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Modulation of Fetal Mouse Liver Cells Cultured on a Pigskin Substrate

Koichi HIRATA,* Yutaka YOSHIDA,* Eva ENGVALL,** Koji SHIRAMATSU,* Aaron E. FREEMAN,*** Michio MORI* and Hiroshi HAYASAKA*

* *Department of Surgery and Pathology, Sapporo Medical College S-1, W-16, Sapporo, 060 Japan*

** *La Jolla Cancer Research Foundation, 2445 Science Park Rd, La Jolla, CA, U. S. A.*

*** *Center for Neurologic Study, 11917 Sorrento Valley Rd., Suit H, San Diego, CA, U. S. A.*

SUMMARY

Mid term mouse fetal liver was cultured on dead, sterile pigskin as a substrate. Organ bits initiated on the dermal side of the pigskin died within one week. However, the bits placed on the epidermal side survived and grew for at least 7 weeks. The cultured cells retained several enzyme activities i. e. gamma-glutamyl transferase (GGT), and lactic dehydrogenase (LDH). Moreover, the production of alpha-fetoprotein (AFP) and albumin in cultured cells was detected. The activity of GGT was high at the beginning of culture and then decreased rapidly. The activity of LDH was high during the entire 7 week period. AFP levels in media and in cultured cells were high during the early stage, but became undetectable after 4-5 week in culture. The production of albumin became higher and higher during culture. Hydrocortisone prolongs production of AFP and reduces that of albumin for 7 weeks. These results indicate that mid term mouse liver cells were cultured and modulated by hydrocortisone on a pigskin epidermal substrate. The long culture and the in vitro modulation of fetal hepatocytes might be supported with the basement membrane of pigskin, especially laminin which is one of basement membrane matrix.

Key words: Mouse fetal hepatocyte, Pigskin substrate, Culture

INTRODUCTION

Culturing liver-derived cells in vitro as monolayer or organ cultures has been developed recently by using various substrata (Michalopoulos and Pitot, 1975; Sattler *et al.*, 1978; Sirica *et al.*, 1979; Michalopoulos *et al.*, 1979), and selective media (Leffert and Paul, 1972; Bonney *et al.*, 1974; Lin and Snodgrass, 1975; Williams *et al.*, 1977). Michalopoulos, *et al.* (1979) compared the effectiveness of several substrata such as collagen membrane, collagen-coated plates and fibroblasts as feeder layers to grow liver cells identified in terms of their P-450 activity. Sirica

et al. (1979) reported the fetal phenotypic expression of cultured adult rat hepatocytes grown on a collagen gel-coated nylon mesh substrate. Others have used cloning of the cultured cells to obtain a cell line without using particular substratum (Bauscher and Schaeffer, 1974; Tokiwa *et al.*, 1979). One of the difficulties in culturing hepatocytes has been that the cultured cells could not survive long enough for utilization of the cells and that even in short term culture some of the cells failed to express the phenotype of hepatocytes.

We have developed a method for culturing mouse epithelial cells for long time on a pigskin as a substrate (Yoshida *et al.*, 1980; Freeman *et al.*, 1981; Hirata *et al.*, in press). And we reported that full term fetal liver cells grown under these conditions maturate into an adult phenotype as liver cells in vivo (Freeman *et al.*, 1981). The purpose of this study is to present the morphological, histochemical and biochemical evidences that mid term fetal liver cells grow on a pigskin and modulate into mere mature by addition of hydrocortisone to the culture medium.

MATERIALS AND METHODS

1. Tissue culture

Cells were cultured on a pigskin substrate by published procedures (Yoshida *et al.*, 1980; Freeman *et al.*, 1981; Hirata *et al.*, in press). Briefly, the livers of mid term fetal N. I. H. Swiss mice (Microbiological Associates, Bethesda, MD) were minced with scissors into small bits approximately 1 mm in diameter. Twenty liver bits were placed onto the tissue culture quality sterile pigskin (Burn Treatment Skin Bank, Phoenix, Arizona) previously prepared as follows: The pigskin was thawed, then soaked for at least 14 days in Eagle's Minimum Essential Medium with 10% fetal bovine serum (EMEM 10). With a sharp scalpel, many criss-crossing slits were cut into the pigskin epidermis. Then each pigskin square was placed epidermal side up on the grids. Medium was placed into the petri dish so that the fluid level touched, but did not overflow the pigskin substrate. Cultures were incubated in 5% CO_2, 95% air at 37°C. The cells were fed 3 times a week. When hydrocortisone was used, 3.63 μg/ml hydrocortisone (Calbiochem-Behring Corp., La Jolla, CA) was incorporated into the medium.

2. Morphological examination

Tissue specimens were put into either 10% formalin or cold acetone solution. The specimens put into acetone were stirred for 3 hours with three changes of the solution. Fixed tissue then was transferred to benzene solution for 45 minutes with agitation and embedded in paraffiin of low melting point (approx. 52°C). Paraffin blocks were cut to 5 μm in thickness.

3. Histochemical and immunohistological studies

Tissue specimens fixed with cold acetone were used for enzyme staining and immunohistological studies. Enzymatic staining for GGT (Tanaka, 1974) and immunoperoxidase staining with peroxidase and anti-peroxidase complexes (Sternberger and Petrali, 1977) were performed using published methods. Frozen-sectioned specimens were used for LDH staining (Mori, 1975).

Rabbit antiserum to mouse albumin was purchased from Cappel Laboratories (Cochranville, PA). This antiserum gave a single line of precipitation against normal mouse serum. Rabbit antiserum to mouse AFP was produced in this laboratory (Ruoslahti, 1976). This antiserum gave a single line of precipitation against AFP-containing extracts of mouse embryos and purified AFP in immunodiffusion. It did not react with normal mouse serum. Normal rabbit preimmune serum was used as control.

4. Enzyme assays of GGT and LDH

GGT activity was assayed with glutamyl-p-nitroanilide as a substrate and glycylgycin as a acceptor, according to the method of Szasz (1976). LDH activity was assayed by the method of Wroblewski and LaDue (1955). Protein content of cells stripped from the pigskin was determined by the method of Lowry *et al.* (1951).

5. Quantification of AFP and albumin

These cultures were fed three times a week. Two-day supernatants were collected once a week. AFP and albumin were measured by sandwich enzyme immunoassays (Engvall, 1980). Mouse AFP used in these assay was purified by immunoadsorbent chromatography from an extract of mouse fetuses. All antibodies were used the same ones indicated in immunohistochemistry.

6. Tritiated thymidine incorporation

After the selected period of incubation, each pigskin was pulsed for 24 hr in EMEM 10 containing 2.0 μCi/ml tritiated thymidine (New England Nuclear, Boston, MA, 50.5 mCi/mole). Detailed procedures were described in published reports (Yoshida *et al.*, 1980; Hirata *et al.*, in press).

7. Cell attachment assay to basement membrane matrix

Preparation of fibronectin, laminin and type IV collagen was described before (Carlsson *et al.*, 1981). Attachment of cells to fibronectin, laminin and type IV collagen was evaluated using polystyrene surfaces coated with these proteins. The coating and washing procedures have been indicated Ruoslahti and Hayman (1979). 5×10^3 liver cells were added to each well and attachment was allowed to proceed for 2 hr at 37°C. The attached cells were fixed with 3% paraformaldehyde in 0.1 M cacodylate buffer solution (pH 7.4) and counted.

RESULTS

1. Morphological examination

The liver organ bits placed on the dermal side of pigskin were all degenerated and dead within a week of cultivation. When liver explants were carefully placed on scalpel blade slits (scores) made in the pigskin epidermis, began to grow along the scores (Fig. 1), and virtually every organ bit in every experiment survived for several weeks. Some of the cells grew under and some of the cells grew over the pigskin epidermis and some of the cells became intermingled with keratin substances of the pigskin epidermis. By 5 weeks of cultivation, the keratin materials had disappeared and cultured cells replaced the entire epidermal layer of the pigskin and

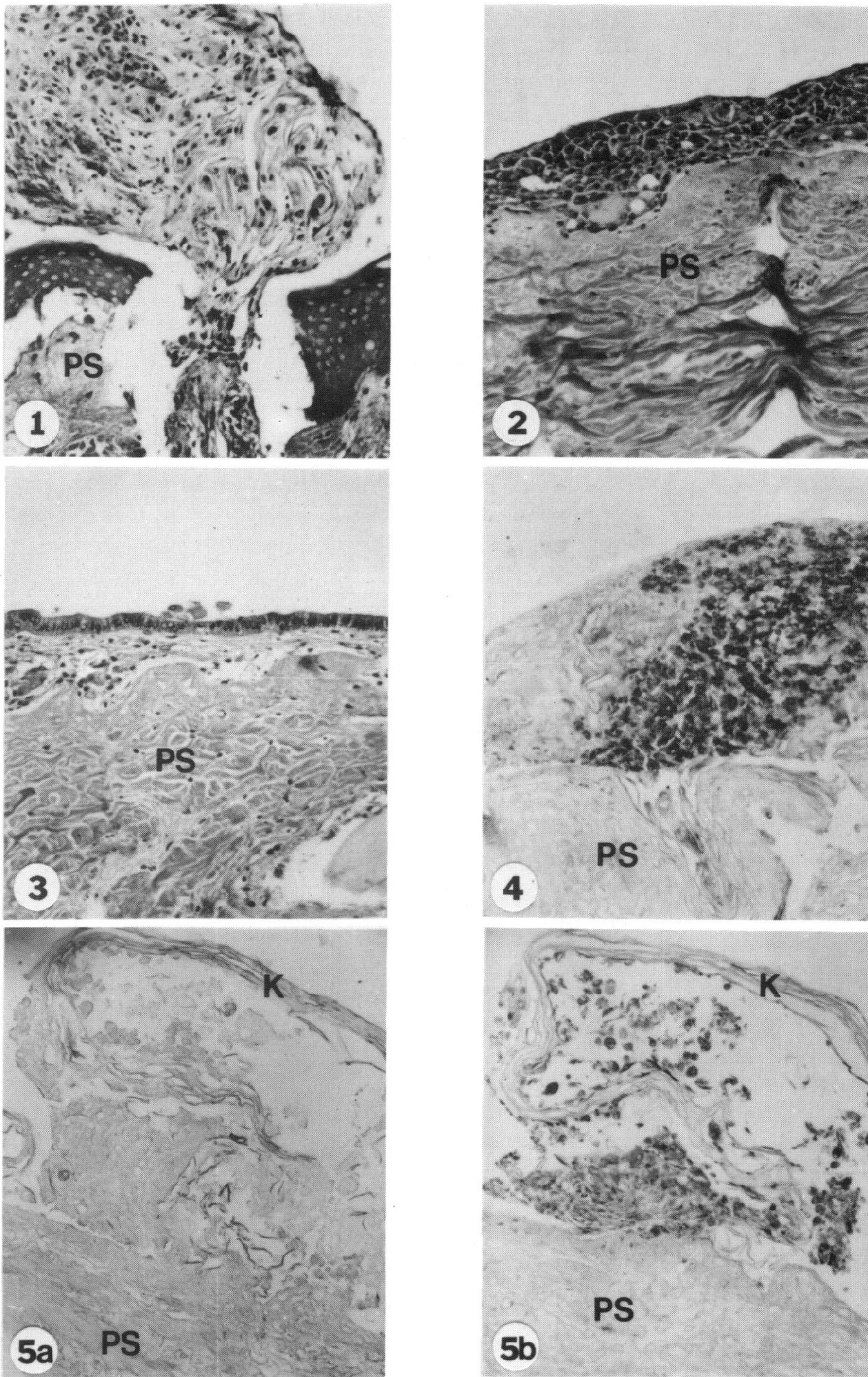

Fig 1−5

Table 1 *Detection of GGT, LDH, AFP and albumin in cultured cells and in media during 7 weeks of culture in vitro.*

Markers	Original explant	Culture Age (Weeks)						
		1	2	3	4	5	6	7
GGT[a,c]	+	+	+	−	−	−	−	−
LDH[a,d]	+	+	+	+	+	+	+	+
AFP[b,d]	++	++	++	+	+	±+	±	−
Albumin[b,d]	+	±+	±+	±+	+	+	±++	+

[a] Activity expressed by staining intensity: −, negative; +, weakly positive; ++, strongly positive.

[b] Results expressed as: −, negative; ±, positive in a few cells; +, positive in a moderate number of cells; ++, positive in most of cells.

[c] The localization of activity was confined to the cell wall of hepatocytes.

[d] The localization of enzymes or proteins was confined to the cytoplasm.

spread widely (Fig. 2). Regular lining with tall columnar cells was sometimes observed (Fig. 3). Under the condition used, the cultured cells survived and grew for 7 weeks.

2. Histochemical characterization of the cultured cells

GGT activity was expressed in 100% of the original organ bit explants in a strong canalicular pattern for up to two weeks, but the activity gradually decreased, until three weeks, no positive cells could be found. LDH activity was found in most of the cells through out 7 weeks of culture. AFP production was found in more than half of the cells (Fig. 4) as cytoplasmic products of diaminobenzidine for 4 weeks in culture, but in few percent of the cells in the 6 week culture (Fig. 5a). Albumin was found very weakly in less than 50% of the cells in culture up to two weeks. In older cultures, albumin production was strongly found in more than 80% of the cells which had migrated between the epidermal and dermal layers (Fig. 5b). The results of enzyme and immunohistochemical studies are summarized in Table 1.

Figure Legends

Fig. 1 3 week culture. An explant on the epidermal side with scores, note cell growth with invasion into the score. PS: Pig skin, H. E. (×510)

Fig. 2 5 week culture. A fairly thick and wide growing cellular sheet has replaced the epidermal layer. H. E. (×510)

Fig. 3 6 week culture. Regular lining arrangement with tall columnar cells like ductular cells. H. E. (×320)

Fig. 4 2 week culture. AFP staining of the cells on the pigskin epidermis. (×510)

Fig. 5 6 week culture. AFP staining (a) and albumin staining (b) of serial sections. K: keratin layer (×510)

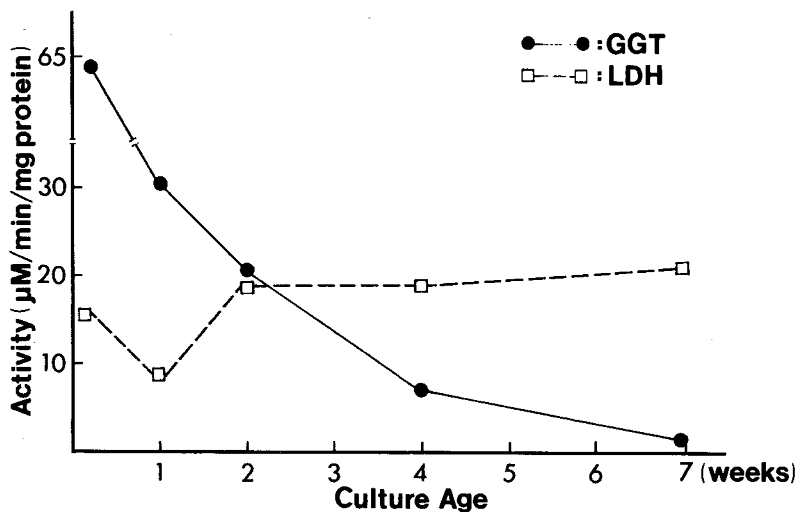

Fig. 6 Gammaglutamyl transferase activity and lactic dehydrogenase activity in fetal liver cells cultured on pigskin.

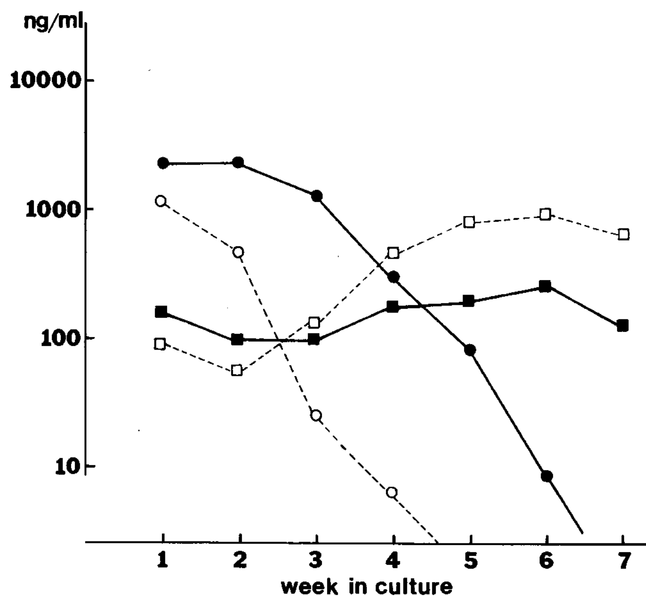

Fig. 7 Effect of hydrocortisone on AFP (○, ●) and albumin (□, ■) synthesis as fetal liver cells age in culture. AFP and albumin in the medium were quantitated (ng/m*l*) by sandwich enzyme immunoassay at various times of culture (weeks). Open symbols, data from regular cultures; solid symbols, data from cultures grown in presence of hydrocortisone.

Table 2. *Incorporation of tritiated thymidine into DNA during 7 weeks culture period of mouse fetal liver cells on pigskin.*

	days in culture							
	0-1	6-7	13-14	20-21	27-28	34-35	41-42	48-49
Net raw counts per minute	57,427	42,798	63,766	67,917	72,906	70,125	68,719	72,167
Relative number in division	1.0	0.8	1.1	1.2	1.3	1.2	1.2	1.3

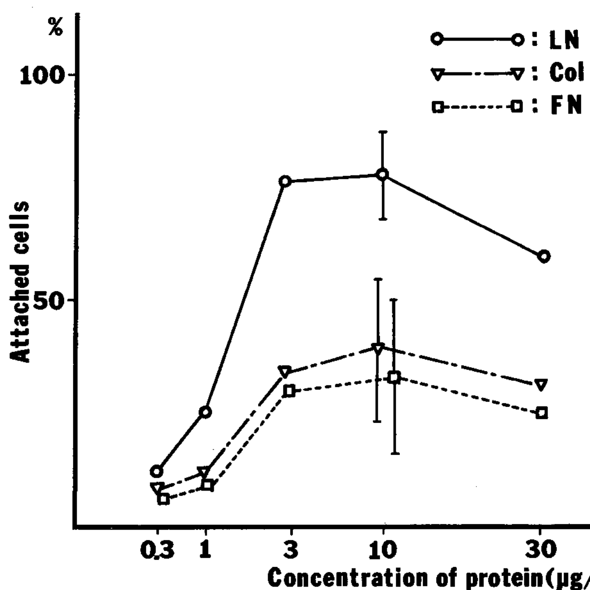

Fig. 8 Attachment of fetal liver cells to microtiter wells coated with various amounts of laminin (LN), type IV collagen (Col) and fibronectin (Fn). Bars show the variation in duplicate tests.

3. Changes of GGT and LDH activities in cultured cells and of AFP and albumin secretions in culture media

The activity of GGT per unit protein in cultured cells rapidly decreased after the start of culture and could hardly be detected after the third week (Fig. 6). However, the activity of LDH remained mostly constant during culture (Fig. 6). AFP secretion into culture media was detected at the beginning of culture but gradually decreased and could not be detected from 5 to 6 weeks after the initiation of culture (Fig. 7). Albumin secretion could be detected very weakly until the third week but gradually increased until the 7th week (Fig. 7). Above results are similar to the histochemical evidences. We could find that hydrocortisone did modulate AFP and albumin excretion. The effect of hydrocortisone was to delay loss of AFP

production. Increase in albumin production was not delayed but was reduced in the presence of hydrocortisone.

4. Tritiated thymidine uptake into cultured cells

The incorporation of tritiated thymidine into DNA has yielded relative information about the growth characteristics of the cells. By calling the number of counts incorporated during the first 24 hr pulse 1, all other counts were expressed as the relative number. From the data (Table 2), the incorporation increased until 3rd week of culture and was thereafter mostly constant.

5. Fetal hepatocytes attachment to basement membrane matrix cells obtained from fetal liver showed 65–85% attachment to plastic surfaces coated with laminin but only about 20–55% attachment to those coated with fibronectin (Fig. 8). Type IV collagen was slightly more effective than fibronectin, but somewhat less effective than laminin for the attachment of fetal liver cells (Fig. 8).

DISCUSSION

The pigskin substrate method is extremely useful for applying cytochemical techniques of analysis since sections of pigskin containing growing cells can be periodically cut from the total culture, fixed, stained and read by light microscopy. GGT, LDH, AFP and albumin were selected because they are specific markers of liver function and because they may have an ontogenetic relationship with each other. There are strong references in the literature to support the selection of these four gene products as evidence that the cultured cells are functional fetal liver hepatocytes. GGT has been found to be a marker enzyme for fetal and transformed liver cells both in vivo and in vitro (Sirica, 1979; Laishes *et al.*, 1978; Huberman *et al.*, 1979).

Production of AFP is a characteristic function of immature hepatocytes. AFP is produced by hepatocytes in fetal and regenerating liver (Sell *et al.*, 1974; Sell *et al.*, 1979; Ruoslahti and Seppala, 1979) as well as in livers or animals treated with carcinogens.

Production of albumin is a characteristic function of fetal and adult hepatocytes in vivo (Sell *et al.*, 1974), but in general albumin production increases as AFP production decreases after birth (Sell *et al.*, 1979). Our cultures in vitro parallel the in vivo ontogenetic development since albumin production increases and AFP production decreases as the cultured age. In contrast, others have found that adult rat hepatocytes tend to aquire fetal characteristics after five to ten days in culture (Sirica *et al.*, 1979; Leffert *et al.*, 1978).

In summary, each of the four gene products is a good marker of hepatocyte function and each of the markers was found in early cultures in exactly the same pattern as expected by fetal hepatocytes in vivo, i. e., strong GGT, LDH and AFP activities and weak albumin production. In older cultures each of the markers is also expressed exactly as expected if the cultures maturate as if the animal had been born, i. e., low GGT activity and AFP production, strong LDH activity and albumin production. Our results show that the culture system lends itself to study of

hormonal control of differentiated functions of liver cells. Addition of hydrocortisone to the medium prolongs AFP production but at times, AFP and albumin are synthesized at high levels. Thus the AFP and albumin genes operate independently and maturation need not occur for all genes at the same time. Freeman *et al.* (1981) interpreted this phenomenon that the effect of glucocorticoids is not due to increased rate of catabolism of AFP, but seems to depend on a decrease in the cellular level of mRNA for AFP.

One of the interesting aspects of this system was that the organ bits explanted on the pigskin dermis could not survive, whereas organ bits explanted on the epidermis did survive. We observed that some of the cultured cells had close contact with degenerated basement membrane-like materials of the pigskin epidermis. The obvious components of basement membrane which might support liver cell attachments and/or growth are type IV collagen, fibronectin and laminin. It is of interest that Wicha *et al.* (1979) describes the requirement of mammary epithelium for Type IV collagen for attachment and growth. Recently, Carlsson *et al.* (1981) have found that fibronectin and not laminin promotes in vitro attachment of normal adult mouse liver cells, but that laminin and not fibronectin promotes attachment of regenerating liver cells. This appearance of laminin in regenerating livers could be interpreted as one of their regenerative reactions. In the present study, our result shows that laminin can promote the attachment of fetal liver cells. Therefore, it is possible that laminin of the basement membrane of pigskin might influence on the long-term culture and the differentiation of liver cells.

This cell system has a number of advantages for this type of experiment. First, the cultures begin with organ bit explants which are metabolically functional for a short term not exceeding two weeks. Subsequently the cell culture outgrowth is multilayered and may express certain functions because of close cell to cell interaction. Function of the cellular outgrowth continues for at least 7 weeks allowing time for various experimental treatments and munipulations.

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