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ISOZYME SHIFT IN CULTURED FETAL HUMAN HEPATOCYTES : A STUDY OF PYRUVATE KINASE AND PHOSPHOFRUCTOKINASE.

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SUMMARY : Hepatocytes from a 4-month old fetus were cultured for 15 days. We found that fetal hepatocytes contained some R_1 (precursor) form of L-type pyruvate kinase. Culture was associated with a considerable increase of the M_2-type pyruvate kinase activity, but some L-type enzyme could be detected even after 10 days.

Isozyme shift of phosphofructokinase seemed to be a progressive rather low phenomenon. Fetal hepatocytes showed an increase of the F-type form and a disappearance of the M-type form during culture. However, by day 10, the L-type enzyme remained predominant ; this is in striking contrast with the findings reported on cultured fibroblasts.

From these results, pyruvate kinase can be considered as a "strong" marker of cell differentiation, while phosphofructokinase is rather a "weak" marker.

INTRODUCTION.

Attempts to maintain specific hepatocytic functions in culture over a long period have been usually unsuccessful (1-3), except in the case of some hepatoma cell lines which remained indefinitely capable to exhibit some specific functions (4-6). However, cultured fetal rat hepatocytes displayed specific properties for a longer time than cultured adult cells (7). In this work, we studied two isozyme markers of liver differentiation (pyruvate kinase and phosphofructokinase) in isolated fetal human hepatocytes and their isozymic shift occuring during culture.

MATERIAL AND METHODS.

Cell isolation and culture.

Hepatocytes were isolated from a 4-month-old fetus following therapeutical abortion, as described elsewhere for fetal rat hepatocytes (7). The liver was excised, minced and washed three times with Hepes buffer. Then the liver fragments were stirred gently at 37° C in the dissociating medium used by Seglen for the isolation of adult rat hepatocytes by perfusion (8). This medium was slightly modified : it contained 0.025 % collagenase CLS (Worthington Saint Louis, USA) and $CaCl_2$ in Hepes buffer at pH 7.6.

Parenchymal cells were maintained in the Ham-F_{12} medium supplemented with 10 % fetal calf serum (normal medium). In some flasks, this cul-

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ture medium was enriched with bovine pancreas crystalline insulin (8 mg/liter), 0.2 % bovine serum albumin and 10^{-5}M hydrocortisone hemisuccinate which is known to inhibit fibroblastic cell growth (7).

Enzymatic studies.

Isozymic analysis of phosphofructokinase was performed by immunoprecipitation using monospecific anti-human M-type, L-type and F-type phosphofructokinase antisera (9-12).

Polyacrylamide slab gel electrophoresis of pyruvate kinase was carried out according to the slightly modified Imamura's technique (13, 14). In some experiments the extracts to be analyzed by electrophoresis were first treated by anti human L-type or M_2-type pyruvate kinase antisera (14-16) or by trypsin (14, 17).

RESULTS.

Hepatocyte culture characteristics.

Isolated hepatocytes attached to the substratum within 4 h and formed monolayers of granular epithelial cells within 24 h (fig. 1). In the presence of insulin and albumin, the plating efficiency was increased and the cells were more spread over the polystyrene surface. Hepatocytes divided actively during the first 4 days as demonstrated by radioautography using ^{3}H-thymidine (not shown) but stopped dividing thereafter. These cells were maintained for two weeks. Fibroblastic cells were few in number during the first 4 days then proliferated actively. Hydrocortisone strongly inhibited their growth and furthermore induced development of intercellular spaces comparable to bile canaliculi between hepatocytes, as previously reported in cultures of fetal rat liver (18).

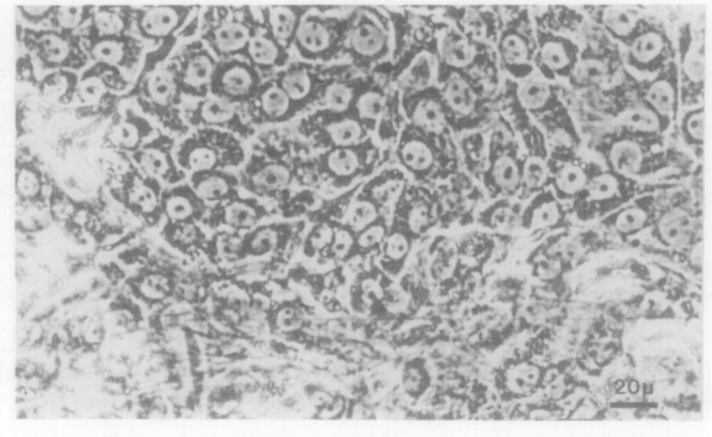

Figure 1. Human fetal hepatocytes after 5 days in culture under phase-contrast microscopy ; they form a monolayer of granular epithelial cells ; most of them contain one nucleus with 1 to 3 nucleoli. X 370.

	Specific activity		Residual PFK activity after neutralization with excess anti PFK anti-sera (%)			Residual PK activity after neutralization with excess anti PK anti-sera (%)	
	PK	PFK	anti-M	anti-L	anti-F	anti-M_2	anti-L
Freshly isolated hepatocytes	0.22	0.062	50	17	94	20	80
15 h cultured hepatocytes	0.19	0.071	55	14	98	20	80
5-day cultured hepatocytes . without hormones	1.4	0.053	62	28	54	0	100
. in hormone-enriched medium	0.94	0.033	55	28	40	0	100
10-day cultured hepatocytes . without hormones	4.4	0.095	88	28	18	0	100
. in hormone-enriched medium	1.9	0.046	68	16	35	0	100
Cultured fibroblasts	2-4	0.05-0.15	85-100	40-60	20-30	0	100

Table 1. ENZYMATIC AND IMMUNOLOGIC CHANGES OF PYRUVATE KINASE AND PHOSPHOFRUCTOKINASE FROM FETAL HUMAN HEPATOCYTES IN CULTURE. The results on cultured human fibroblasts were obtained from 4-10 measures whose the extreme values are given.

Enzyme studies (table I).

Phosphofructokinase : Specific activity of phosphofructokinase was stable during the 10 first days of hepatocyte culture (0.03-0.09 IU/mg protein). Fetal hepatocytes were characterized by the predominance of L-type isozyme ; the F-type form was virtually absent. The culture was associated with the appearance of some F-type phosphofructokinase, especially in cultures performed in the absence of hormones. After 10 days of culture, L-type phosphofructokinase remained clearly the predominant form, in striking contrast with the isozymic pattern of cultured fibroblasts.

Pyruvate kinase : Freshly isolated and 15 h cultured hepatocytes contained M_2-type as well as small amounts of L-type (fig. 2). The immunological titrations of L-type and M_2-type pyruvate kinases confirmed that both forms were present. They accounted for 20 and 80 % of the total enzyme activity respectively. Total enzyme activity was strongly increased at 5 and 10 days of cell culture ; by day 10, it was 25-fold increased in cultures without hormones and about 10-fold increased in hormone-enriched cultures. At these dates all the activity could be neutralized by anti-M_2-type antiserum and electrophoretically, M_2-type pyruvate kinase was the only form observed. However, when the sample subjected to electrophoresis had first been treated by excess anti M_2-type antiserum, a faint band migrating as normal liver L-type pyruvate kinase could be observed (fig. 3). In freshly isolated and 15 h

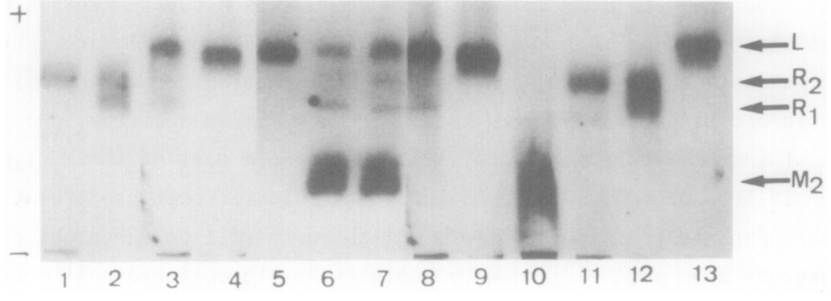

Figure 2. Polyacrylamide slab gel electrophoresis of pyruvate kinase from fetal human hepatocytes ; specific staining for pyruvate kinase activity.

1 and 11 : purified R_2 pyruvate kinase from red cells ; 2 and 12 : partially purified red cell enzyme ; 3 : 15 h cultured hepatocytes pretreated with anti M_2-type antiserum, 4 :idem 3 + treatment with trypsin ; 5 and 13 : purified liver L-type enzyme ; 6 : isolated fetal hepatocytes ; 7 : 15 h cultured hepatocytes ; 8 : idem 7, pretreatment with anti M_2-type antiserum ; 9 : idem 8 + treatment with trypsin ; 10 : idem 7, pretreatment with anti L-type antiserum.

The arrows indicate the position of the pyruvate kinase isozymes L, R_2, R_1 and M_2.

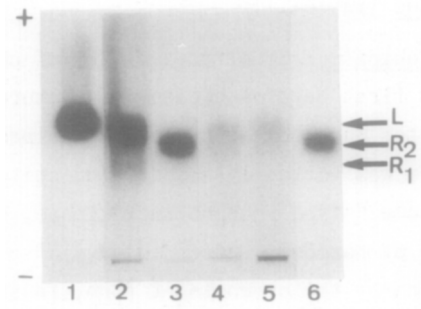

Figure 3. Detection of L-type pyruvate kinase in cultured fetal human hepatocytes. Polyacrylamide slab gel electrophoresis, specific staining for pyruvate kinase activity.

1 : purified liver L-type pyruvate kinase ; 2 : 15 h cultured hepatocytes, pretreatment with anti M_2-type antiserum ; 3 and 6 : purified R_2 isozyme ; 4 : 5-day cultured hepatocytes, pretreatment with anti-M_2 antiserum ; 5 : 10-day cultured hepatocytes, pretreatment with anti M_2-type antiserum.

cultured hepatocytes, two intermediate bands were observed between the positions of M_2-type and adult liver L-type pyruvate kinases ; they exhibited approximately the same migrations as both forms of red cell pyruvate kinase (fig. 2). These bands persisted after treatment with excess anti-M_2-type antiserum, but disappeared after treatment with excess anti-L-type antiserum. They were also suppressed by trypsin treatment (fig. 2).

DISCUSSION.

Our results show that both M_2- and L-type pyruvate kinases which have been already found in fetal human liver extracts (15, 19), are synthesized by the hepatocytes. The first phenomenon occuring during hepatocyte culture was a rapid and strong increase in total pyruvate kinase activity, due to an increase of the M_2-type form. By day 10 of culture, a part of the M_2-type enzyme could be ascribed to the associated fibroblastic cell proliferation which could explain the higher activity measured in cell cultures not enriched with hydrocortisone which is known to inhibit the fibroblastic cell growth (7).

After 5 days of culture, L-type pyruvate kinase was no longer detectable by usual immunological and electrophoretic methods, which confirms the classical view that this isozyme is readily repressed during dedifferentiation in vitro. A shift towards a more fetal-like state has also been demonstrated in non-dividing cultured hepatocytes from normal adult rats, after a few days in culture (20), indicating that there is no direct link between production of fetal antigens and cell division. However it appeared that small amounts of L-type enzyme still existed after 10 days of culture and consequently after hepatocyte proliferation but were masked by the highly predominant M_2-type form. After specific immunoneutralization of this latter form, electrophoresis

clearly showed a band corresponding to L-type enzyme. Thus it can be concluded that if synthesis of M_2-type pyruvate kinase is increased, that of L-type remains incompletely depressed in cultured hepatocytes.

In freshly isolated hepatocytes as well as in short-term cultured cells two extra bands were detected between the M_2- and L-isozymes ; they migrated in the same position as the isozymes of red blood cells, i.e R_1 and R_2 . These extra bands were precipitated by anti-L-type but not by anti-M_2-type antiserum. Moreover, they seemed to be converted into a form migrating as liver L-type enzyme by a mild proteolytic attack with trypsin. Therefore it may be assumed that they correspond to R_1 and R_2 respectively (14, 17). This assumption agrees with the data reported by several authors in animals (21, 23). However, in some of these reports, it was difficult to ascribe these minor isozymes to hepatocytes since hemopoietic cells are abundant in fetal liver. In contrast, such a contamination can be excluded in our system since hemopoietic cells were separated from parenchymal cells by differential centrifugation and did not adhere to the bottom of culture flasks. If, as previously hypothesized (14, 17), R_1 -type corresponds to the precursor form of L-type isozyme, our findings could signify that the proteolytic system responsible for the cleavage $R_1 \rightarrow L$ is relatively "immature" in the fetus.

The isozymic shift observed for phosphofructokinase was more progressive than for pyruvate kinase. It is known that the liver contains predominantly L-type isozyme, associated in fetus with small amounts of M-type phosphofructokinase (24). In culture, F-type enzyme progressively increased. Although these changes could reflect a dedifferentiation towards a fibroblastic like pattern, cultured hepatocytes remained clearly different from fibroblasts even after 10 days of culture, especially when the culture medium was enriched with hormones. This observation indicates that phosphofructokinase can be regarded as a "weak" marker of cellular differentiation and could be very useful to allow a "step to step" appreciation of different stages of differentiation in cell culture, development and malignancy.

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