SYNTHESIS OF α-FETOPROTEIN *in vitro* BY HUMAN HEPATOCYTES, SINGLY AND IN MICROCOLONIES

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By a combination of microelectrophoresis and precipitation in polyacrylamide gel, α-fetoprotein (α-FP) produced by single hepatocytes and by microcolonies of hepatocytes was determined. Liver cells from 6-13-week-old human fetuses were cultivated *in vitro* for 2-5 days. α-FP was found to be produced in amounts of between 70 and 800 pg per cell by 23 of 28 single hepatocytes and by 89 of 91 microcolonies consisting of 2 to 35 cells

KEY WORDS: *α-fetoprotein; microelectrophoresis and precipitation of polyacrylamide gel; human fetus; culture of hepatocytes.*

α-Fetoprotein (α-FP) is a blood serum protein synthesized by embryonic and fetal hepatocytes, with a characteristic dynamics during individual development. In man α-FP appears in the fourth to fifth week of intrauterine life, its level reaches a maximum (3-4 mg/ml) in the 12th-16th week of intrauterine development, after which it falls gradually toward its "background" level (2-10 ng/ml) during the first months after birth [1, 4]. The mechism controlling α-FP synthesis in ontogeny has not yet been studied. It is not known whether what is regulated is the intensity of α-FP synthesis by all hepatocytes of the embryonic liver or the ratio between the numbers of hepatocytes producing and not producing α-FP, which varies in the course of ontogeny [1]. To solve this problem, the profile of synthesis of the serum proteins by individual hepatocytes at the early stages of development must be known.

The writers previously developed an ultramicromethod of determining antigens, involving a combination of microelectrophoresis and precipitation in polyacrylamide gel (microEPAG) [2] by means of which α-FP can be determined in picograms. The conditions for obtaining primary cultures of hepatocytes from human fetal liver also were worked out [3].

In this investigation the microEPAG method was found to be suitable for the determination of α-FP synthesis by human fetal hepatocytes, singly, and in microcolonies, cultivated *in vitro*.

EXPERIMENTAL METHOD

Experiments were carried out on primary cultures of human hepatocytes. The material used was obtained from therapeutic abortions (at 6-13 weeks).

The media used in different experiments were F-12, Eagle's medium in Dulbecco's modification, and RPMI-1640 with 20% calf or calf embryonic serum. The duration of survival of the cells in all the media was about the same.

A suspension of liver cells was washed three times with balanced salt solution and a small part of it was diluted with culture medium to a concentration of 100 cells/ml.

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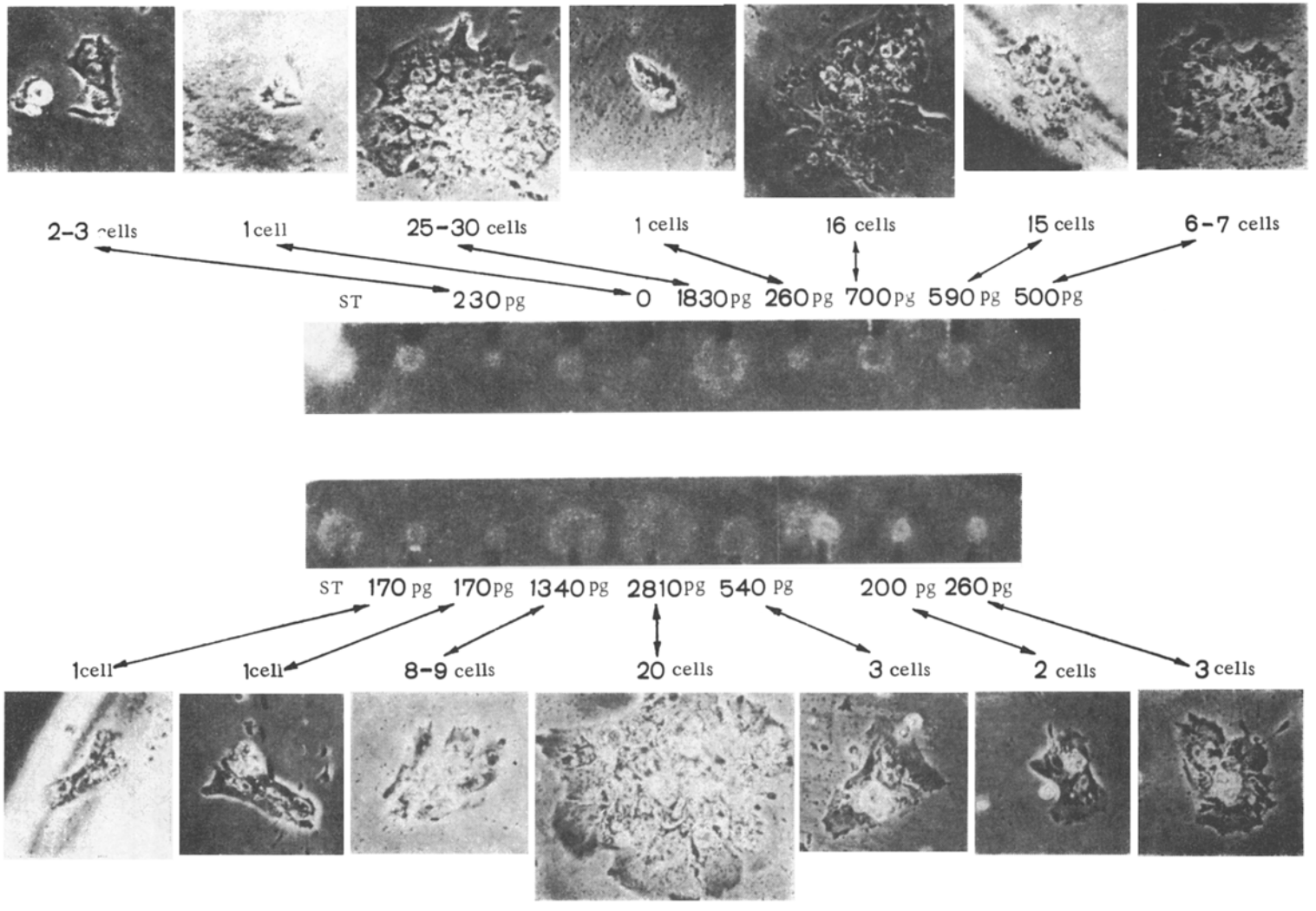

Fig. 1. α-FP production by single hepatocytes and microcolonies of hepatocytes. Top and bottom rows are photomicrographs of cells in wells. Middle rows are autoradiographs of corresponding analyses; ST denotes standard, 875 pg α-FP per sample.

TABLE 1. Content of α-FP in Growth Media of Single Hepatocytes and Microcolonies of Hepatocytes from Human Fetuses (in pg per cell)

Number of cells in specimen	First removal of medium			Second removal of medium		
	No. of experiments	samples with αFP / total No. of samples	αFP content (M ± m)	No. of experiments	samples with αFP / total No. of samples	αFP content (M ± m)
1	9	23/28	$n=28$ 220±32	7	7/19	$n=19$ 47±16
2—5	10	52/53	$n=53$ 206±38	7	16/31	$n=31$ 24±5
6—35	8	37/38	$n=38$ 108±19	5	14/21	$n=21$ 15±4

To isolate single hepatocytes or aggregates (of 2 to 35 cells) the diluted suspension of washed hepatocytes was poured into wells in microplates containing 60 conical wells with a base 0.8 mm in diameter and a volume of 10 µl (Falcon Microplates 3014, USA), kept in an atmosphere containing 7% CO_2 at 37°C. Next day the base and the walls of each well were examined in an inverted MBI-11 microscope under low power. Usually at this time the cells had already flattened out. By scanning wells with single isolated cells and wells with discrete colonies could be observed. The cells were counted and photographed in phase contrast under a magnification of 140×. Removal and change of medium were carried out at different times of cultivation: from 2 to 8 days. Depending on the ability of the cells to survive, one or two changes of growth medium were used.

To determine α-FP in the growth media, the microEPAG method described earlier [2] was used. The capillary tubes had a volume of 7 µl.

The specimens of growth media were stored in the frozen state. Before analysis they were dried in air to a volume of 3-4 µl, measured with calibrated capillary tubes, and treated with Cyanogum solution taken in a concentration to give a final dilution of 4%. The resulting solution was drawn up into a capillary tube and the α-FP was determined after polymerization.

All the material thus obtained was analyzed by means of the series II "Immunological diagnostic serum for primary human liver carcinoma and teratoblastoma" obtained from the N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR.

Antibodies against α-FP were taken in a dilution of 1:1000. A dilution of antigen of 1:400, containing 875 pg α-FP in 7 µl, was used in each experiment as the standard. Absolute quantities of α-FP were calculated by comparing the areas of precipitation rings formed by the test material and the standard dilution of α-FP.

Antibodies against rabbit immunoglobulin, labeled with ^{125}I, were obtained from Izotop (Moscow).

EXPERIMENTAL RESULTS

Hepatocytes from 6-13-week-old human fetuses were cultivated. The fetal liver cells were viable in isolation for 3-7 days. The spread-out cells had the morphology of hepatocytes. No attachment of hematopoietic cells evidently takes place under these conditions. The single isolated hepatocytes preserved their characteristic polygonal form, their granular cytoplasm, and their large circular nucleus. Heteropycnosis and cytolysis then took place as a general rule.

The initial "background" level of α-FP in the suspension of hepatocytes on seeding was verified in the supernatant after removal of the cells by centrifugation. In samples taken from nine separate suspensions of hepatocytes no α-FP was found. No α-FP likewise was found in samples from wells in microplates not containing cells.

Altogether 190 samples of growth medium were investigated for the presence of α-FP. At the first removal of growth media obtained from wells with single cells after culture for 2 and 5 days, α-FP was found in 23 of 28 samples. The α-FP content determined in the growth media of single isolated cells fluctuated from 100 to 600 pg per cell (Table 1; Fig. 1). Under these circumstances the α-FP content determined in the growth media of hepatocytes obtained from fetuses of different ages, and also in growth media taken on the second and fifth days of cultivation, did not differ significantly.

Of 91 samples from the first removal of growth media obtained from wells with microcolonies of hepatocytes, no α-FP was found in only two. The mean amount of α-FP produced by one cell of a colony was rather less than the amount obtained for single isolated cells. The α-FP level varied within wide limits — from 70 to 800 pg per cell. Just as for single cells, α-FP production was not found to depend on the age of the embryos or the time of removal of the growth media.

At the second removal of growth media the ratio between the samples with and without α-FP was considerably changed: α-FP was found in only 7 of 19 samples of growth media of single isolated cells and in 30 of 52 samples of growth media from wells with microcolonies of hepatocytes.

The main object of this investigation was to discover how suitable the microEPAG method is for determining α-FP production by single hepatocytes and by microcolonies of hepatocytes consisting of a small number of cells. Positive results were obtained in all experiments with microcolonies of hepatocytes consisting of 2 to 10 or more cells, and also with most of the single hepatocytes studied. The absence of α-FP in the cell suspension when diluted highly (100 cells/ml), the presence of "zero" samples (wells without cells) or of samples with cells not containing α-FP in nearly every experiment, and also the fact that the quantity of α-FP in the sample was proportional to the number of cells in the well are all evidence against the possibility of passive accumulation of α-FP, at least in detectable amounts. It is more difficult to rule out the possibility of passive accumulation of α-FP from dead or dying cells which do not attach themselves to the base of the well and undergo lysis and which are not therefore taken into account when the cells are counted.

The proportionality (even though only approximate) between the α-FP level in the sample and the number of cells it contained, and also the presence of α-FP in the second removals in individual experiments are arguments against any serious effect of this type of passive accumulation of α-FP, although it cannot be ruled out completely.

Most probably, therefore, in these experiments the production of α-FP by individual liver cells or by microcolonies of them was in fact observed

The quantitative results are of course only a rough guide. The results reflect the error of determination by the microEPAG method as a result of measurement of areas of very small diameter, differences in the duration of survival of the single cells and colonies, and differences in the original material. Nevertheless, in most experiments reasonably close mean results, giving some idea of the synthesis of α-FP by single cells, were obtained. With a further increase in the sensitivity of the method of determination, a stricter quantitative analysis of the production of α-FP and other serum proteins by single hepatocytes may probably be achieved.

If the results so far obtained are regarded as qualitative, the preliminary conclusion can be drawn that the overwhelming majority of hepatocytes in the liver of 6-13-week-old fetuses produce α-FP. This conclusion is based on the fact that 23 of 28 (82%) single cells and practically all (98%) of the colonies consisting of two to five (or more) cells accumulated α-FP during cultivation.

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