Localization of alpha-1-fetoprotein and DNA-synthesis in liver cell populations during experimental hepatocarcinogenesis in rats

W. D. KUHLMANN

Immunocytochemistry SFB 136 and Institut für Nuklearmedizin, DKFZ Heidelberg, Germany

Int. J. Cancer 21, 368-380, 1978

Summary

Six-, 12- and 20-week-old rats were fed N-nitrosomorpholine (NNM) at low concentrations (6 mg/kg/day) or high concentrations (20 mg/kg/day) for 6 or 12 weeks. Irrespective of the age of the rats, both NNM schedules resulted in development of hepatomas and during the early stages of hepatoma induction, liver histotoxic patterns depended only on the dose of carcinogen employed. Necrosis of hepatocytes and proliferation of small, oval-shaped cells occurred when high doses of NNM were applied. Parallel to the proliferation of oval-shaped cells, resurgence of alpha-1-fetoprotein (AFP) in rat sera was observed and production of this protein was confined to the oval-shaped cells as shown by immunoperoxidase staining.

During proliferation of bile duct epithelium, induced by galactosamine injections, those cells could also stain for AFP, and proliferation of oval-shaped cells concomitant with intracellular AFP staining resulted from restitution of heavily damaged liver.

In pulse-chase labelling experiments with $[^3]H$thymidine, oval-shaped cells were seen in continuous development towards hepatocytes, and distinct areas of hyperplastic appearance occurred. Normal hepatocytes and hyperplastic areas did not stain for AFP. Upon low-dose feeding of NNM, no cellular AFP and no serum AFP were detected unless hepatoma cells had developed.

At the stage of malignant conversion, distinct AFP-staining nodules were localized which consisted of neoplastic hepatocytes. AFP-staining and non-AFP-staining nodules were seen concomitantly in the same animal. AFP was localized only in neoplastic hepatocytes. Pulse-labelling experiments with $[^3]H$thymidine showed the proliferative character of hepatoma cells from which the AFP-staining population in particular was involved.

No correlation was found between the presence of AFP and histological grading of hepatomas. The wide range of both serum AFP levels and their rising rates in individual rats indicated the highly heterogenous character of the induced hepatomas with respect to AFP production.

Table 1. Experimental hepatocarcinogenesis
Cellular fluctuations during hepatocarcinogenesis

At high doses of NNM, prominent changes in livers occurred as megalocytic degeneration of adult hepatocytes and concomitant proliferation of PAS-negative, small, oval-shaped cells within 3 weeks of NNM feeding. By $[^{3}H]$thymidine labelling in pulse-chase experiments, oval-shaped cells were shown to be actively engaged in DNA synthesis and, furthermore, their development into hepatocytes was demonstrated. These cells constituted areas with hyperplastic appearance still active in thymidine incorporation, but during further progression DNA synthesis came to a standstill and glycogen accumulation comparable to that seen in normal hepatocytes was observed. Hence, proliferation of oval-shaped cells, of unknown origin, was a significant response to the acute hepatotoxic damage, and restitution of the liver occurred by subsequent maturation of these cells into hepatocytes. Our findings were in accordance with observations made with other carcinogens where development of proliferating “oval cells” into hepatocytes was suggested (Price et al., 1952; Farber, 1956; Inaoka, 1967; Iwasaki et al., 1972; Onoe et al., 1973). Finally, cirrhotic distortion of the original liver structure was a regular feature. The fact that no age differences were found in the sequential fluctuations of liver cells indicated common pathways of liver regeneration in rats during and after the provoked liver damage.

In contrast, neither proliferation of oval-shaped cells nor extensive necrosis of hepatocytes were detected when low concentrations of NNM were fed. Consequently, areas of hyperplastic appearance as described above were not seen. During later stages of hepatocarcinogenesis, but before hepatomas developed, we were able to distinguish areas of enhanced glycogen storage which may be regarded as irreversible precancerous lesions (Bannasch, 1975). In our material, such cells did not show signs of malignancy (like enhanced proliferation), and their role in later development of hepatomas was not clear.
Fig. 1: $[^3]$Hthymidine incorporation in pulse-labelled rat liver. (a) Liver from day 28, HE stained preparation; note numerous small, oval-shaped cells. (b) Same liver, note pulse-label in proliferating oval-shaped cells. (c) A distinct area of oval cell maturation is indicated. (d) Same liver after a $[^3]$Hthymidine pulse of 1 h; note DNA labelling in an area of hyperplastic appearance (----) and in oval-shaped cells (arrowhead).
Localization of AFP during the induction phase

By routine microscopy and immunoperoxidase staining of serial sections it was clearly shown that the emerging oval-shaped cells were responsible for AFP production during the early stages of carcinogenesis with high doses of NNM. These results support immunofluorescence work (Dempo et al., 1975; Tchipysheva et al., 1977) in which transitional cells and “small hepatocytes” were thought to produce AFP in the early stages of azo-dye and 2-acetylamino-fluorene carcinogenesis. From the 1-h pulse labelling experiments with \(^{3}\text{H}\)thymidine we found that a small proportion of the AFP-staining oval-cell population was actually in S-phase, whereas the majority of AFP-staining cells were not labelled by \(^{3}\text{H}\)thymidine. The importance of this observation is still under study and may reflect AFP synthesis after one or two mitotic cycles of the oval-shaped cells which in turn retained the capacity for DNA replication during stages preceding hepatocyte maturation (see “\(^{3}\text{H}\)thymidine incorporation in pulse-chase experiments”). Finally, a correlation between the reappearance of AFP in sera and intensity of oval-cell proliferation concomitant with AFP-positive staining was noted in individual rats. Similar serological observations were reported with other chemicals (Kitagawa et al., 1972).

In contrast to these observations we found that, during carcinogenesis with low doses of NNM, no significant proliferation of oval-shaped cells, no AFP staining of liver cells and no reappearance of AFP in sera (by the method employed) were seen. Thus, serological, histological and immunocytochemical data suggested that oval cells were indeed the cells which produced AFP at his stage of carcinogenesis. Since both NNM schedules (low and high doses) led to hepatomas, oval-cell proliferation with concomitantly transitory AFP synthesis was not regarded as a prerequisite for conversion to cancer. Merely, oval cells resulted from restitution of damaged liver due to acute toxic injury.
These conclusions were supported by immunocytochemical AFP staining in rats with galactosamine-induced experimental hepatitis. One of the prominent morphologic features was the intensive proliferation of bile-duct epithelium as already described by Lesch et al. (1970) and which was followed by the appearance of AFP in serum (Sell et al., 1974). In our animals, we also observed bile ductular proliferations from day 2 after injections of galactosamine. Such proliferations could be stained for intracellular AFP. Rats in which few regeneration plates were observed contained AFP serum levels that were below the detectable limit of the method employed. It could be that a minimal proliferation rate of oval-shaped cells was necessary for the reexpression of AFP.

When NNM feeding was stopped, oval-cell proliferation came rapidly to a standstill and, furthermore, AFP was no longer detected afterwards until hepatomas developed. In contrast to other reports (Okita et al., 1974) we did not observe AFP staining in hyperplastic nodules.

Fig. 3: Detection of AFP in liver during early stages of hepatocarcinogenesis. (a) Liver from day 28; note intracellular AFP in grouped oval-shaped cells. (b) Liver from day 35; distinct AFP staining in small cells; inset: higher magnification view of AFP-positive oval-shaped cells.
Cell producing AFP at the hepatoma stage

At the hepatoma stage, AFP was localized in the cytoplasm of cells which by routine histology were typical basophilic and PAS-negative neoplastic hepatocytes, and which were clearly distinguished by size and shape from normal adult hepatocytes. These neoplastic hepatocytes did not accumulate glycogen as was usually the case in normal hepatocytes. PAS-positive globules within intracellular spaces might occur in malignant and normal hepatocytes of individual rats, but these findings did not follow a regular pattern. Recently, intracellular PAS-positive globules were found to be related to the deposition of α-1-antitrypsin in human primary liver carcinoma cells (Palmer and Wolfe, 1976). However, in our NNM-induced hepatoma cells, no clear-cut relationship between production or storage of AFP and PAS-positive globules could be established: upon immunocytochemical control incubations, such PAS-positive globules exhibited enhanced contrast, a phenomenon which needs further consideration.

It is important to note that AFP staining never occurred in adjacent normal liver cells. In the usual hematoxylin/eosin preparations, AFP-positive and AFP-negative nodules were seen as circumscribed areas of liver cell carcinoma of grade II/III with trabecular pattern or as grade IV liver cell carcinoma (Edmondson and Steiner, 1954), but no correlation between histology and serum AFP levels was possible.

Our studies support the findings of others (Goussev et al., 1971; Nishioka et al., 1972) that not every hepatoma cell was an AFP producer cell. Rather, AFP-staining cells represented popu-
lations of distinct neoplastic hepatocytes with a certain degree of retrodifferentiation (clones). This suggestion was supported by the fact that AFP-staining and non-AFP-staining nodules could be observed side by side. Following pulse labelling of hepatoma-bearing rats with $[^3]$H]thymidine for 1 h, 10-20% of the hepatoma cells, whether AFP-positive or not, were observed to be in S-phase. However, after a further chase of 24 h, >50% of the AFP-staining cells exhibited DNA replication, whereas the number of labelled nuclei from non-AFP-staining hepatoma cells in the same animal was much lower. It must be mentioned, however, that in other animals non-AFP-staining nodules could be also heavily engaged in DNA synthesis. The importance of these observations is not yet clear.

Preferential localization of AFP-staining cells at the border of sinusoid capillaries, along bile ducts, blood or lymphatic vessels (Goussev et al., 1971; Uriel et al., 1973) was not observed in distinct nodules, but occurred when huge tumor masses were reached. In the latter, more or less necrotic areas and damaged cells were also present which stained for both AFP and IgG. This staining pattern was considered to be atypical (Engelhardt et al., 1971).

**Reappearance of AFP during hepatocarcinogenesis**

Early elevated AFP levels were described as being a response to the carcinogenic diet, and this phenomenon was correlated with carcinogenicity of the chemical agent, accumulated dose, and subsequent liver alteration, including oval cell proliferation (Watabe et al., 1971; Kitagawa et al., 1972; Kroes et al., 1972, 1973, 1975; de Néchaud and Uriel, 1973). Because elevated serum AFP levels without oval-cell proliferation could be measured during and after feeding of very small quantities of N-2-fluorenlyacetamide, hepatocarcinogenic agents were also shown to have metabolic effects in common which lead to selective derepression of AFP synthesis (Kroes et al., 1972; Becker and Sell, 1974). Because the method employed for AFP measurements in the sera of our rats did not permit the detection of very low amounts of AFP (<0.1 µg/ml) it was possible that in the present studies low amounts of AFP also resulted from a selective derepression of AFP synthesis. But, more importantly, the elevated serum AFP concentrations described here were only observed in rats given high doses of NNM, which resulted from AFP synthesis by oval-shaped cells.

Reappearance of serum AFP in the later stages of carcinogenesis was unequivocally caused by hepatoma cells as demonstrated in the present work by immunoperoxidase staining. The occurrence of AFP in primary liver cancer was considered to be the direct consequence of retrodifferentiation during neoplastic change in which stages may be reached where synthesis of AFP takes place (Uriel, 1969, 1976). Then, it may be argued that various degrees of retrodifferentiation would generate neoplastic hepatocytes of clonal origin leading to AFP-positive and AFP-negative hepatomas. This suggestion was supported by our immunoperoxidase staining technique which permitted a clear-cut distinction between AFP-staining and non-AFP-staining hepatoma nodules. The phenotype of neoplastic hepatocytes was of ancillary importance and did not reflect the potential of a neoplastic hepatocyte to produce AFP.

Serum AFP levels resulted from synthesis, secretion and turnover of this protein, and the rising rates of serum AFP in the course of hepatoma development were useful indicators for production and especially for secretion of AFP by the responsible cells. In rats with steeply rising AFP levels, signs of pronounced secretion could be observed, and in these cases the Golgi complexes were heavily stained for AFP.
From the foregoing it can be deduced that immunoperoxidase labelling of AFP in combination with autoradiographic studies of proliferating cells enabled us to give a concise description of the cellular basis of AFP synthesis. Furthermore, results from measurements of serum AFP levels and from monitoring their rising rates in the course of hepatoma progression strongly indicated that all these diagnostic procedures together were useful tools to elucidate phenomena of production and secretion of AFP. This may be also true for other tumor-relevant substances in other systems.

Fig. 5: Localization of AFP at the hepatoma stage. (a) Low magnification view of a hepatoma nodule after staining for AFP; note strong AFP reaction in neoplastic hepatocytes (←). (b) Serial section from same liver was reacted for glycogen by PAS; normal hepatocytes in the liver are strongly stained, whereas the AFP-positive hepatoma cells remained PAS-negative (→).
Fig. 6: Localization of AFP at the hepatoma stage. (a) Higher magnification from the border of a hepatoma nodule and stained by HE. (b) Serial section from same hepatoma region and reacted for AFP; neoplastic hepatocytes penetrate normal liver, and AFP reaction is only in the hepatoma cells. (c) Another view from an AFP-staining hepatoma. (d) Thymidine incorporation in AFP-staining hepatoma cells (→); adjacent normal liver is not labelled.
Fig. 7: Hepatoma with steep rising rates of serum AFP. (a) HE stained preparation; note prominent peri-nuclear halo (→). (b) Serial section from same hepatoma stained for AFP; note strong AFP reaction in perinuclear area (→).

References


Farber, E.: Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetyl-aminofluorene and 3’-methyl-4-dimethylaminoazobenzene. Cancer Res., 16, 142-148 (1956)


Inaoka, Y.: Significance of the so-called oval cell proliferation during azo-dye hepatocarcinogenesis. Gann, 58, 355-366 (1967)

Iwasaki, T. et al.: Fluctuation of various cell populations and their characteristics during azo-dye carcinogenesis. Gann, 63, 21-30 (1972)

Kitagawa, T. et al.: α-Fetoprotein and hepatocarcinogenesis in rats fed 3’-methyl-4-(dimethyl- amino) azobenzene or N-2-fluorenylacetamide. Int. J. Cancer, 10, 368-381 (1972)


Uriel, J. et al.: Localization of rat liver α-foetoprotein by cell affinity labelling with tritiated oestrogens. *Nature (Lond.)*, 244, 190-192 (1973)