# CHARACTERISTICS OF HUMAN EMBRYONIC HEPATOCYTES CULTIVATED IN VITRO

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A simple and reproducible method of monolayer cultivation of hepatocytes from 7-12-week human fetuses is suggested. Such cultures are capable of limited growth, they can be maintained without subculture for 4-6 weeks, and they preserve certain specific characteristics: synthesis of such characteristic serum proteins as albumin and $\alpha$-fetoprotein, the presence of a high glycogen content and high monoamine oxidase activity, and the characteristic reaction to high concentrations of glucocorticoid. These properties can be used in experimental genetics as markers or for selection purposes.

KEY WORDS: human fetus; hepatocytes in culture; $\alpha$-fetoprotein.

For the study of many problems connected with the control of cell differentiation and certain special problems, many workers in recent years have attempted to use proliferating specialized human cells in culture in vitro. Parenchymatous cells of human embryonic liver are particularly important in this respect for these cells have a number of effectively controllable metabolic mechanisms.

The following types of human liver cells are described in the literature as suitable for culture: 1) aneuploid transplantable lines, with some tendency to malignant change [6], obtained from spontaneously transformed cells of a primary human liver culture – these cells preserve certain specific functions relatively unchanged; 2) diploid cultures consisting of morphologically changed cells or a mixture of cells of different types [5, 7, 8, 9, 10] – these cultures preserved certain specific properties for a varied period of time.

The objective of this investigation was to obtain a monolayer culture of hepatocytes satisfying the following demands: maintenance of viability as long as possible; homogeneity as regards cell type; preservation of specific characteristics. The requirements as regards method were simplicity and reproducibility. Furthermore, so that such cultures could be used in experimental genetics, they must also possess properties by means of which they could serve as markers or for positive selection.

## EXPERIMENTAL METHOD

The liver from human fetuses aged 7-12 weeks (from therapeutic abortions) was cultured. The liver was washed with medium, the surrounding connective tissue was removed, and the organ was cut up into small pieces in 2-3 volumes of a 1:1 mixture of trypsin and versene. After incubation for 30 min at 36.5° C the minced liver was vigorously pipetted, covered with excess of medium, and centrifuged for 10 min at 2000 rpm. The residue was resuspended in medium and the cells were counted in a Goryaev's chamber, using Türck's fluid. The hepatocytes under these conditions appear as the largest cells, retaining their cytoplasm. From $1 \cdot 10^6$ to $3 \cdot 10^6$ cells (depending on the age of the fetus) in 4 ml medium were transferred to a Petri dish (diameter 5.5 cm) with coverslips (9 × 18 mm) on the bottom. The cells were cultivated at 36.5° C in an atmosphere with 7% $CO_2$. The medium was changed on the third day. Fragmented and nonadherent red cells were carefully washed off with a jet of medium. The medium was then changed every

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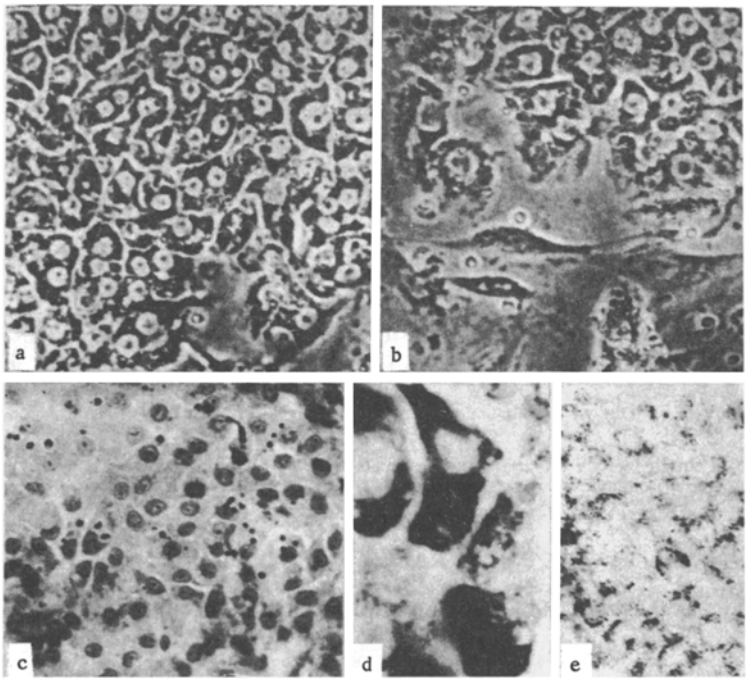

Fig. 1. Human hepatocytes in vitro: a) culture with addition of prednisolone (phase contrast, 140×); b) cells in culture without prednisolone (phase contrast, 140 ×); c) stained with hematoxylin–eosin; 160 ×; d) reaction for glycogen, 720 ×; e) reaction for monoamine oxidase, 160 ×.

3 days. The composition of the nutrient medium for seeding was: Eagle's medium with glutamine 80%, embryonic calf serum (GJBCO) 20%, prednisolone 150 mg/ml. Further cultivation was carried out in the same medium without prednisolone. On the third day of cultivation insulin (0.12 unit/ml) and glucose (to a final concentration of 0.1%) were added.

Intravital phase-contrast microscopy, using an inverted microscope and fixed preparations, was adopted for the cytomorphological investigation. The material was fixed with Bouin's solution and stained with hematoxylin–eosin. Cytochemical tests for glycogen, lipids, alkaline and acid phosphatases, monoamine oxidase, nonspecific esterase, and dopa-oxidase were carried out by the usual methods [1, 3].

For the autoradiographic investigation the material was incubated for 24 h with thymidine-$H^3$ (USSR) in a dose of 0.5 Ci/ml. The pool of labeled cells was determined by counting 2–4 cultures, with at least 500 cells in each culture, at each point. $\alpha$-fetoprotein and serum albumin were determined in the culture medium by Ouchterlony's double immunodiffusion test in gel in the micromodification [2] using a standard test system [4]. To determine $\alpha$-fetoprotein, an immunodiagnostic serum for primary carcinoma of the liver and teratoblastoma, prepared by the N. F. Gamaleya Institute of Epidemiology and Microbiology, was used. The test system for human serum albumin was kindly provided by A. I. Gusev and A. K. Yazova.

## EXPERIMENTAL RESULTS

Altogether 87 specimens were taken for culture. Cultivation was successful in all cases. The seeding efficiency was 50–65% depending on the age of the fetus. The cultures were maintained without subculture for 4–6 weeks. Observations on evolution of the cultures gave the following results. Single cells and conglomerates of 10–12 cells settled to the bottom and became attached there in under 12 h after seeding. Phase-contrast microscopy after 24 h revealed numerous islands of polygonal epithelial cells with large nuclei and granular cytoplasm. Most cells had one nucleus, but cells with two nuclei were fairly common and those with three nuclei less so. The nucleus contained one or two compact, round nucleoli (Fig. 1a, c). In cultures to which no prednisolone was added during seeding, cells with different morphology could be seen between the islands of hepatocytes – ranging from single polygonal cells to fusiform bipolar cells of fibroblast-like type (Fig. 1b). After seeding with prednisolone the islands of epithelial cells were much larger and on the 7th–10th day they were almost confluent.

In the experiments in which thymidine-$H^3$ was used, 18 ± 3% of labeled nuclei were found on the third day of cultivation, 21 ± 4% on the 4th day, 31 ± 5% on the 5th day, and 21 ± 6% on the 6th day. On the 10th day the cells ceased to incorporate thymidine-$H^3$, and approximately from that day cells with labeled nuclei appeared in dense zones of the culture. On the 22nd day labeled nuclei also were found in the dense parts of the layer. The appearance of labeled cells in the late periods of cultivation may perhaps indicate that death of the hepatocytes in the stationary stage does not take place critically, as in the case of fibroblasts, but by gradual death and separation of individual cells, while neighboring cells start to replicate. This could be a manifestation of physiological regeneration in vitro. Despite the obvious increase in the number of cells and the large proportion of cells synthesizing DNA, mitoses were rarely seen in these cultures and an exact count of the mitotic index was difficult. Some binuclear cells were seen in which one nucleus was in mitosis (Fig. 1c).

The results of the cytochemical tests were as follows. The reaction for glycogen was strong in all cells. Glycogen filled the whole cytoplasm in large masses. The strongest reaction was observed in the dense parts of the monolayer. It was weaker in the stratified cells and at the periphery of the colonies (Fig. 1d). In the tests for lipids Sudan-positive material was uniformly distributed throughout the cytoplasm to form networks or vesicles. The reaction for alkaline phosphatase was positive in all cells, but its activity varied. The reaction for acid phosphatase was strong in all cells. The reaction for esterase (nonspecific) was weaker than in fibroblasts. The reaction for monoamine oxidase (tryptamine oxidase) was strong in all cells (Fig. 1e). A marked reaction for dopa-oxidase was observed in the dense areas.

On the 7th and 10th days of cultivation, immunochemical analysis of the sections showed the presence of serum proteins synthesized in vivo by parenchymatous cells of the embryonic liver – $\alpha$-fetoprotein and serum albumin. All cultures grown under standard conditions actively synthesized these proteins. In order to protect these proteins in the medium, cultivation for several days without a change of medium was required.

A primary culture of human embryonic liver cells adapted to growth in vitro was thus obtained. The specificity of the cells in this investigation was established on the basis of a combination of features: production of serum proteins, a high glycogen content, high monoamine oxidase activity, and a reaction to high concentrations of glucocorticoid.

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