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α_1-FETOPROTEIN PRODUCTION DURING THE HEPATOCYTE GROWTH CYCLE OF DEVELOPING RAT LIVER

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SUMMARY : The relation of AFP production to DNA synthesis was investigated in newborn rat liver and in primary cultures of fetal rat hepatocytes, by combining immunoperoxidase AFP localization and autoradiography after ^{3}H-thymidine labelling. The vast majority of AFP-positive hepatocytes did not incorporate ^{3}H-thymidine after $\leq$ 4-h isotope pulses, suggesting that in the developing liver, essentially no production of AFP occurs in S, G_2 or M phases of the hepatocyte cell life cycle. Serial or continuous thymidine labelling experiments further indicated that post-mitotic hepatocytes constitute a sizable fraction of AFP-producing cells.

INTRODUCTION.

The production of α_1-fetoprotein (AFP) by the liver is generally associated with parenchymal cell proliferative growth activity. However, the exact relationship of AFP synthesis with the liver cell replication cycle remains incompletely delineated (1, 2, 3). The purpose of this study was to investigate, by combined ^{3}H-thymidine labelling and AFP immunolocalization, in which phase(s) of the hepatocyte cell life cycle is AFP produced during normal liver development in rat.

MATERIAL AND METHODS.

The study was conducted on 6- and 12-day old Wistar rat livers and on primary cultures of fetal hepatocytes 48 h after cell seeding. Cultures were established from 18-20-day old Wistar rat fetuses as described previously (4).

For thymidine labelling, newborn rats received intra-peritoneal injections of 1 µCi per g ^{3}H-methyl-thymidine (15 mCi/mole) (CEA, Saclay, France). One group of rats received a single injection and was sacrificed after 0.5, 2, 4 or 24 h ; a second group of rats received 4 consecutive injections at 4-h intervals and was sacrificed 24 h after the first injection. Cultured cells were incubated with 1 µCi of tritiated thymidine per ml of medium, and processed 0.5, 4 or 24 h later.

For AFP immunolocalization, liver sections and cultured cells were fixed in alcohol-acetic acid at 4°C, incubated with immunopurified peroxidase-labelled sheep anti-rat AFP IgG antibodies, and then stained for peroxidase

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activity, following the procedures described in detail previously (5). Controls of reaction specificity included immunolocalization with peroxidase-labelled normal sheep gammaglobulins or with peroxidase-labelled rabbit immunoglobulins anti-rat gammaglobulins ; reaction with a 0.1 % peroxidase solution ; and direct incubation with the peroxidase-staining reagent.

Autoradiographies were done with Ilford K-5 film emulsion. AFP was measured by radioimmunoassay (6).

RESULTS AND DISCUSSION.

In newborn liver sections as well as in fetal liver cultures, AFP was localized only in the cytoplasm of hepatocytes. All other cell types were AFP-negative. AFP-positive and AFP-negative hepatocytes appeared morphologically similar (7).

In the newborns, the proportion of AFP-positive hepatocytes was highest between day 6 and day 12, reaching up to 25 % at day 12, as we observed before (8). Serum AFP levels decreased only slightly during that period, from 3.5 to 2.3 mg/ml. Autoradiographies of ^{3}H-thymidine-labelled livers indicated that the fraction of liver cells in S phase was also maximum and relatively constant during the day 6 to 12 period, with 4 to 5 % of cells incorporating the tracer during a 2 h pulse : these data are also in agreement with previous observations (9).

The results of double labelling experiments in 6-and 12-day old animals are presented in Table I. In rats injected with a single dose of ^{3}H-thymidine, essentially no liver cell (< 0.1 %) was positive for both AFP and the radiolabel at 0.5 and 2 h. At 4 h, about 0.4 % AFP-positive cells had incorporated ^{3}H-thymidine, and after 24 h, the number of doubly labelled cells reached about 1.2 %. In rats which had received 4 consecutive injections of tracer at 4-h intervals, as many as 10 % of AFP-positive cells were thymidine-labelled 24 h after the first injection.

In fetal liver cultures, the number of AFP-stained hepatocytes also labelled with ^{3}H-thymidine increased from 1 % to 5 % to 15 %, 0.5, 4 and 24 h after addition of the isotope to the culture medium (table I).

Our observations demonstrate that, in vivo as well as in vitro, the vast majority of AFP-positive perinatal rat liver cells do not incorporate ^{3}H-thymidine after ≤ 4 h isotope pulses. Since the duration of G_2+M cell cycle phases of rat hepatocytes is 1.5 to 5 hours (9, 10, 11), our results therefore strongly indicate that in the developing liver, essentially no production of AFP occurs in S, G_2 or M phases of the hepatocyte cell life cycle. The presence of a few AFP-positive hepatocytes labelled with thymidine after ≤ 4 h isotope pulses, also observed by Kuhlmann in primary fetal rat liver cultures (12), could be accounted for by incomplete AFP release after

TABLE I

PERCENTAGE OF AFP-CONTAINING HEPATOCYTES LABELLED WITH ^{3}H-THYMIDINE

	Time after ^{3}H-thymidine injection (in h)				
	0.5	2	4	24	24[a]
6-day old rat liver		0.05 (4)[b]	0.4 ± 0.1[c] (4)	1.2 ± 0.7 (6)	10.3 ± 3.5 (7)
12-day old rat liver		0.05 (4)	0.4 ± 0.1 (4)	1.4 ± 0.2 (4)	10.4 ± 1.5 (8)
Primary culture of fetal rat hepatocytes (2 or 3 days of culture)	1.0 ± 0.5 (6)		5.6 ± 2.7 (5)	15.2 ± 7.5 (4)	

a) The rats were given 4 injections of ^{3}H-thymidine, at intervals of 4 h and were sacrificed 24 h after the first one.
b) The figures in parentheses represent the number of experiments
c) The values are given as mean ± SE.

synthesis in some cells -an idea which is reinforced by our previous findings suggesting reduced protein secretion by newborn rat hepatocytes (13, 14)- or by AFP synthesis in some cells which reentered a post-mitotic AFP-producing stage.

Our double-labelling data with short-term thymidine pulses thus seem to rule out previous suggestions that AFP production by fetal rat hepatocytes might take place in G_2 (1) or that synthesis might occur prior to S with secretion taking place during or after S, G_2 or M (1, 2). On the other hand, the conclusion that AFP is synthesized and secreted before S is consistent with the rapid cellular transit of AFP after synthesis (6, 15), and with other observations on the kinetics of AFP production in relation to DNA synthesis in fetal liver cultures (16) and during chemical liver injuries (17, 18, 19) and hormone-induced AFP suppression (15, 16).

Our data with 24-h multiple thymidine labelling are in agreement with those obtained with short-term thymidine pulses : since the cell cycle of newborn rat hepatocytes ranges between 50 h and 7.1 days (11), these results also suggest that AFP is synthesized after mitosis and before S phase, i.e AFP production is a G_1 and/or a G_0 (or some intermediate state (20)) event.

The exact position of AFP synthesis in the overall life cycle of the hepatocyte remains incompletely elucidated at present. In certain growth states (e.g. tumor cells, exponentially growing fetal liver in vivo (21)), the production of AFP, and other secretory proteins as well, must occur in continuously cycling hepatocytes. However in normal post-natal liver, or in adult liver injuries, AFP synthesis may be quantitatively unrelated to DNA synthesis and mitotic activities (18, 22). Furthermore, certain liver toxins induce AFP apparently without significant DNA synthesis and vice-versa (23, 24). The present experiments do not completely answer the question as to whether the production of AFP in the developing liver is dependent upon a cycling or a non cycling cell population. The 24-h multiple thymidine labelling data in vivo and the 24-h pulse culture results, showing up to 10 to 15 % thymidine-labelled AFP-positive hepatocytes, do indicate that a sizable fraction of AFP-producing cells was involved in DNA synthesis and mitosis prior to AFP synthesis. However, the exact size of the growth fraction of fetal rat liver in vitro and of post-natal rat liver in vivo is unknown (11), and thus our data do not reveal whether the double-labelled cells subsequently reentered the division cycle or left the proliferating pool, i.e. whether they returned to G_1 or shifted toward G_0. Whether AFP synthesis is a cell replication event at all stages of normal liver development thus remains at this time an open question.

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