Immunoperoxidase Labelling of Alpha$_1$-Fetoprotein (AFP) in Normal and Regenerating Livers of a Low and a High AFP Producing Mouse Strain

W. D. KUHLMANN[*]

Laboratory of Experimental Medicine and Immunocytochemistry, Institut für Nuklearmedizin
DKFZ Heidelberg, Germany

Summary

Serum AFP concentrations in normal adult BALB/c/J and in normal adult C3H/He mice were in the order of 0.6 µg/ml and 0.1 µg/ml, respectively. In BALB mice, AFP was localized in the cytoplasm of differeniated mono- and binucleated hepatocytes in centrolobular and intermediate zones of normal adult liver. No cellular AFP could be detected in liver sections of normal adult C3H mice.

CCl$_4$ intoxication was accompanied by increase of serum AFP levels. A maximum was reached on day 4. Afterwards, concentrations declined. In sera of BALB/c/J mice, AFP levels reached values 10-fold higher and more than in sera of C3H/He mice.

From day one after CCl$_4$ intoxication, cellular AFP was detected in hepatocytes of portal and periportal areas including intermediate zones adjacent to the necrosis. The intensity of AFP staining reached a maximum between the days 3 and 4. Hepatocytes in front of the necrotic areas usually contained the strongest AFP reactions. In both mouse strains, cellular AFP pattern was comparable, but strongest immunoreactivity was observed in liver sections of BALB/c/J mice.

Liver injury and subsequent regeneration occurred to the same extent in both studied strains. The much higher serum AFP levels and the stronger AFP immunolocalizations in BALB mice were thought not due to increased numbers of AFP producing and releasing cells during liver regeneration. Additional mechanisms must play a role in increased AFP synthesis per single cell. C3H/He was a low AFP-inducible and BALB/c/J was a high AFP-inducible mouse strain.

Introduction

Alpha$_1$-fetoprotein (AFP) is a normal constituent of fetal serum and amniotic fluid in many species (Bergstrand and Czar, 1956; Gitlin and Boesman, 1967a, b). In late embryonic life, the liver is the main organ of AFP production. After birth the serum level of this protein decreases rapidly to values which can be only detected by methods such as radioimmuno-assay (Ruoslahti and Seppälä, 1971; Sell, 1973).

AFP can reappear in substantial quantities in adult individuals in consequence of liver regeneration and processes of malignant conversion (Abelev, 1974; Laurence and Neville,

1972). Hence, this protein is of considerable value in clinical and experimental medicine. The question is posed of how gene regulation of AFP differs under normal and pathological conditions. Recently, the genetic regulation of AFP in a great variety of mouse strains was studied (Olsson et al., 1977). It was found that a single recessive Mendelian gene called *Raf* (regulation of alpha-fetoprotein) was involved in the regulation of AFP synthesis in normal adult mice from which low and high AFP-producing mice strains could be distinguished.

The intention of the present study was to add information to the cellular basis of AFP production in a low and a high AFP-producing mouse strain under normal adult conditions and during liver regeneration after carbon tetrachloride intoxication. For this purpose, the amount of AFP in sera was measured, and cellular AFP was localized by immunoperoxidase labelling techniques.

Materials and Methods

Animals. C3H/He mice were purchased from the Zentral-Institut für Versuchstiere, Hannover, Germany. BALB/c/J mice were obtained from The Jackson Laboratory, Bar Harbor, USA. Twelve week-old animals of male sex were used throughout and fed Altromin standard diet (Altromin, Lage, Germany) and tap water.

Between 9:00 and 10:00 A.M., overnight fasted mice were given orally one dose of 1 ml of 10% $CC1_4$ in liquid paraffin/100 g body weight (100 µl CCl_4/100 g mouse) (Pihko and Ruoslahti, 1974), directly into the esophagus, by means of a curved trochar and syringe. Three series of each mouse strain were scheduled for liver intoxication. From each series, three mice were bled and livers dissected out: 1, 2, 3, 4, 5 and 6 days after $CC1_4$ ingestion. Control mice were kept on the standard diet and tap water.

Preparation of Immune Sera. Mouse AFP was isolated from amniotic fluid by use of preparative polyacrylamide gel electrophoresis and immunoadsorption techniques. AFP was pure as shown by SDS polyacrylamide gel electrophoresis and immunodiffusion methods (Kuhlmann, 1975 and 1976).

Anti-AFP immune sera were obtained by hyperimmunization of rabbits with isolated AFP, and anti-rabbit IgG immune sera were produced in sheep by hyperimmunization with rabbit IgG (Miles, Frankfurt, Germany) (Kuhlmann, 1975). A rabbit anti-mouse IgG immune serum was prepared in the same manner.

Quantitative Electroimmunodiffusion. AFP content in individual mouse sera was quantitated by the procedure of electroimmunodiffusion (Laurell, 1966) with a slight modification: the immune serum was used in a high dilution (50 µl anti-AFP/100 ml gel) in 1% agarose; after electrophoresis, gel plates were washed for 2 days, then incubated with sheep anti-rabbit IgG antibodies being conjugated with glucose oxidase (Kuhlmann, 1978); after repeated washings in PBS plates were dried and reacted by cytochemical stain for glucose oxidase activity (Kuhlmann and Avrameas, 1971).

Immunocytochemical Reagents. Cellular AFP was detected by an indirect (Sandwich) staining method. For this purpose, pure rabbit anti-AFP and pure sheep anti-rabbit IgG antibodies were isolated from crude immune sera by means of immunoadsorbents. IgG molecules of the "Sandwich" antibodies (sheep anti-rabbit IgG) were conjugated with horseradish peroxidase (HRP, RZ 3; Boehringer-Mannheim, Germany) by use of glutaraldehyde and purified on a Sephadex G 200 column (Kuhlmann, 1975; Kuhlmann, 1977).

Tissue Processing and Immunocytochemistry. Liver slices of about 0.5 cm thickness were fixed in 96% ethanol - 1% acetic acid for 12-15 h at 0-4° C, dehydrated in absolute ethanol, cleared in benzene and embedded in paraffin (Kuhlmann, 1975).

Sections, 5-7 μm thick, were mounted on acetone-cleaned slides, deparaffinized in xylene and passed from absolute ethanol into 0.1 M phosphate-buffered saline pH 7.2 (PBS). Endogenous peroxidases were irreversibly inhibited by treatment with 1% hydrogen peroxide in PBS for 1 h (Kuhlmann, 1975). Then, slides were washed in PBS supplemented with normal sheep serum (1:30) and reacted by an indirect staining method: incubation was first carried out with unlabelled anti-AFP antibodies (0.1 mg/ml PBS) for 20 min and followed by HRP conjugated anti-rabbit IgG antibodies (0.1 mg/ml PBS) for 20 min. Antibodies that did not react were removed by three successive washings for 5 min each, in PBS supplemented with 1% bovine serum albumin and 0.5 M NaCl.

Peroxidase activity was assayed by incubation in 3,3'-diaminobenzidine (Graham and Karnovsky, 1966), 0.5 mg per 1 ml Tris-HCl buffer pH 7.2 (0.2 mol Tris/liter buffer) containing 0.01% H_2O_2. After washings in PBS, sections were treated for 1 min with 0.1% OsO4 in PBS, dehydrated and mounted under coverglass.

Controls. For routine histology, sections were stained using hematoxylin/eosin.

Specificity of the immunocytological reactions was controlled on serial tissue sections by incubation in (1) normal rabbit IgG globulins (0.1 mg/ml PBS); (2) rabbit anti-AFP absorbed with an immunoadsorbent which was prepared by coupling AFP to cyanogen bromide-activated Sepharose 4B (Cuatrecasas, 1970); (3) rabbit anti-mouse IgG antibodies. Either procedure was followed by HRP conjugates of sheep anti-rabbit IgG antibodies and enzyme substrate.

Results

Liver Injury and Regeneration. Livers from control mice did not show pathological changes. Each of the experimental mice of the three series received the same amount of $CC1_4$, but the extent of necrosis and subsequent regeneration varied slightly in individual mice.

Hydropic degeneration was found within 24 h after poisoning, and focal lesions with mononuclear cell infiltrates were present on the second day. Necrosis occurred in centrolobular areas. Necrotic hepatocytes and associated cell infiltrates remained for up to four days. No difference in histotoxic pattern was observed in BALB/c/J and C3H/He mice.

In both strains subsequent liver regeneration was similar. A marginal zone was seen between viable peripheral zones of the lobule and the necrotic central area. This marginal zone contained most of the dividing hepatocytes. Increased mitotic activity was found between day 2 and day 6 after $CC1_4$ ingestion.

Detection of AFP in Sera. Serum AFP concentrations in normal BALB/c/J and C3H/He mice were in the order of 0.6 μg/ml and 0.1 μg/ml, respectively. $CC1_4$ intoxication was regularly accompanied with an increase of AFP levels. Data are shown in Fig. 1. Serum AFP was slightly increased 24 h after poisoning, then it rose steeply and reached a maximum on day 4. Afterwards, concentrations declined steadily. In both mouse strains, variations in serum AFP levels followed the same time scale. However, AFP concentrations in BALB/c/J reached levels 10-fold higher and more than in C3H/He mice.

Localization of AFP in Normal Livers. In BALB/c/J mice, AFP was detected in the cytoplasm of mono- and binucleated hepatocytes in centrolobular areas and in intermediate zones (Fig.

2). The AFP staining intensity was moderate but clearly distinguished. In C3H/He mice no AFP was localized.
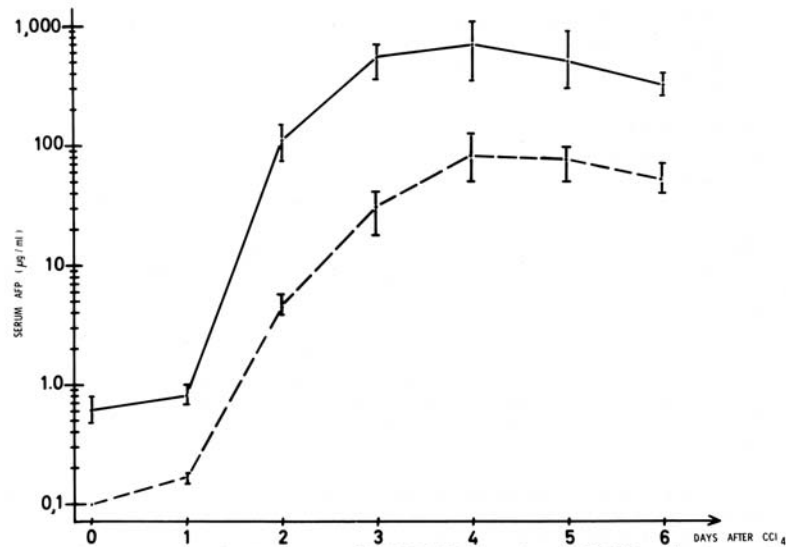

Fig. 1. Concentrations of AFP in sera of BALB/c/J (●——●) and C3H/He mice (●---●). Each point represents the mean of all experiments. The upper and lower serum AFP values are given in the brackets

Cellular Detection of AFP in Regenerating Livers. From day one after CCl_4 poisoning of BALB/c/J mice, the AFP staining pattern changed. Now, AFP positive hepatocytes were found in portal and periportal areas as well as in intermediate zones adjacent to the necrosis (day 2). The intensity of immunocytochemical reactions increased and reached a maximum between the days 3 and 4. The front between necrosis and viable liver was clear-cut, and AFP was limited to hepatocytes in the non-damaged zone (Figs. 3, 4). In many liver blocks, eminent strong AFP reactions were seen in hepatocytes which either formed groups of several cells or were disseminated randomly and intermingled with less stained hepatocytes. Also, hepatocytes in front of the necrotic areas contained the strongest AFP reaction (Figs. 3, 4). Five to 6 days after CCl_4 intoxication, intensity of AFP staining declined, but its cellular localization was still comparable to that of day 3 and day 4.

In C3H/He mice, cellular AFP was observed for the first time on day 2 after CCl_4 intoxication. Nearly all hepatocytes in the intact zones of the liver lobules reacted distinctly. The strongest AFP reaction could be observed in hepatocytes of the marginal zones immediately adjacent to necrotic areas (Fig. 5). On day 3, AFP staining intensity increased (Fig. 6). No changes were found on day 4. Afterwards, AFP staining decreased.

In both mouse strains, cellular distribution of AFP was the same. The only difference encountered was the higher staining intensity for AFP in livers of BALB/c/J mice.

Immunocytochemical Controls. Cellular uptake of plasma proteins from extracellular spaces due to diffusion was controlled by immunocytochemical reaction for serum IgG. This type of staining was regarded as non-specific and occurred in cells of necrotic areas (Fig. 7). No reaction was seen in cells of normal liver, in zones free of necrosis and in AFP staining hepatocytes. Liver sections did not stain upon incubation in normal rabbit IgG globulins and absorbed rabbit anti-AFP antibodies.
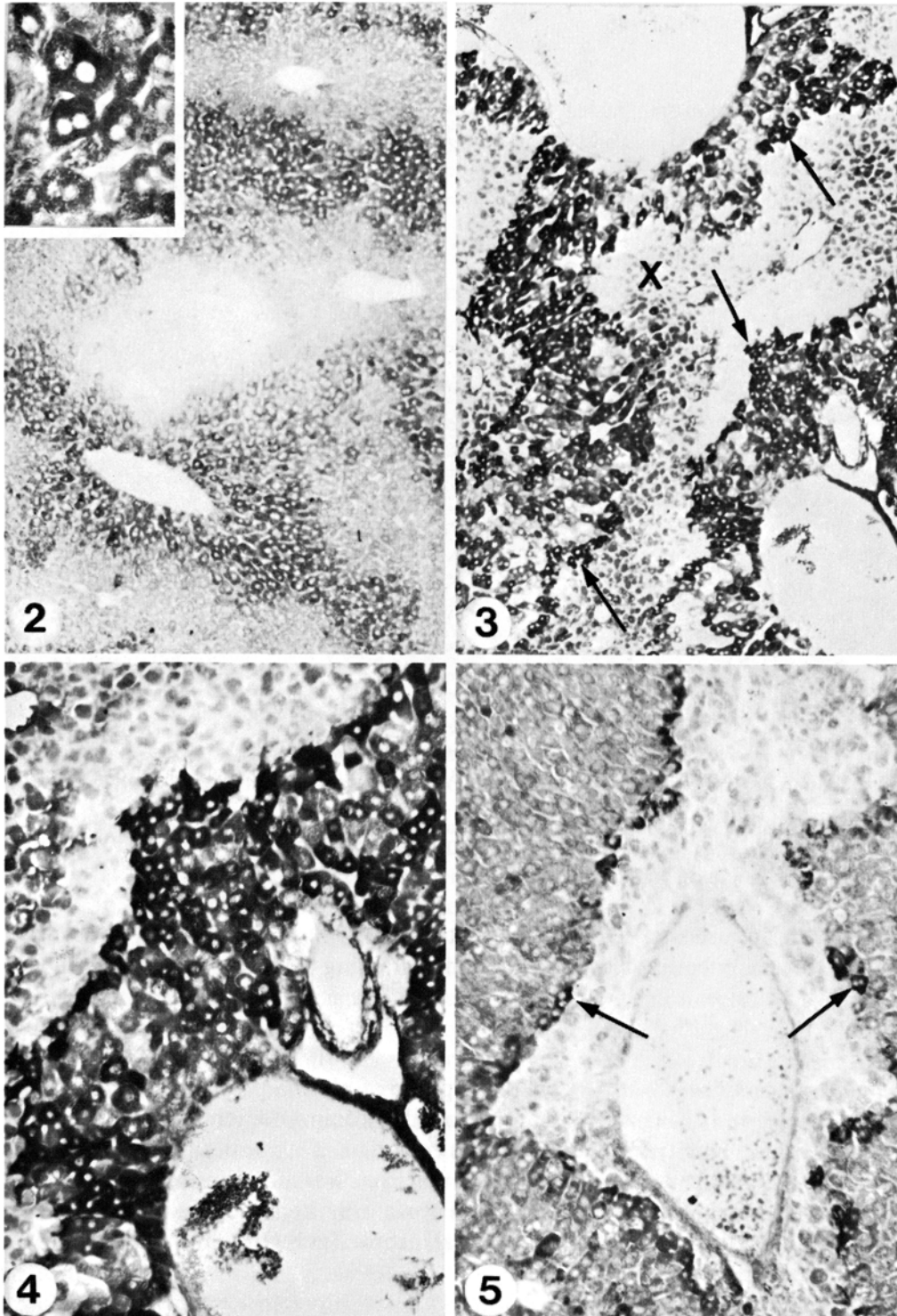
Fig. 2. Detection of AFP in normal liver of adult BALB/c/J mouse, original ×63. Inset: higher magnification of AFP staining mono- and binucleated hepatocytes, original ×225

Fig. 3. Localization of AFP in hepatocytes of regenerating BALB liver; 3 days after CCl₄ intoxication. Note strong AFP reaction (←) in cells adjacent to the necrosis (×), original ×56.7

Fig. 4. High magnification view of a portal area on day 3 after CCl₄ intoxication; BALB mouse. Strong AFP reaction in hepatocytes of non-damaged zone, original ×144

Fig. 5. Detection of AFP in C3H liver on day 2 after CCl₄ intoxication. The majority of the hepatocytes reacted distinctly; note strong AFP staining in hepatocytes adjacent to the necrotic area (←), original ×144
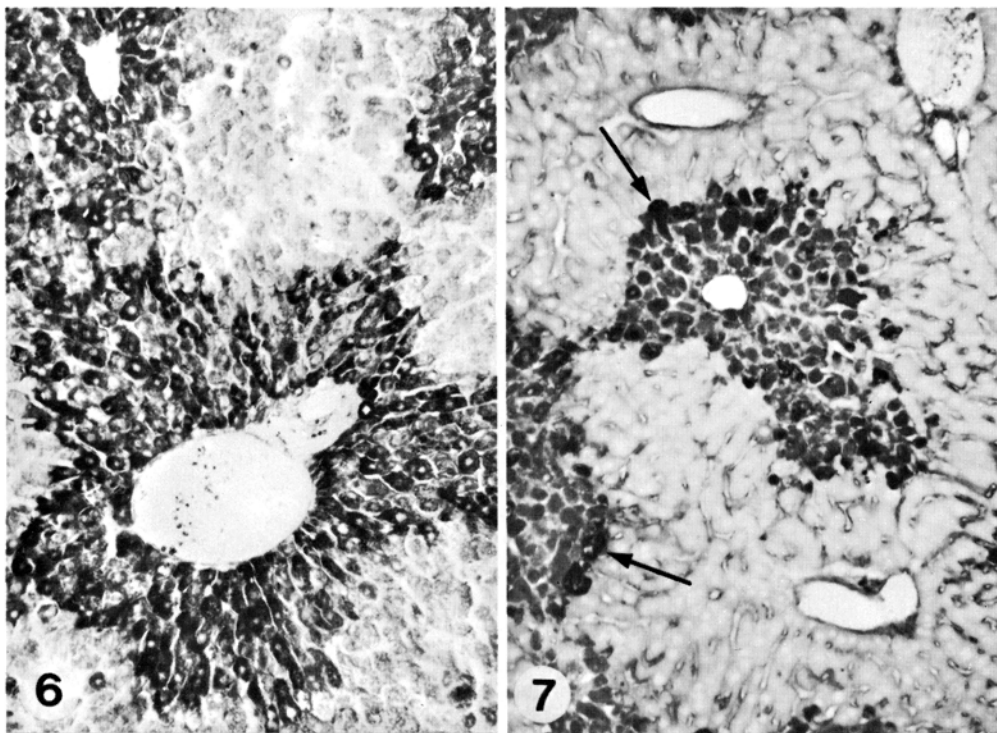
Fig. 6. Intense AFP staining in C3H liver on day 3 after CCl$_4$, original ×160

Fig. 7. Control reaction on BALB mouse liver; 3 days after CCl$_4$. Non-specific staining in damaged liver zones. Note cellular uptake of plasma IgG (←), original ×144

Discussion

Liver regeneration was found to be associated with an increase in serum AFP levels (Pihko and Ruoslahti, 1974; Bakirov, 1968). Since Olsson et al. (1977) described a *Raf* gene to be responsible for the maintenance of a certain AFP synthesis in normal adult mice, we were interested to follow AFP production under conditions of liver regeneration in two distinct strains, i.e., in C3H/He known as a low, and in BALB/c/J known as a high, AFP producing mouse strain. This study was thought to give information on AFP regulation under conditions of enhanced but non-malignant liver cell proliferation. Hence, comparison of serum AFP levels and cellular localization of AFP with liver regeneration was made.

The best conditions for light microscopic immunolocalization of AFP were defined (Kuhlmann, 1975; Kuhlmann, 1978). Passive uptake of plasma proteins from extracellular spaces, known as an artifact, was controlled by reaction of serial sections for mouse IgG. Hepatocytes in normal liver and those in regenerating zones did not stain for serum IgG which is usually not present in hepatocytes. Non-specific absorption of IgG was found to occur only in necrotic areas. The latter, however, were readily distinguished by such control incubations.

No immunolocalization of AFP was seen in normal adult C3H/He mice. Although the even low basal level of AFP was detected by serological methods, the cellular basis of AFP production could not be related to distinct populations of liver cells. It appeared most probable that the amount of synthesized AFP per single cell was too low to be detected by the immunoperoxidase technique employed.

The mouse strains studied differed inasmuch as under normal adult conditions, AFP synthesis was at least 5 times higher in BALB than in C3H mice. Such increased serum AFP concentrations were shown to be due to an increased rate of AFP synthesis rather than to a

decreased rate of catabolism of AFP (Olsson et al., 1977). The present immunocytological results clearly demonstrated that in BALB mice differentiated adult mono- and binucleated hepatocytes produced AFP. The histological pattern of AFP immunoreactivity indicated that AFP production preferentially occurred in hepatocytes of centrolobular and intermediate zones of the normal liver. It was in these hepatocytes that AFP synthesis was not efficiently turned off. Hence, continuation of AFP production at that high level in this special population of adult hepatocytes led to enhanced basic AFP amounts in sera of normal BALB/c/J mice. Moreover, the homogenous cytoplasmic staining indicated that the amount of AFP per single cell within the AFP positive population was quite the same to contribute to the elevated serum AFP concentrations. However, in consideration of the already mentioned non-immunoreactivity of sections from normal C3H mice, we could not establish whether non-stained BALB hepatocytes either do not contain AFP, or whether they contained too small amounts of AFP to be detected.

Detailed histological description of mouse liver damage due to administration of carbon tetrachloride, and subsequent regeneration, will not be repeated here (for references see Stowell and Lee, 1950; Tsuboi and Stowell, 1951; Wahi et al., 1955; Stenger, 1966). In both mouse strains, hepatic regeneration was accompanied by high serum AFP levels. Synthesis of this protein was seen in practically all hepatocytes of non-damaged liver, i.e., in portal, periportal and marginal zones. It is important to note that due to CCl_4 intoxication, hepatocytes in portal and periportal areas immediately started with significant AFP production whereas hepatocytes in centrolobular zones no longer stained for AFP. The latter were degenerated and necrotic. In BALB/c/J as well as in C3H/He mice, the marginal zone adjacent to the central necrosis not only appeared important in the regeneration process (most of the dividing hepatocytes), but also contributed significantly for AFP production. Impressively, cells of this zone usually contained the strongest AFP reactions.

In both mouse strains, the intensity of the immunocytochemical reactions and quantity of AFP in sera correlated well. Maximum immunolocalization was observed on days 3 and 4 after CCl_4 intoxication. Later on, serum AFP levels and cellular AFP reactions declined constantly.

With respect to AFP immunolocalization, the main difference between the two mouse strains studied was the regularly observed stronger staining in BALB/ c/J than in C3H/He mice. This observation was a quantitative criterion and reflected a higher rate of AFP synthesis in BALB hepatocytes than in those of C3H/He. Consequently, serum AFP levels occurred in BALB mice which could be 10 times and more higher than in C3H mice.

In BALB/c/J and C3H/He mice, growth-controllling hepatic environment favored activation of AFP production. However, the fact that liver injury and subsequent regeneration occurred to the same extent in both mouse strains suggested that increased AFP levels were not merely due to increased numbers of AFP producing and releasing cells as a result of liver regeneration. From the above we deduced that additional mechanisms must play an important role in increased AFP production per single cell. Because AFP production already occurred in quiescent adult hepatocytes of normal liver and since not every hepatocyte proliferated at any given time during liver regeneration, en-hanced synthesis of AFP must be causally related to both non-proliferating and proliferating hepatocytes. Our results support the speculation that AFP synthesis may be induced in preexisting differentiated hepatocytes and cells from the proliferating pool in regenerating liver (Engelhardt et al., 1976). In the course of liver regeneration after single CCl_4 intoxication, C3H/He mice proved to be a relatively "low AFP-inducible" and BALB/c/J mice to be a "high AFP-inducible" mouse strain.

References

Abelev, G.I.: α-Fetoprotein as a marker of embryo-specific differentiations in normal and tumor tissues. Transplant. Rev. **20**, 3-37 (1974)

Bakirov, R.D.: Appearance of embryonal serum α-globulin in adult mice after inhalation of carbon tetrachloride. Byull. Eksp. Biol. Med. **2**, 45-47 (1968)

Bergstrand, CG., Czar, B.: Demonstration of a new protein fraction in serum from the human fetus. Scand. J. Clin. Invest. **8**, 174 (1956)

Cuatrecasas, P.: Protein purification by affinity chromatography. Derivatizations of agarose and polyacrylamide beads. J. Biol. Chem. **245**, 3059-3065 (1970)

Engelhardt, N.V., Lazareva, M.N., Abelev, G.I., Uryvaeva, I.V., Factor, V.M., Brodsky, V.Ya.: Detection of α-foetoprotein in mouse liver differentiated hepatocytes before their progression through S phase. Nature (London) **263**, 146-148 (1976)

Gitlin, D., Boesman, M.: Fetus-specific serum proteins in several mammals and their relation to human α-fetoprotein. Comp. Physiol. **21**, 327-336 (1967 a)

Gitlin, D., Boesman, M.: Sites of serum α-fetoprotein synthesis in the human and in the rat. J. Clin. Invest. **46**, 1010-1016 (1967b)

Graham, R.C., Karnovsky, M.J.: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. **14**, 291-302 (1966)

Kuhlmann, W.D.: Purification of mouse alpha$_1$-fetoprotein and preparation of specific peroxidase conjugates for its cellular localization. Histochemistry **44**, 155-167 (1975)

Kuhlmann, W.D.: Untersuchungen zur zellulären Lokalisierung von alpha$_1$-Fetoprotein unter normalen und pathologischen Bedingungen. In: Tumorantigene in der Gastroenterologie. Lehmann, F.G. (ed.), pp. 27-32. München: Demeter Verlag 1976

Kuhlmann, W.D.: Ultrastructural immunoperoxidase cytochemistry. Prog. Histochem. Cytochem. **10**, 1-57 (1977)

Kuhlmann, W.D.: Localization of alpha$_1$-fetoprotein and DNA-synthesis in liver cell populations during experimental hepatocarcinogenesis in rats. Int. J. Cancer **21**, 368-380 (1978)

Kuhlmann, W.D., Avrameas, S.: Glucose oxidase as an antigen marker for light and electron microscopic studies. J. Histochem. Cytochem. **19**, 361-368 (1971)

Laurell, C.B.: Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem. **15**, 45-52 (1966)

Laurence, D.J.R., Neville, A.M.: Foetal antigens and their role in the diagnosis and clinical management of human neoplasms: a review. Br. J. Cancer **26**, 335-355 (1972)

Olsson, M., Lindahl, G., Ruoslahti, E.: Genetic control of alpha-fetoprotein synthesis in the mouse. J. Exp. Med. **145**, 819-827 (1977)

Pihko, H., Ruoslahti, E.: Alpha fetoprotein production in normal and regenerating mouse liver. In: L' alpha-foetoprotéine. Masseyeff, R. (ed.), pp. 333-336. Paris: INSERM 1974

Ruoslahti, E., Seppälä, M.: Studies of carcino-fetal proteins. III. Development of a radioimmunoassay for alpha-fetoprotein. Demonstration of alpha-fetoprotein in serum of healthy human adults. Int. J. Cancer **8**, 374-383 (1971)

Sell, S.: Radioimmunoassay of rat α-fetoprotein. Cancer Res. **33**, 1010-1015 (1973)

Stenger, R.J.: Ultrastructural alterations within hepatic parenchymal cells after carbon tetrachloride poisoning. Methods Archiv. Exp. Pathol. **1**, 677-700 (1966)

Stowell, R.E., Lee, C.S.: Histochemical studies of mouse liver after single feeding of carbon tetrachloride. Arch. Pathol. **50**, 519-537 (1950)

Tsuboi, K.K., Stowell, R.E.: Enzyme alterations associated with mouse liver degeneration and regeneration after single carbon tetrachloride feeding. Cancer Res. **11**, 221-228 (1951)

Wahi, P.N., Tandon, H.D., Bharadwaj, T.P.: Acute carbon tetrachloride hepatic injury. Composite histological, histochemical and biochemical study. Part 1. Histological and histochemical studies. Acta Pathol. Microbiol. Scand. [A] **37**, 305-314 (1955)