reconstructed livers with areas of hyperplastic appearance. In this regenerative stage, the proliferating cell population showed more abundant cytoplasm than the early oval cells. At withdrawal of NNM, necroses disappeared, and oval cell proliferation ceased.

Results from serum AFP measurements in liver injury by feeding high NNM doses are shown in Figure 17 with numerical data in the table given below. AFP increased significantly between the second (day 14) and third bleeding (day 21). Highest levels were reached around the days 28 to 42 from beginning of NNM feeding.



	8	14	21	28	35	42
Rat No. 1	0,05	0,07	1,10	1,80	2,25	2,27
Rat No. 2	0,06	0,06	0,95	1,60	2,10	2,16
Rat No. 3	0,07	0,08	1,05	1,68	2,00	2,06
Rat No. 4	0,06	0,06	1,02	1,45	1,75	1,90
Rat No. 5	0,08	0,08	1,22	1,65	1,91	2,01
Rat No. 6	0,09	0,12	1,20	1,50	2,20	2,33
Rat No. 7	0,09	0,12	1,30	2,00	2,20	2,18

Figure 17: Serum AFP levels from animals during early stages of hepatocarcinogenesis at high dose NNM feeding. Blood samples (from rat No. 1 to rat No. 7) were taken at weekly intervals from day 8 through day 42. Numerical AFP data are tabulated below the graph. Abscissa: days of NNM feeding. Ordinate: AFP µg/mL serum.

AFP immuno-expression was detected at the same time in the cytoplasm of proliferating small, oval-shaped cells. AFP was present either in single oval cells or in oval cells forming strings and pseudotubular structures of bile ductular aspect (Figures 18-21). Their morphology was comparable to the described bile ductular cell reaction in the GalN experiments. Also comparable to the GalN experiments, typical bile ducts in portal spaces did not stain for AFP. Liver sections with Gomori's silver impregnation demonstrated a gain of bile ductular cross-sections corresponding with an increase of ductular constructs or an arborization of ductular structures. Serum AFP dropped to normal values within two weeks after finishing NNM application.

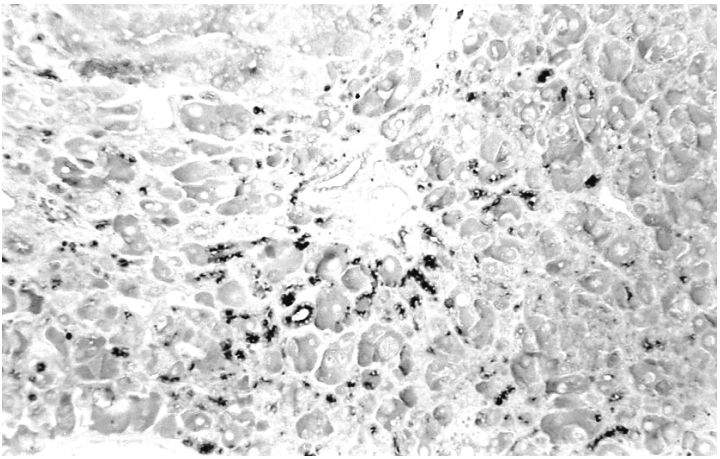


Figure 18: Rat liver tissue at high dose NNM feeding (day 28). AFP-positive canalicular epithelial cells form distinct cellular groups and tubular structures. Immuno-staining 160 x.

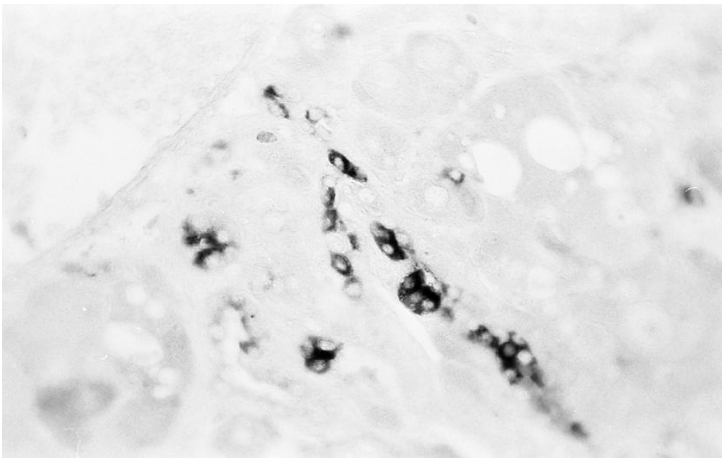


Figure 19: Rat liver tissue at high dose NNM feeding (day 28). Detection of AFP in proliferating biliary epithelial cells (oval cells) which form distinct cellular groups. Immuno-staining 540 x.

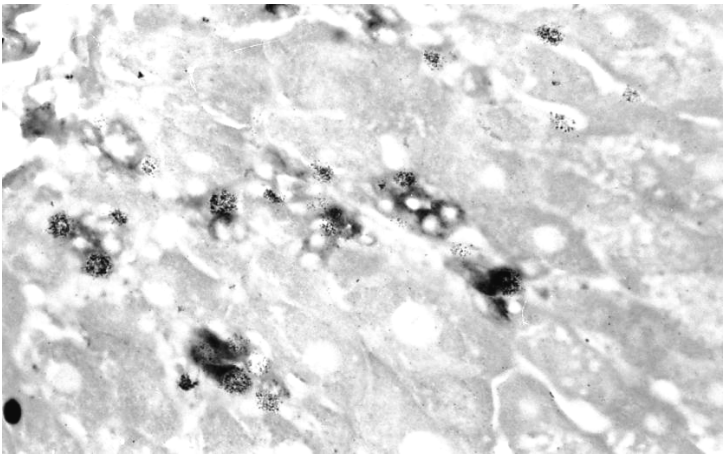


Figure 20: Rat liver tissue at high dose NNM feeding (day 28). [^{3}H] thymidine incorporation in pulse-labelled rat liver. AFP-positive and [^{3}H] thymidine labelled oval cells form structures with ductular appearance. Immuno-staining and autoradiography 540 x.

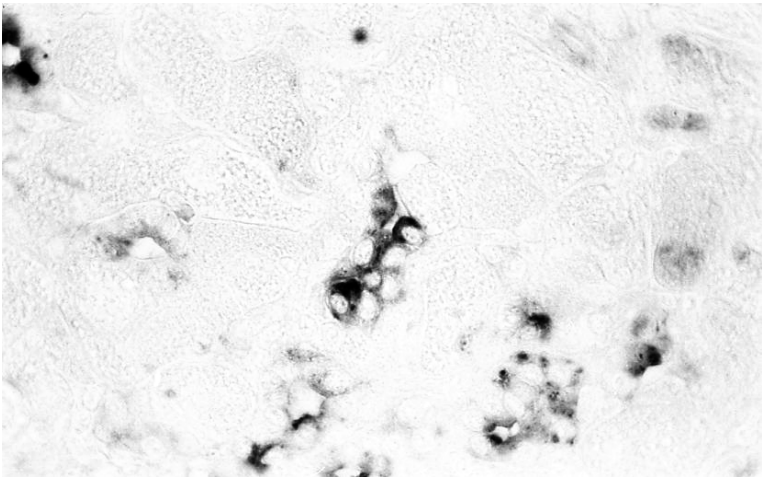


Figure 21: Rat liver from day 28-35 at high dose NNM feeding. Detection of AFP in grouped oval cells which form tubular structures. Immuno-staining 540 x.

3.3.5 Late recovery phase

Livers were marked by hyperplastic areas (nodular hyperplasias) with heavy distortion of the original lobular structure and changing to cirrhosis. Hyperplastic areas consisted of foci of acidophilic hepatocytes. Then, PAS-positive hepatocytes featuring glycogen storage appeared. Cellular changes occurred in rats being fed with high NNM doses much earlier than in rats which were maintained at low NNM doses. It deems worthy to note that in this stage of hepatocarcinogenesis the hyperplastic areas with acidophilic hepatocytes and PAS-positive cells did not stain for AFP (Figure 22). With time in progress, likewise small-sized hepatocytes emerged in such areas, and we detected AFP immunoexpression (Figures 23-24). This population might indicate a shift to neoplasia.

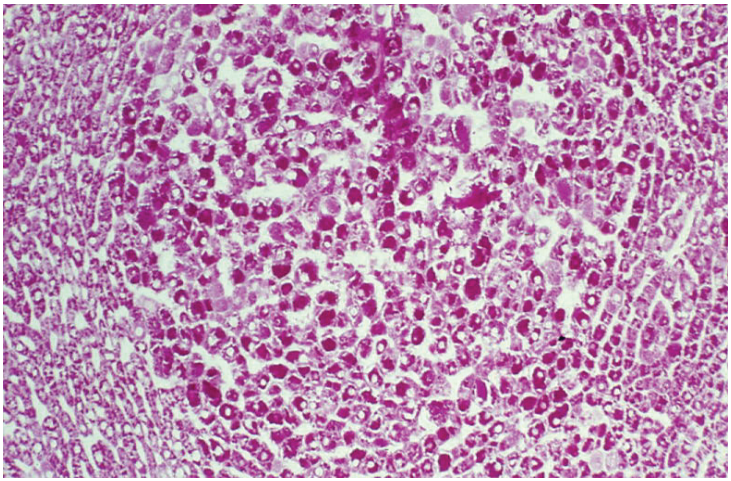


Figure 22: Rat liver, low dose NNM feeding on day 45. Hepatocytes with glycogen storage in hyperplastic liver area. PAS stained tissue section 160 x.

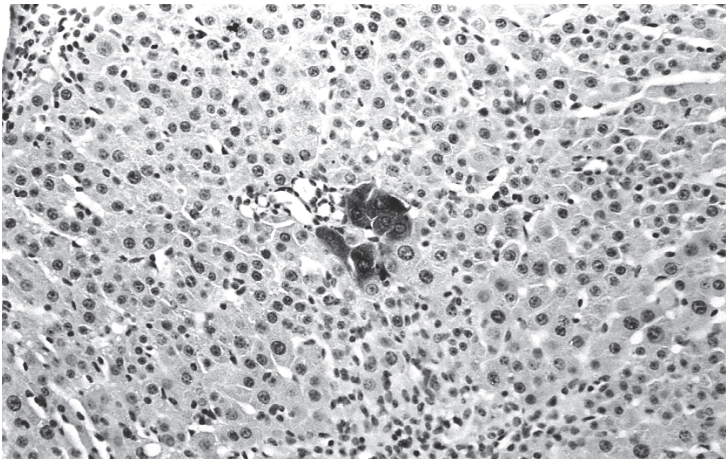


Figure 23: Rat liver tissue on day 60 (high dose NNM feeding) with AFP-positive cell group. Immuno-staining and hematoxylin 160 x.

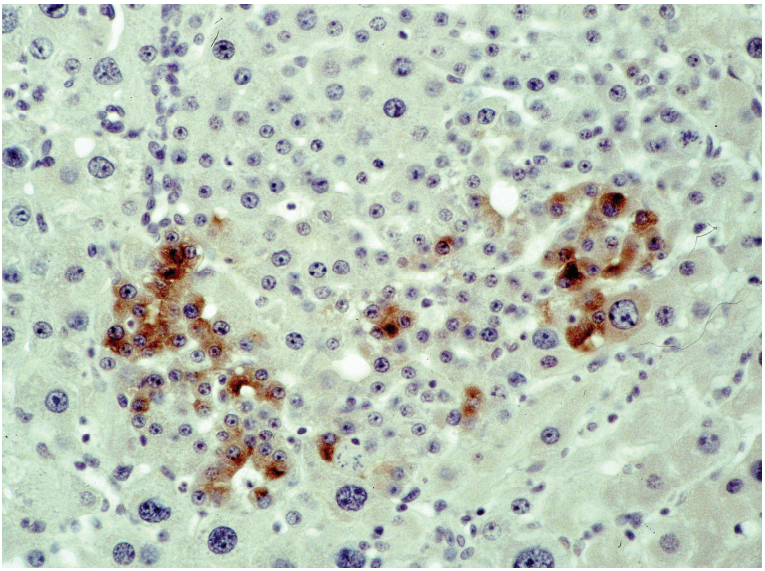
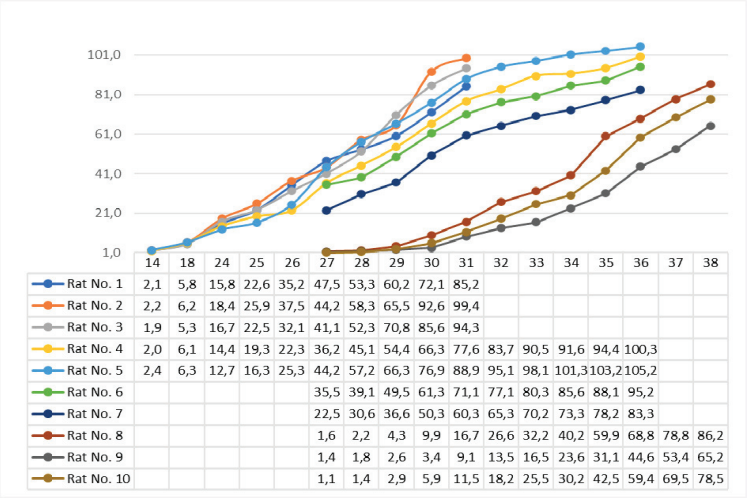


Figure 24: Rat liver at high dose NNM feeding. Tissue section with a nodule of hyperplastic appearance. Ductular epithelial cells (oval cells) are seen in transition towards hepatocytes. Note strong immuno-expression of AFP in both oval cells and in cells appearing as intermediate-sized hepatocytes. Transition of AFP-positive hepatocytes towards neoplasia is also possible. From Kuhlmann WD and Peschke P [114]. Immuno-staining and hematoxylin 250 x.

3.3.6 Liver cancer

Hepatocellular carcinomas developed in both experimental groups whether applying the genotoxic carcinogen NNM at low or high doses. However, the induction period was variable. Carcinoma development and AFP expression were monitored by histology and blood collections at regular intervals. In high-dose carcinogen experiments, liver cancer was already observed about 14 weeks after onset of carcinogen application, while in low-dose carcinogen experiments, cancer development needed longer periods (about 27 to 30 weeks).

Measurable AFP concentrations coincided with the incidence of distinct tissue nodules, microscopically hepatocellular carcinomas. Livers contained one or more nodules, quite small ones in the early stages of cancer development. At the beginning, the AFP amount was low in the range of 1-2 µg/mL serum. AFP concentrations were steadily rising during the following weeks (Figure 25). At autopsy, not all the developing tumors were found to stain for AFP. Dynamics of AFP levels differed over the time and reflected variability of AFP immuno-expression. Animals were sacrificed at periodic intervals for histologic and serologic screenings. Endpoint studies were not done. AFP levels could exceed concentrations of several mg AFP/mL of serum. In accordance with guidelines of animal care, experiments were terminated when animals had apparent signs of illness.



	14	18	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Rat No. 1	2,1	5,8	15,8	22,6	35,2	47,5	53,3	60,2	72,1	85,2							
Rat No. 2	2,2	6,2	18,4	25,9	37,5	44,2	58,3	65,5	92,6	99,4							
Rat No. 3	1,9	5,3	16,7	22,5	32,1	41,1	52,3	70,8	85,6	94,3							
Rat No. 4	2,0	6,1	14,4	19,3	22,3	36,2	45,1	54,4	66,3	77,6	83,7	90,5	91,6	94,4	100,3		
Rat No. 5	2,4	6,3	12,7	16,3	25,3	44,2	57,2	66,3	76,9	88,9	95,1	98,1	101,3	103,2	105,2		
Rat No. 6						35,5	39,1	49,5	61,3	71,1	77,1	80,3	85,6	88,1	95,2		
Rat No. 7						22,5	30,6	36,6	50,3	60,3	65,3	70,2	73,3	78,2	83,3		
Rat No. 8						1,6	2,2	4,3	9,9	16,7	26,6	32,2	40,2	59,9	68,8	78,8	86,2
Rat No. 9						1,4	1,8	2,6	3,4	9,1	13,5	16,5	23,6	31,1	44,6	53,4	65,2
Rat No. 10						1,1	1,4	2,9	5,9	11,5	18,2	25,5	30,2	42,5	59,4	69,5	78,5

Figure 25: Serum AFP levels were measured over the time of hepatocarcinogenesis until carcinoma developed. Rat No. 1 up to rat No. 7 were given high doses of NNM, while animals No. 8, No. 9 and No. 10 received low doses of NNM. Time points of serum collection with the respective AFP concentrations are depicted in the graph. AFP levels and their rising rates differed significantly due to carcinogen burden and carcinoma development. From each NNM group, animals were set aside to control tumor growth and AFP immuno-expression. Numerical AFP data are tabulated below the graph. Abscissa: period of observation from 14 to 38 weeks. Ordinate: AFP µg/mL serum.

No strict correlations existed between serum AFP concentrations and histological type of carcinomas. Some carcinoma nodules were AFP immunoreactive while others were not. In all cases, however, AFP staining was restricted to carcinoma cells. Penetration of carcinomas into normal liver tissue was seen at cancer borders. Normal tissue and tissue areas with dysplastic and hyperplastic appearance did not stain for AFP. AFP-positive cells stained basophilic with conventional dyes and, moreover, they were free of glycogen (PAS negative). Another typical sign of cancer cells was their proliferative activity. Most carcinoma cells became labelled by [^{3}H] thymidine whereas DNA precursor labelling of normal tissue was negligible (Figures 26-28).

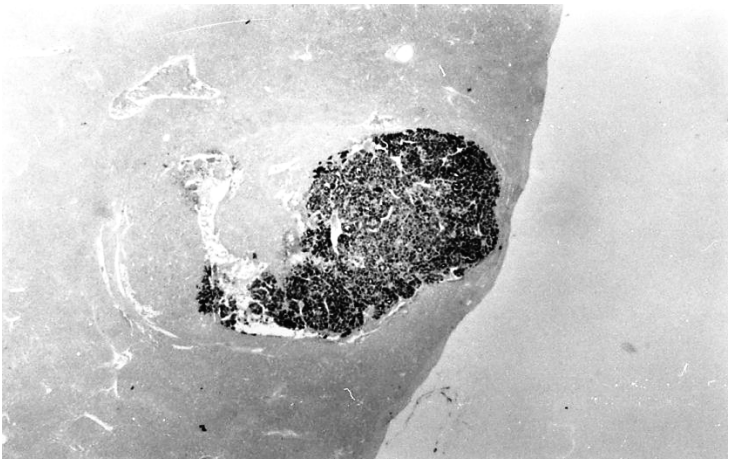


Figure 26: Rat liver tissue on day 150 with hepatocellular carcinoma. Tissue section was stained for AFP immuno-expression, note strong AFP reaction in carcinoma cells. From Kuhlmann WD and Peschke P [114]. Immuno-staining, low microscopic magnification 16 x.

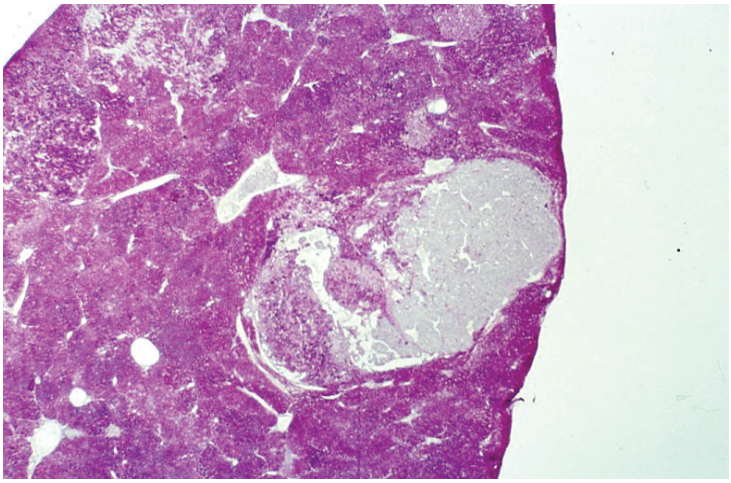


Figure 27: Paraffin section from same liver area as in Fig. 23 with glycogen staining by periodic acid Schiff reaction (PAS). Strong PAS staining in hepatocytes of carcinoma free tissue area. AFP-positive carcinoma cells are PAS-negative. From Kuhlmann WD and Peschke P [114]. PAS stained tissue section at low microscopic magnification 16 x.

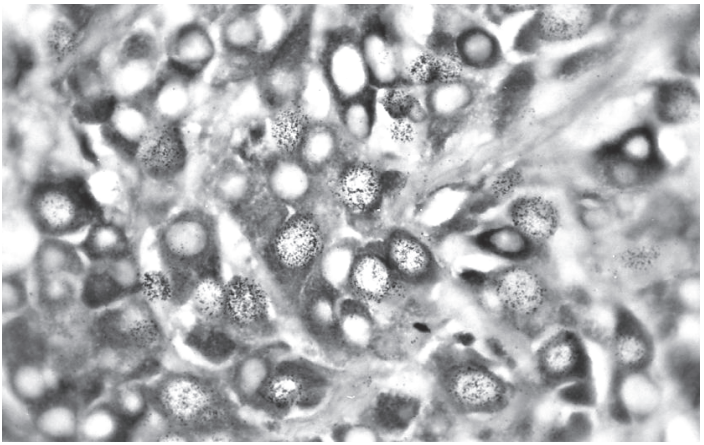


Figure 28: Rat liver tissue with hepatocellular carcinoma. [^{3}H] Thymidine pulse-labelling of rat liver. Autoradiography of AFP immuno-stained paraffin section. Note AFP-positive carcinoma cells and their proliferative activity. [^{3}H] thymidine labelling and immuno-staining. Hematoxylin 540 x.

4 Discussion

Liver regeneration is an all-important topic in biomedicine and clinical hepatology. The broader term liver regeneration is applied here to all types of liver growth after tissue loss, regardless of the reasons for which the loss arose. This also includes the case of partial hepatectomy, although in the strict sense, the repair process involves compensatory hyperplasia, namely growth from the amputation stump after tissue ablation. Another comment regarding liver repair is that new hepatocytes will not just arise by self-replication. Then, differences in repair mechanisms must be expected due to lack of uniform environmental conditions caused by the type of tissue damage. Such aspects are certainly important but will not be considered in detail. Instead, attention is paid to processes of liver repair by mature hepatocytes and by biliary epithelial cells.

AFP is the classic example of a marker of fetal liver development. After birth and during normal postnatal life, AFP expression is physiologically suppressed. Re-emergence occurs in pathological conditions and has made this fetal protein a useful marker in processes of liver regeneration and in the development of malignancies. These observations brought about the term "oncodevelopmental marker". The search for progenitor and stem-like cells of the liver has been the aim of many investigations using a variety of experimental designs. It has been suggested that the cellular localization of AFP by immunohistology would be worthwhile. In this context, immunoperoxidase techniques appeared to be a valuable tool to study cellular fluctuations during liver repair and hepatocarcinogenesis [58, 86]. The most challenging factors in immunohistology concern the immunological reagents and problems of tissue sampling. In this context, technical procedures, theoretical and practical aspects of immuno-staining were described [124].

4.1 AFP regulation

AFP is physiologically downregulated after birth and during hepatocyte maturation. The extent of AFP expression depends especially on the animal species and the postnatal age. The capability of AFP synthesis in postnatal hepatocytes still exists within limits. Intracellular sites of AFP synthesis correspond to structures which are typically found in cells synthesizing secretory proteins. Typical cell structures comprise organelles such as the rough-surfaced endoplasmic reticulum (RER), the perinuclear space and the Golgi apparatus with its central role in the secretory pathway and the sites of protein modification. The subcellular allocation is much the same in fetal, neonatal, and postnatal hepatocytes which is equally found in liver carcinoma cells [87, 157-160]. With postnatal time in progress, AFP immuno-staining is fading in a graded fashion with reduced numbers of hepatocytes being at random distribution. Fewer numbers of RER segments are stained until AFP is no longer detected; time periods depend on the species examined. In parallel, a continuous decline of serum AFP is observed. Finally, and species-dependent, AFP becomes hardly or eventually no longer detected by immunological means in adult life. Thus, highlighting an efficient repression of AFP.

Age-related, normal (low) adult serum AFP levels are suggested to reflect AFP production from a small proportion of hepatocytes undergoing cell division at any time in the

liver [84]. AFP synthesis is temporarily resumed in situations of regeneration which will also include liver homeostasis. An association with increased mitotic and cell cycle activities was evident [62, 161-168]. Besides mitotic and cell cycle activities, there is evidence that AFP synthesis is regulated by genetics. For example, AFP gene regulation could be shown to play a role in mice with strain dependent strictness [169]. Further experiments revealed that AFP levels are likely controlled by a Mendelian gene which was named by the authors *Raf* as for regulation of alpha-fetoprotein [100]; *raf* and *Rif* genes (*raf* gene for regulation of AFP, *Rif* gene for regulation of induction) act on-site of transcription [170]. These two loci (*raf* and *rif*) will determine the AFP level in normal adult life and in liver regeneration, respectively [171-173]. The action of *raf* seems to be restricted to the liver [174]. Differences in basal AFP levels can be expected according to different mRNA levels. Anyhow, additional mechanisms of AFP regulation must be respected [99].

The impact of regulatory processes became evident from quantitative serology and especially from immunohistology [59]. In histology, zonation of AFP immuno-expression in adult liver is readily observed in mice of the BALB/cJ and C3H/He strain. The midlobular hepatocytes are strongly immuno-stained for AFP. In contrast to mice, the BD X rat strain used by us was quite different and revealed under physiological conditions only very low AFP concentrations and zonal reactions could not be observed. This feature was ascribed to the very low amounts of AFP per cell. In any case, zonal expression of liver-characteristic molecules such as drug metabolizing enzymes and other molecular systems for liver cell functions is not surprising and a known common trait [143, 175-178].

4.2 Hepatic progenitors and AFP

Stem and progenitor cells may be important sources for cell renewal and homeostatic balance in organs. Despite efforts in research, many questions remain as to the interpretation of repair mechanisms in disease and pathways in carcinogenesis. In this context we aimed to use AFP as marker in liver injury models in the evaluation of progenitor and stem-like cells. Normally, AFP expression is effectively downregulated in postnatal life, but significant re-expression of AFP occurs under certain conditions. Hence, this protein offers a powerful tool as marker of cell regeneration and carcinogenesis. Our experiments included partial hepatectomy as a classical non-toxic approach, and toxic models such as injuries induced by CCl_4, GalN or NNM. These models differ in some characteristics.

Significant amounts of AFP occur in liver regeneration and in liver cancer, but serum AFP concentrations and cellular sites of AFP synthesis show differences depending on the experimental setting. We observed that (a) postnatal AFP expression is regulated with certain strictness in species and strains; (b) adult hepatocytes can be prolific and re-express AFP during regeneration as for example in partial hepatectomy and following intoxication by carbon tetrachloride; (c) regenerative capacity of adult hepatocytes can be blocked in severe injury so that reconstitution of livers with concomitant AFP synthesis occurs through progenitor cells which proliferate from biliary epithelial cells; (d) biliary epithelial cells acting as stem-like progenitor cells reach levels of maturation with activation of fetal genes and significant AFP synthesis; (e) adult hepatocytes and bile ductular

progenitor cells possess stem-like properties and can be involved in liver repair; (f) biliary epithelial cells and hepatocytes can be targets for genotoxic carcinogens to develop liver cancer with potential AFP expression.

4.3 Liver regeneration

The adult liver is characterized as a non-growing proliferative tissue with balanced cell production when cell loss occurs ("steady state" tissue). During normal turnover and low mitotic index, adult hepatocytes can begin new rounds of the cell cycle [17, 107, 179, 180]. In cases of injury, the liver proves its great potential for regeneration. Partial hepatectomy is a frequently studied regeneration model in small rodents. Remaining livers will restore within about 1 week [107, 181-183]. Resected lobes will not reappear, instead the liver remnant restores liver function by compensatory growth [184, 185]. Hepatocytes have the inherent capacity to restore rapidly lost cell mass. Liver restitution in response to partial hepatectomy or carbon tetrachloride intoxication occurs through proliferation of mature hepatocytes.

Within about 24 hours, quiescent hepatocytes switch to a replicate state with DNA synthesis, mitosis, and cell division. They behave in such situations like stem cells and no progenitors are necessary [88]. Other injury models enable further insights into the process of liver regeneration and, moreover, all models have their special characteristics, notably with respect to the susceptibility to hepatotoxins [182, 186-188].

Correlations exist between hepatocyte mitosis and AFP synthesis. However, serum AFP levels not just reflect the number of hepatocytes engaged in repair. From our experiments we deduced that species and strain specific AFP genetics (see above) are important and influence on a great extent the different amounts of AFP, irrespective of the type of injury model. This does not exclude further mechanisms inherent to the type of injury which will also contribute to the expression of AFP.

The use of marker molecules such as AFP highlighted processes of regeneration and in particular the location of stem-like cells, progenitors, and maturation stages. Progenitor cells represent a class of transit-amplifying cells with options of further differentiation. In both partial hepatectomy and CCl_4 intoxication, adult hepatocytes proved their quality as functional and valuable committed stem cell system that can rapidly respond to liver injury [179, 180].

In contrast to partial hepatectomy and CCl_4 intoxication, pathways of liver repair differed in GalN and NNM injuries for reasons of blocked regenerative activity of adult hepatocytes. In such situations, small oval-shaped cells developed from the canals of Hering (canaliculi of Hering) and proliferated extensively. They were regarded as progenitors which can differentiate into mature hepatocytes [58, 60, 189]. During maturation, levels of cytodifferentiation and reactivation of fetal genes were gained with concomitant AFP synthesis. It can be accepted that liver repair is achieved by two different modes, (a) hepatocyte-driven proliferation of remaining mature hepatocytes as in the case of hepatectomy or carbon tetrachloride intoxication, and (b) regeneration by proliferation of ductular

epithelial cells and conducting themselves as progenitors or transit-amplifying cells on the way to hepatocyte maturation as in case of GalN and NNM intoxication. Both regeneration pathways are connected with the synthesis of AFP. The small-sized oval cells and their progeny populations reach levels with fetal gene activation and significant AFP expression that signals stemness. In histology, they can form cell clusters, duct-like structures and AFP-positive immuno-staining with expansion and proliferation into the necrotic areas. The preformed bile ducts did not stain for AFP.

4.4 Ductular epithelial cell reaction and AFP expression

When we compared cellular responses in our experimental models, then it was evident that varying cell lineages become activated with different repair mechanisms. Stem-like progenitor cells are relevant to liver regeneration in special injury models which differ for instance from pathways observed in partial hepatectomy or in carbon tetrachloride intoxication. Such special injuries must be intense or chronic so that regeneration by mature hepatocytes becomes prevented. The injuries by GalN and by the carcinogen NNM in appropriate doses are examples for this type of toxicity. Morphologic features encompass an intense proliferation of bile-duct epithelium [68]. The description of oval cell proliferation with concomitant AFP synthesis in such experiments was a step forward to explain liver regeneration and transitory AFP expression [58, 60, 189-193].

Modalities of repair following GalN and NNM injuries are similar. Regeneration is started up by ductular epithelial reaction with oval cell proliferation and concurrent AFP synthesis. Oval cells were heavily engaged in DNA synthesis as outlined in [^{3}H] thymidine labelling experiments. Pulse-chase labelling revealed their development towards hepatocytes. The term oval cells derived from previous studies of chemical hepatocarcinogenesis and liver injury [13, 194]. Their role in both regeneration and carcinogenesis has been addressed [34, 58, 195-197]. Oval cell populations present themselves as transit-amplifying cells [198] particularly as proliferating biliary epithelial cells and subsequent maturation into hepatocytes enable the recovery of the livers and highlight their potency as hepatic progenitor cells [58, 199, 200].

Hepatic progenitor cells derive from intrahepatic stem cells located in the canaliculi of Hering [77]. The canals of Hering represent a potential reservoir for progenitor cells in adult livers [201]. From these, small-sized cells (oval cells) and maturational lineages originate. When activated to proliferate they give rise to cell populations with bi-potential nature that can differentiate into hepatocytes and biliary epithelial cells [30]. The origin of proliferating cells from tissue-determined stem cells of the adult organ was discussed on several occasions [3, 75, 90, 114, 189, 191-193, 202-209]. In this context, experiments on NNM intoxication, allyl alcohol and cocaine were much instrumental [58, 210-213]. It has been described that liver repair can start from small-sized cells whose progeny differentiate and finally repopulate necrotic zones. The ability of oval cells to differentiate into multiple cell types suggests that they possess a remarkable degree of plasticity and transdifferentiating potential.

If the oval cells are considered as functional progenitors for hepatocytes and cholangiocytes which can differentiate into the mature forms of the two hepatic epithelial lines (hepatocytes and bile duct epithelia), then the canals of Hering and the terminal branches of the bile ductular system are sources of intrahepatic stem cells. With respect to the same embryonic origin of bile ducts and hepatocytes, the biliary epithelium and its proliferating oval cells have defined roles in liver regeneration as transit and amplification compartment (Figure 29). Such progenitor cells are thought to resume differentiation pathways like stem cells. Then, maturation stages are reached with the ability of AFP expression. At higher differentiation stages and maturation, the ability to express this marker will expire.

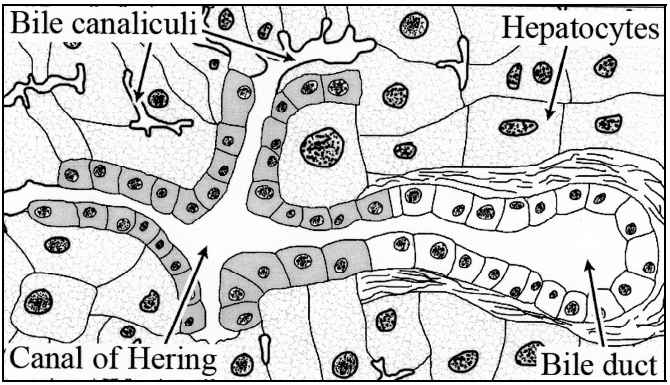


Figure 29: Schematic representation of structure in normal liver lobule with the canal of Hering which drains bile from the bile canaliculi into the bile duct (modified from Junqueira L.C. & Carneiro J. [1980]. In *Basic Histology*, Lange Medical Publications p 350), Kuhlmann WD and Peschke P [114]. Oval cells can proliferate from the canals of Hering and lead to ductular epithelial structures.

Once activated, proliferating oval cells seem to be able to differentiate via intermediate-sized hepatocytes into mature hepatocytes and bile duct epithelium. The exceptional feature of bile ductular epithelial cells and their progeny may be the outstanding quality of plasticity and transdifferentiation. Cellular reprogramming for retrodifferentiation, transdifferentiation and interconversion to distinct cell types has been now and then discussed and proposed as possible sources for restoring liver tissue [214, 215]. These processes can be initiated by mechanisms such as the overexpression of transcription factors or by influencing signalling pathways. The many published aspects on liver regeneration and all the conflicting views on this subject, however, have not yet achieved a conclusive issue [28, 32, 35, 36, 88, 89, 91-94, 96, 216-222]. Finally, controversies relate to the models used. Plasticity and transdifferentiation of oval cells and other stem or progenitor cells, however, open impressive opportunities for regenerative medicine, particularly in the field of liver regeneration.

The fate of progenitor cells when differentiating into hepatocytes or bile ducts will be governed by the liver microenvironment. Progenitor cells committed to differentiate into hepatocytes or bile ducts may have interactions with near-by-cells or may react with paracrine factors of the liver microenvironment [33, 213]. Most probably, inflammatory stress occurs in livers caused by injury and the associated cytokine secretions will certainly play a role in cross-regulation of epithelial and mesenchymal cells and, thus, forming regenerative units in which hepatopoiesis can take place [223]. Paracrine molecules as well as Wnt signal proteins as local mediators and Wnt/β-catenin pathways are important in cellular reactions. For example, Wnt/β-catenin signaling proved its importance in the regulation of hepatocyte proliferation after carbon tetrachloride intoxication [224]. In other cases of liver repair, hepatocyte proliferation was found to be accompanied by increased NF-κB activity [225] that was needed for HGF (Hepatocyte Growth Factor) induced proliferation of hepatic stem-like cells [226]. Other reports elaborated blocked hepatocyte proliferation by toxic agents and oval cell proliferation. When oval cells begin to proliferate, expression of SCF (stem cell factor) and its receptor (c-kit) can be observed in the oval cell compartment [227]. It is proposed that the SCF/c-kit system, several other growth factors, cytokines and signaling pathways are engaged in activating hepatic stem-like cells and in their differentiation [228-230]. Their impact on liver regeneration has been described [231, 232]. A major challenge is to understand how all the necessary genetic and pathway factors interact in a precise sequence to direct stem cells and hepatic lineage cells into functional liver cells.

4.5 Extra-hepatic cells and liver regeneration

There is some evidence that hematopoietic stem cells can contribute to the development of hepatocytes. The possibility of hematopoietic stem cells to contribute to the development of hepatocytes is amazing and let assume the existence of a common stem cell [233]. Aspects of transdifferentiation and plasticity of adult stem cells are of interest for therapeutical reasons. Some experimental findings allowed the hypothesis that reprogrammed adult stem cells can differentiate into other cell types such as hepatocytes and other epithelial cells including hepatic oval cells [1, 234-241].

Relations between liver lineage cells and bone marrow were supposed from the observation that hepatic oval cells and hematopoietic stem cells share common markers such as CD34, Thy-1 and c-Kit mRNA and protein [242-245]. Relationship between hematopoietic stem cells and liver was also deduced from combined transplantation and liver injury studies. In animal experiments, 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 after injury. An extrahepatic source for liver repopulation seemed possible [234, 240, 246, 247].

Other experimental data, however, could not so far endorse the assumption that sources of oval cells in injured liver will arise through transdifferentiation from bone marrow cells [248]. Indeed, the participation of bone marrow cells in liver regeneration would be surprising because hepatocytes and biliary epithelium develop from endoderm

while reticuloendothelial and hematopoietic cells differentiate from mesoderm. Yet, an alternative explanation for the development of plasticity is the formation of hybrids by spontaneous cell fusion giving rise to heterokaryons [249]. Some transplantation experiments and cytogenetic analyses were in favor for the possibility that hepatocytes derived from bone marrow will arise from cell fusion instead of differentiation of haematopoietic stem cells [250-252]. The discussion on extrahepatic sources for liver regeneration and the role of hematopoietic stem cells for liver regeneration continues [240, 247, 253].

4.6 Early stages of hepatocarcinogenesis

Chemical hepatocarcinogenesis includes special situations because genotoxic carcinogens are eventually toxic at the same time. Acute toxicity was clearly dose-dependent and followed by oval cell proliferation. This histological feature was associated with AFP immuno-expression in the proliferating cell population and significant serum AFP increase. Both, oval cell proliferation and concurrent AFP expression are results of repair processes following toxic injury by high doses of carcinogen (NNM). In contrast, low doses of NNM had no toxic effects and oval cell proliferation did not occur. In this setting, the regulatory system of AFP gene expression seemed not to be affected. We have no evidence whether hepatocarcinogens in low and non-toxic doses have a role in increased AFP expression by specific gene amplification as was suggested earlier [254, 255]. Serum AFP levels remained too low to be detected. Also, AFP immuno-expression could not be observed in liver cells by immunohistology. It should be noted, however, that the effects of carcinogens on gene expression can vary significantly. On the one hand, this depends on the selected carcinogen as well as on the dosage and the duration of application. The exact mechanisms underlying the effects of carcinogens on gene expression are not yet fully understood.

Liver cancer arose with each of the two used carcinogen schedules, i.e., low, and high NNM doses. Neither oval-cell proliferation nor AFP synthesis were prerequisites for cancer formation. Hence, hepatocellular carcinoma developed independently of the carcinogen dosage, and toxic injury and subsequent repair were not required. In any case, it is assumed that hepatocytes as well as oval cells are targets for carcinogens. Some authors attributed an important role of oval cells towards neoplastic transformation [23, 256, 257]. Oval cells and foci of altered hepatocytes were discussed to be stages in cancer development with growth advantage over normal cells [258, 259]. Generally, the proliferative activity of oval cells can make them susceptible to carcinogens with risk for transformation [6].

In chemical hepatocarcinogenesis, phenotypic alterations at the microscopical level were attempted for classification purposes. Hyperplastic nodules and altered hepatocytes are deemed as preneoplastic liver lesions with the probability to develop into malignant neoplasms [12, 143, 260-262]. Nodules show an increased number of hepatocytes in a disorganized manner. Altered hepatocytes can show a variety of morphological changes including enlarged cell size and increased nuclear-cytoplasmic ratio. Then, typical pat-

terns are for example excessive glycogen storage as well as decreased or increased activity of enzymes, respectively. Focal enzyme alterations are thought to be the result of genotoxic effects of carcinogens. Recently, important aspects of regulation, expression and oncogenic signalling of relevant genes for drug-metabolism have been reviewed [263].

Hepatic lesions are assessed as preneoplastic changes with the possibility to develop into cancer. Phenotypic instability is given to occur in early stages of the cancerous disease with drawbacks for their exact classification. The many morphological attributes described in histology can be also seen in unrelated disease cases. At this point we could not find AFP immuno-staining in “hyperplastic nodules” and in “foci of altered hepatocytes” independent of the mentioned phenotypic patterns. So far, this oncofetal protein is not a suitable marker for “foci of altered hepatocytes” and the described preneoplastic lesions.

Up to now, it is impossible to predict which cell or cellular lesion is the true premalignant one during experimental hepatocarcinogenesis. The underlying mechanisms of liver cancer development are complex and multifactorial, and probably based on interactions between genetic, environmental and lifestyle factors. The differing responses to carcinogenic diets used for the initiation of liver cancer may contribute to the variability in premalignant lesions that develop. Then, different types of carcinogens or different doses of the same carcinogen may result in different types and numbers of cellular lesions, each with varying potential for malignant transformation. Therefore, while plasticity and transdifferentiating potential of oval cells may be important in hepatocarcinogenesis, the described observations of so-called premalignant lesions are possibly biased findings.

Based on AFP expression by oval cells and their developing progeny it is suggested that these populations behave as liver stem-like cells. During experimental hepatocarcinogenesis, oval cells can follow further differentiation and maturation stages and can give rise to small and intermediate-sized hepatocyte cell clusters that have the potential to become carcinoma cells. When this happens, AFP immuno-expression can be observed in cell clusters as they undergo transition towards carcinoma cells. These populations can acquire clonogenic properties under certain conditions. Like that, AFP immuno-expression is a sign of neoplasia (neoplastic transformation) during hepatocarcinogenesis. Further research is needed to fully understand the relationship between oval cell proliferation, AFP expression, and hepatocarcinogenesis.

4.7 AFP and hepatocellular carcinoma

The many known tumor types in human liver can be seen in animals alike, and most of them can be induced by appropriate agents in experimental hepatocarcinogenesis. From the heterogenous group of malignant tumors, hepatocellular carcinomas belong to the primary liver cancers of high malignancy. Their histological features may comprise solid and glandular growth patterns, they can also include a mixture of hepatocellular carcinoma and intrahepatic cholangiocarcinoma. It must be emphasized that the phenotype is not an exact reference to histogenesis. Major sequences in malignancy covered concepts suchlike altered hepatocytes, foci and nodules with varied cellular and biochemical chan-

ges which might lead to carcinomas. Basic ideas of transformation, dedifferentiation and retro-differentiation have been discussed.

Adult hepatocytes are often thought to have a leading role as origin in hepatocellular carcinomas. A review summarized hypotheses for evidence that hepatocytes can transform directly through genetic alterations into hepatocellular cancer cells or perhaps hepatocytes dedifferentiate into hepatocyte precursor cells which then become carcinoma cells. Furthermore, hepatocytes may transdifferentiate into biliary-like cells to give rise to cholangiocarcinoma cells [264]. But there again, biliary epithelial cells are thought to act as progenitors in hepatic differentiation and to undergo malignant transformation. The multiple roles of duct cells as liver progenitors in regeneration and malignancy were guessed in early papers [194, 265]. The participation of oval cells in the process of hepatocarcinogenesis, however, was for a long time contradictory.

AFP expression is a classical feature of hepatocellular carcinomas. With the appearance of serum AFP in our animals, livers could carry one or more carcinoma nodules. Some but not all were AFP-positive in immunohistology. In the absence of hepatotoxic agents in the later stages of hepatocarcinogenesis, AFP expression was likewise not due to a toxic response. Re-appearance of AFP is the sign of an immature state in which hepatocytes may down- or up-regulate genes to express this oncofetal protein. This step compares with processes of dedifferentiation.

Generally, AFP-positive carcinoma cells were basophilic in classical histology. The PAS-negative behavior was another typical feature of carcinoma cells. While carcinomas with and without AFP immuno-expression could occur side by side in the animal's liver, this observation indicates the heterogeneity in the development of hepatocellular carcinomas with distinct genetic characteristics. Genetic mutations during tumor development have an impact on the resulting cancer cells and lead to the selection of cells with different properties.

AFP-positive and AFP-negative carcinoma cells proliferated actively as seen from [^{3}H] thymidine pulse labelling experiments. It remained unclear where and why AFP expression came along the path of cancer development. The different serum AFP levels in animals with carcinomas are ascribed to the numbers of AFP positive cells and their synthetic output, respectively. This heterogeneity applies to differences in cell differentiation perhaps associated with differences in malignant potency.

Capacities for renewal, differentiation and tumorigenesis reside in a small proportion of cells being called cancer stem cells [6, 266, 267]. Cancer stem cells (CSC) can be found in human hepatocellular carcinomas as well as in animal models. They are a kind of subpopulation of cancer cells with the ability to differentiate into multiple cell types within the tumor, for example into hepatocyte-like and cholangiocyte-like cells. CSC cells can be resistant to different forms of therapy, and they will contribute to tumor progression.

For reasons that cancer cells derive from tissue-determined stem-like cells or from transit-amplifying cells, oval cells and their progeny can have a leading role in the deve-

lopment of hepatocellular carcinomas. This, however, is still under study. Their role as facultative stem-like cells and their role in the carcinogenic process may apply to the assumption that transit amplifying cells can fail to differentiate normally. At a certain differentiation stage, these cells will undergo maturation arrest and acquire the potential for proliferation which may give rise to cancer [6, 22, 24, 268, 269]. Some epigenetic mechanisms may further modulate carcinogenesis.

AFP-positive tumor cells represent neoplastic developments with degrees of differentiation and maturity. Fetal gene expression in adult life strengthened the hypothesis that re-appearance of fetal proteins in tumors is in correlation with dedifferentiation or retrodifferentiation processes [270-272]. As an additional aspect, expression of fetal proteins in liver cancer may be part of the concept of "blocked ontogeny" as proposed by V. R. Potter [273]. This theory suggests that cancer cells may re-express genes and proteins that are usually only expressed during fetal development, as they are in a less differentiated state (reverted to a less differentiated state, like fetal liver cells) and may be more likely to divide rapidly and to form tumors. Thereby, synthesis of AFP can be the result of blocked hepatocyte maturation during cancer development [24]. It must be emphasized that AFP re-expression in liver cancer is not an exclusive trait of malignancy because AFP will appear in situations which are not in conjunction with malignant growth. The term "transitory cell antigens" is therefore appropriate due to the adherence or restriction to a transitional stage of cell differentiation. Despite all this, clonality, maturation arrest or retrodifferentiation can merge in selecting cell populations with high autonomy. While the expression of AFP and other fetal proteins may be indicative of dedifferentiation or retrodifferentiation processes, these molecules are not necessarily definitive markers of cancer or cancer progression. Anyway, the relationship between fetal protein expression and cancer development remains complex.

5 Conclusion

Animal studies have shown stem-like properties of adult hepatocytes and stem-like properties of biliary epithelial cells with the latter originating from the canaliculi of Hering. In partial hepatectomy and after carbon tetrachloride injury, adult hepatocytes are the main source for liver restitution. Stem cells with the ability of multilineage differentiation are not required in these cases of liver repair. Increase of serum AFP concentration is associated with the proliferative activity of hepatocytes. The degree of AFP synthesis largely depends on both animal species and respective strains. Regenerative capacity of hepatocytes can be blocked in defined injury conditions. Like this, liver repair follows other pathways which involve the proliferation of biliary epithelial cells (oval cells). Oval cells and their progenies are descendants from the canaliculi of Hering and the small interlobular bile ducts. They act as stem-like cells and are considered the specific progeny of liver stem cells. During proliferation and maturation, they reach levels with fetal gene activation leading to AFP synthesis as sign of reversal ontogeny. In hepatocarcinogenesis cancer cells may derive from oval cells or from mature hepatocytes. AFP resurgence in hepatocellular carcinomas reflects traits of retro-differentiation. Maturation arrest during cell differentiation is also possible. AFP re-expression is not confined to malignancy because such molecules can re-appear in cells during non-malignant growth. The broader term “transitory cell antigens” is the appropriate naming.

6 Conflicts of Interest

The authors declare no conflict of interests.

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