ALPHA-FETOPROTEIN SYNTHESIS IN PRIMARY CULTURES OF ADULT MOUSE HEPATOCYTES

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Synthesis of the embryo-specific serum protein alpha-fetoprotein (AFP) is renewed in the regenerating mouse liver [1]. Studies of the cellular basis for AFP synthesis in the regenerating mouse liver have shown that it is renewed in mature, differentiated, pre-existing hepatocytes, quite unconnected with and, indeed, in the total absence of proliferation [4, 5, 8]. The main sign which distinguishes AFP-containing cells from the remaining hepatocytes in the mouse liver after exposure to hepatotoxins or after local mechanical injury is the localization of these cells in the perinecrotic regions [5, 8]. On these grounds the writer suggested that renewal of AFP synthesis is directly linked with local disturbances of interaction between hepatocytes and that the basic condition for repression of AFP synthesis at the tissue level is integrity of the trabecular structure of the liver [3]. It was accordingly decided to determine whether AFP is synthesized in primary cultures of adult hepatocytes in which the normal trabecular structure is completely disturbed.

Studies on primary monolayer cultures of hepatocytes from adult rats showed that these cells preserve many of their differential features *in vitro*. In particular, they synthesize albumin and certain other serum proteins. As a rule proliferation of hepatocytes is not found in these cultures. Only in one or two reports was it stated that hepatocytes can proliferate in principle *in vitro*, and in these cases small quantities of AFP were found in the medium [10, 13]. Primary cultures of adult mouse hepatocytes have so far received little study. Recently the presence of perceptible quantities of AFP was demonstrated in culture fluids of such cultures, together with serum albumin [11]. However, the localization of AFP was not studied in the hepatocyte cultures, so that it was still not clear whether this synthesis is linked with all or only with some hepatocytes.

In the investigation described below AFP synthesis by primary cultures of adult mouse hepatocytes was confirmed, the conditions for reproduction of this phenomenon were established, and the localization of AFP was studied immunohistochemically.

EXPERIMENTAL METHOD

Mice of lines C57BL/6J, SWR, BALB/cJ, and (C57BL/6J × CBA)F_1 aged 8-20 weeks were used. The liver was perfused through the inferior vena cava by means of a peristaltic pump at the rate of 2.5 ml/min consecutively with 12-15 ml of a 1 mM solution of EGTA in Hanks' solution without Ca^{++} and Mg^{++}, and 16-20 ml of a 0.3% solution of collagenase (from Serva, West Germany) in lactalbumin hydrolysate. At the end of perfusion the liver was mechanically disintegrated and the cell suspension was washed twice in serum-free medium and transferred to plastic Petri dishes 9 cm in diameter (Leningrad Biopolymers Factory), each containing 10 ml of L-15 medium (Flow Laboratories, England) with glutamine, 10% embryonic calf serum (Gamaleya Institute of Experimental Microbiology, Academy of Medical Sciences of the USSR), and gentamycin. The number of hepatocytes added to each dish was 2×10^6 to 5×10^6. To wash off nonadherent cells the medium was changed twice — after 4 and 24 h. The cultures were fixed at different times in 4% paraform in 0.1 M phosphate buffer, pH 7.2, for 30 min, washed off for at least 1 h with buffered physiological saline, and then studied either by the indirect immunoperoxidase method or by the unlabeled antibodies immunoperoxidase method

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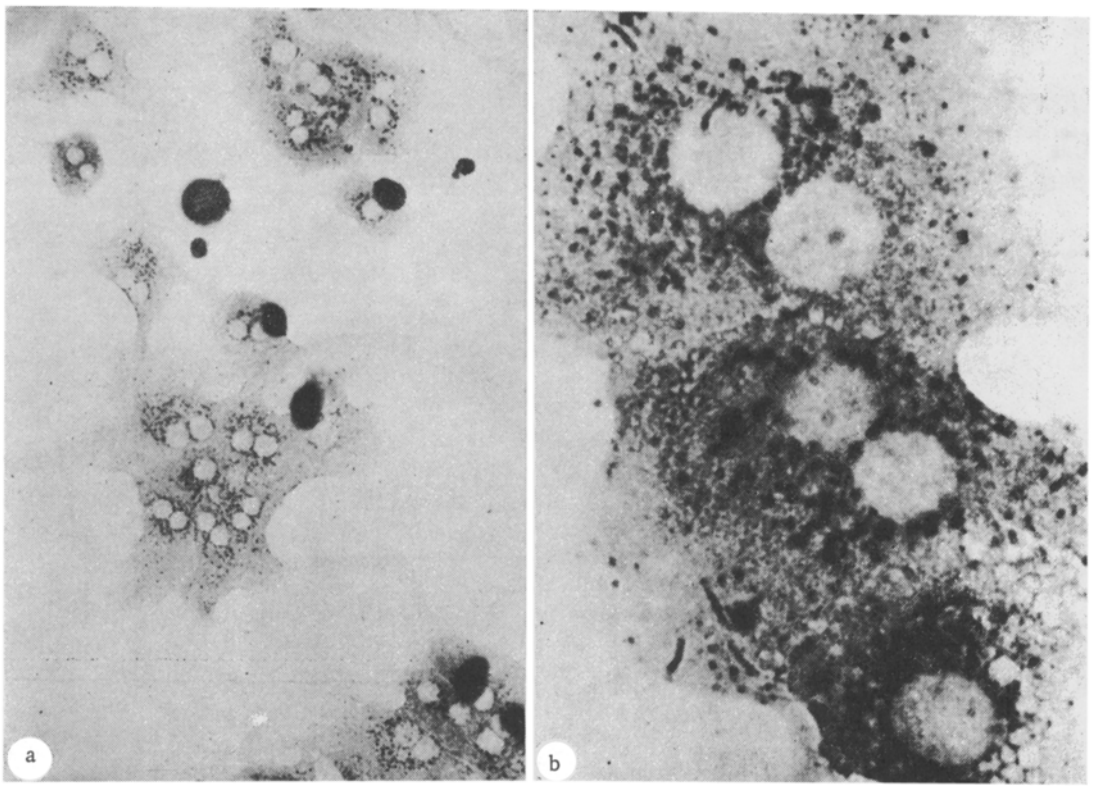

Fig. 1. Detection of albumin in 2-day culture of adult mouse hepatocytes by unlabeled antibodies immunoperoxidase method (location of antibodies determined by their characteristic dark brown staining). a) Albumin in hepatocytes of islets, 20 ×; b) the same, under high power (100 ×). Granules evidently correspond to cisterns of Golgi apparatus and secretory vacuoles.

TABLE 1. Detection of AFP in Adult Mouse Hepatocytes Cultured *in Vitro*

Expt. No.	Line of mice	Detection of AFP in culture on day: 1	2	3	4	5	6	7	8
1	F_1(C57Bl/6j× ×CBA)		—						
2	The same		—	—					
3	" "			+/—	+	+			
4	" "				+	+	+		
5	C57Bl/6j				+/—		+		+
6	The same			+/—		+			
7	" "			+		+		+	
8	" "				+		+		
9	SWR			—	+				
10	The same	—		+		+			
11	" "				+				
12	BAlB/cj			+		+			

Legend. Each sign corresponds to one dish; —) absence of cells, +/ —) very few cells, +) appreciable number of cells.

[14]. Before treatment with antibodies, endogenous intracellular peroxidase was inhibited with 0.3% H_2O_2 in methanol, and at the same time this facilitated subsequent penetration of antibodies into the cell cytoplasm. After washing off with physiological saline the cultures were then treated with preparation of monospecific rabbit antibodies against AFP and albumin, followed by pig antiserum against rabbit IgG and a complex of horseradish peroxidase with rabbit antiperoxidase antibodies (from DAKO Immunoglobulins, Denmark) or with donkey

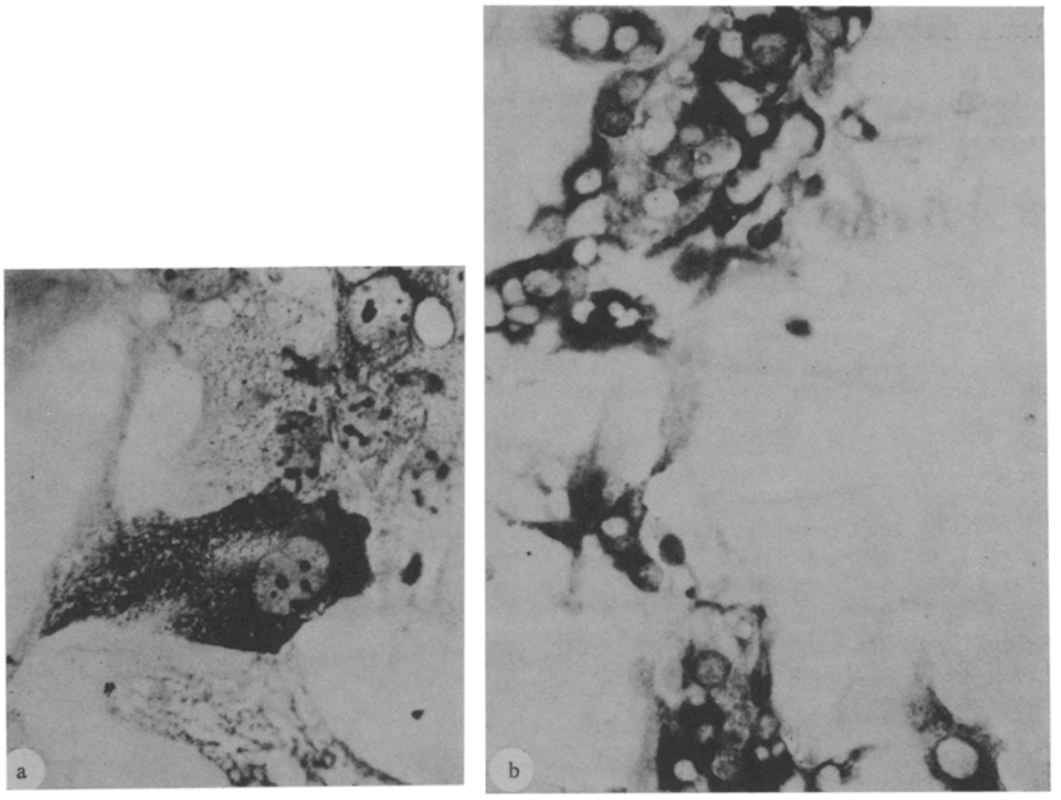

Fig. 2. Detection of AFP by indirect immunoperoxidase method in 3-5 day-cultures of adult hepatocytes: a) single AFP-containing hepatocyte in 3-day culture. Counterstained with hematoxylin. AFP-containing hepatocyte stands out clearly against other cells because of brown staining of its cytoplasm, 40 ×; b) AFP present in most hepatocytes of 5-day culture, 20 ×.

antiserum against rabbit IgG, conjugated with horseradish peroxidase. Peroxidase activity was detected by the usual method [9]. As a rule the preparations were then weakly counterstained with hematoxylin. Preparations of specific antibodies exhausted by the corresponding pure antigens served as the controls. In certain cases the presence of AFP was determined in culture fluids by means of aggregate hemagglutination [7] and immunoisotachophoresis [2].

EXPERIMENTAL RESULTS

Isolated adult mouse hepatocytes adhered comparatively well to the plastic within a few hours. By 24 h in culture they formed islets or even extensive sheets consisting of a single layer of cells or more. The cells in the islets and sheets were polygonal and, in their morphology, they resembled hepatocytes of the adult liver *in vitro*. On the 1st-2nd day of culture all hepatocytes contained albumin in their cytoplasm (Fig. 1). No AFP was found in any culture at these times (Table 1). Starting with the 3rd day of culture appreciable changes were found in the morphology of the hepatocytes in culture: They grew longer, often they became irregular in shape, fusiform, or fibroblast-like. As before, albumin was found in their cytoplasm. In some cases AFP also was detected. In two experiments there were solitary cells of this nature (Fig. 2a), in three experiments there was an appreciable but small number of these cells, and in one experiment approximately half of the hepatocytes were of this kind (Table 1, experiment No. 7). In two experiments no AFP-containing cells were found on the 3rd day of culture. By the 5th day of culture there was an appreciable number of AFP-containing hepatocytes (Fig. 2b). Usually about half of the total number of hepatocytes were of this kind. Albumin was detected in all hepatocytes, but with varied intensity, at these times. AFP continued to be found in the culture until it died, which was usually on the 6th-8th day in culture. Despite the decrease in the absolute number of hepatocytes, there was no decrease in the percentage of cells containing AFP.

In some experiments (Nos. 4, 7, 8, 10) dividing hepatocytes were found on the 3rd-5th day of culture. In experiments No. 4 on the 5th day and No. 7 on the 3rd day of culture the level of proliferation was fairly high — the number of mitoses without previous addition of cytostatics reached about five per 1000 cells. The fact that it was hepatocytes which proliferated was confirmed by the presence of albumin and sometimes of AFP in the cytoplasm of the dividing cells. Besides hepatocytes, connective-tissue cells also were regularly seen in the cultures. In experiment No. 7 the AFP content in the culture fluids was determined. It was found to be fairly high, namely 0.25 μg/ml on the 5th day and 0.5 μg/ml on the 7th day of culture. The AFP content in the medium in experiment No. 11 was about 0.03 μg/ml and the number of AFP-containing cells in experiment No. 11 was 1 order of magnitude less than in experiment No. 7.

Unfortunately, despite a sometimes fairly high level of proliferation, it was impossible to obtain survival of significant numbers of hepatocytes for more than 8 days in culture.

To judge from the number of AFP-positive hepatocytes, AFP synthesis proceeded equally in cultures of all the lines of mice used, including C57BL/6J. This is interesting because during regeneration of the liver *in vivo* the level of AFP synthesis in mice of this line was 1 order of magnitude less than in SWR mice and 2 orders of magnitude less than in BALB/cJ mice [7, 12]. The background level of AFP synthesis in the adult animal also was 1 order of magnitude less in C57BL/6J than in BALB/cJ mice [12]. To judge from the number and brightness of hepatocytes staining for AFP in cultures of liver cells from adult C57BL/6J mice, induction of its synthesis took place much more intensively *in vitro* than *in vivo* during regeneration.

On the basis of these results the possible mechanisms of regulation of AFP synthesis at the cellular level can be judged. Data showing that adult hepatocytes as a rule do not proliferate *in vitro* suggested that in the present experiments, just as during regeneration of the mouse liver, AFP synthesis was unconnected with proliferation of the hepatocytes. In that case it would be highly tempting to suppose that AFP synthesis in primary cultures of adult liver is directly linked with a disturbance of interaction between hepatocytes, the absence of a trabecular structure, and the increased mobility of the hepatocytes. On the other hand, the presence of an appreciable number of dividing hepatocytes in the cultures indicates that it is too early to draw any such conclusions. Undoubtedly the differences in the manifestation of genetic regulation of AFP synthesis *in vitro* and *in vivo* requires further study.

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LITERATURE CITED

1. G. I. Abelev, Transplant. Rev., 20, 3 (1974).
2. G. I. Abelev and É. R. Karamova, Byull. Éksp. Biol. Med., No. 9, 334 (1979).
3. A. S. Gleiberman, N. I. Khramkova, and T. D. Beloshapkina, Byull. Eksp. Biol. Med., No. 6, 576 (1979).
4. N. M. Lazareva, Byull. Éksp. Biol. Med., No. 2, 346 (1980).
5. V. S. Poltoranina, Byull. Éksp. Biol. Med., No. 2, 212 (1981).
6. V. S. Poltoranina and T. V. Sorokina, Byull. Éksp. Biol. Med., No. 7, 71 (1978).
7. A. M. Olovnikov, Dokl. Akad. Nauk SSSR, 169, 1180 (1966).
8. N. V. Engel,gardt, V. S. Poltoranina, et al., Byull. Éksp. Biol. Med., No. 10, 1251 (1976).
9. R. C. Graham and M. J. Karnovsky, J. Histochem. Cytochem., 14, 291 (1966).
10. H. Leffert, T. Moran, S. Sell, et al., Proc. Natl. Acad. Sci. USA, 75, 1834 (1978).
11. B. De Nechaud, A. Viron, and E. Puvion, C. R. Acad. Sci. (Paris), 289, Ser. D, 737 (1979).
12. M. Olsson, E. Ruoslahti, and C. Lindal, J. Exp. Med., 145, 819 (1977).
13. A. E. Sirica, Y. Richards, T. Tuscada, et al., Proc. Natl. Acad. Sci. USA, 76, 283 (1979).
14. L. Sternberger, Immunocytochemistry, Wiley (1979).