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Properties and identification of rat liver cells in long-term cultivation*

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Abstract

When cultured cells are used in experiments, It is very important to know from what kinds of cells the cultured cells are originated, and what characteristics the cultured cells maintain continuously in vitro Some properties of rat liver cells in long-term cultivation were examined for the purpose of identifying the cultured cells with parenchymal liver cells by investigating their functions. The production of rat serum albumin and α-globulin which is regarded as specific functions of liver parenchymal cells was detected in these cultured rat liver cells with the method of immunoelectrophoresis. Histochemically, acid phosphatase, glucose-6-phosphate dehydrogenase, succinic dehydrogenase, lactic dehydrogenase, and adenosine triphosphatase were demonstrated in the cultured rat liver cells which were morphologically epithelial. Alkaline phosphatase showed little activity in these cells. Glycogen was recognized by the periodic acid-Schiff technique, when bovine serum concentration in the culture fluid was reduced to 5 per cent. These histochemical findings of cultured rat liver cells were identical with those of parenchymal liver cells in vivo. These facts suggest that there is a poibility of the continuous cultivation of liver cells by the present methods and of the identification of the cultured cells with the parenchymal liver cells from their functions.

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PROPERTIES AND IDENTIFICATION OF RAT LIVER CELLS IN LONG-TERM CULTIVATION

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Though epithelial cell lines are obtained as a continuous culture from liver tissues by the present culture methods, these cells can hardly be identified with the parenchymal liver cells from the morphological observation. Many other types of cells are expected to grow from liver, because liver contains endothelial cells of blood and lymphatic vessels, bile-duct epithelium cells, blood cells, fibrous tissue cells and reticulo-endothelial cells (Kupffer cells). As summarized in the previous paper (1), the epithelial cell lines have been established from the livers of various animals, but no specific identification of these epithelial cells with parenchymal liver cells has been possible. EVANS and her associates investigated the estradiol inactivating capacity with their cell line from mouse liver but the cells did not inactivate estradiol (2). The albumin production, as one of specific functions of parenchymal liver cells, was demonstrated in the rat and the bovine cells cultured from liver tissues respectively by the immuno fluorescent technique and these results suggested that the cultured cells were identical with liver cells (1, 3).

The cells in long-term tissue culture generally lose their specific cellular characteristics and a dedifferentiated state of the cultured cells emerges. The investigation of cell differentiation in culture is related to the synthesis of somatic proteins by the cells and will offer many advantages to the experiments of developmental biology and identification of the cells in culture. The differentiation of the cultured cells (4, 5, 6) or the maintenance of differentiated cells *in vitro* (7, 8, 9) has been reported. The purpose of this work is to demonstrate the differentiated functions of rat liver cells in long-term cultivation and to identify the cultured cells as the parenchymal liver cells. As one of differentiated functions, the production of serum proteins of the cultured rat liver cells is examined by immunoelectrophoretic method, using the extracts from the homogenates of the cultured cells. At the same time, histochemical staining reactions, phagocytic and pinocytic activities and some other cellular characteristics are

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investigated with these cells.

MATERIALS AND METHODS

Cells and culture methods: The cultivation of rat liver cells was established from Donryu 5-day-old male rats. The method of culture was described by KATSUTA and TAKAOKA (10). The medium of the primary culture consisted of 20 per cent bovine serum, 0.4 per cent lactalbumin hydrolysate and buffered saline (mixture D), but the cells of the established line were cultured in the medium composed of 20 per cent bovine serum, 0.4 per cent lactalbumin hydrolysate, 0.08 per cent yeast extract and Earle's solution. The cells were subcultured with 0.2 per cent trypsin in phosphate buffered isotonic saline, pH 7.6.

Fractionation of cultured liver cell homogenates: Cultured rat liver cells were collected from 554 to 612 culture days and homogenized with a glass homogenizer in phosphate buffered saline, pH 7.4 and centrifuged for 30 minutes at 12,000 rpm. After ammonium sulphate was added to the supernate at the final concentration of 50 per cent, the fluid was again submitted to 20 minutes centrifugation at 6,000 rpm. The sediment, designated as Fraction 1, was dissolved in physiological saline, and dialysed against phosphate buffered saline for several days at 4°C. The proteins remaining in the supernate were precipitated further with ammonium sulphate at 65 per cent concentration and then treated in the same manners as Fraction 1. This fraction was designated as Fraction 2. For the detection of serum proteins produced in these cultured rat liver cells, the two fractions were condensed and submitted to microimmunoelectrophoretic analysis. Electrophoresis of the fractions was performed on microscope slides in agar gel with barbital buffer, 0.1μ, pH 8.6. Precipitation lines were then formed by diffusion against the rabbit anti-rat serum antiserum.

Immunization of rabbits with rat serum: Two rabbits were immunized with whole rat serum in Freund's complete adjuvant by subcutaneous and intramuscular injections. These sensitized rabbits' sera were used as antibody to whole rat serum after the examination of the excellent appearance of immunoprecipitin arcs against rat serum by the method of microimmunoelectrophoresis.

Histochemical stainings: Cultured rat liver cells were stained histochemically by the following techniques; acid phosphatase by GOMORI's (11), glucose-6-phosphate dehydrogenase and lactic dehydrogenase by NACHLAS, WALKER and SELIGMAN's (12, 13), succinic dehydrogenase by NACHLAS, TSOU, DE SOUZA, CHENG and SELIGMAN's (14), adenosin triphosphatase by WACHSTEIN and MEISEL's (15), and alkaline phosphatase by TAKAMATSU's method (16). For the demonstration of glycogen the cells were stained by the periodic acid-Schiff technique (17). The positive-reacting materials in the cells were examined to prove to be glycogen by its disappearance following digestion with saliva at 37°C for 60 minutes.

Demonstration of phagocytosis and pinocytosis: To test the possibility that these cells might be phagocytic, the colloid of chondroitin sulfuric iron was added to the culture medium at the final concentration of 0.2 per cent. The cells cultured on cover slips were stained after 24, 48 and 72 hours of the incubation by the

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method of prussian blue reaction. For the investigation of pinocytosis, the incorporation of bovine serum albumin (BSA) in the medium into the cultured cells was studied by immunofluorescent technique; namely, the cultured cells were reacted with rabbit anti-BSA γ-globulin which had been labelled with fluorescein isothiocyanate (1).

RESULTS

As illustrated in Fig. 1, each of the fractions 1 and 2 from cell homogenates produced one precipitation line. When these two lines were com-

Figure 1. The immunoelectrophoretic pattern of Fractions 1 and 2. The Fraction 1 (Frac. 1) was precipitated with ammonium sulfate at the 50% concentration from the extract of cultured rat liver cells and the Fraction 2 (Frac. 2) at 65% concentration. The top pattern shows α-globulin line and the lower one represents albumin line. The anode is to the left.

pared with the control immunoelectrophoretic patterns of normal rat serum, they were consistent with the reactions of α-globulin and albumin. Lipoprotein was not revealed in these two lines by the staining of oil red 0. The results furnished evidence that the rat liver cells in long-term cultivation maintained some functions of liver cells to produce two kinds of serum proteins, albumin and α-globulin.

Morphologically, flat, pavement-like sheets of epithelial cells were always observed. The cells spread widely on glass surface with abundant pale staining cytoplasm and their oval or round nuclei contained generally one to several nucleoli. The cells as well as the nuclei varied considerably in size, some being two to four times larger than others. Binuclear, trinuclear or multinuclear cells were sometimes observed (Fig. 2).

Histochemically, acid phosphatase, glucose-6-phosphate dehydrogenase, succinic dehydrogenase, lactic dehydrogenase, and adenosine triphosphatase were elicited in the cultured rat liver cells. The positive-reacting enzymes except acid phosphatase and adenosine triphosphatase were distributed as fine granules in the cytoplasm of many cells, but acid phosphatase was stained in large granular bodies in all cells. Adenosine triphosphatase was also found in all cells in mitochondria and cell membrane as the form of rods or granules. Little alkaline phosphatase activity was

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present in these cells. When the cells were cultured in the medium containing 4-dimethyl-aminoazobenzene at the final concentration of 10γ/ml for 48 hours, the staining activity of lactic and succinic dehydrogenases decreased prominently.

Glycogen was recognized by the periodic acid-Schiff reaction, when bovine serum concentration in the culture medium was reduced to 5 per cent (Fig. 3). The positive staining materials of the periodic acid-Schiff reaction disappeared from the cells after the digestion with saliva. Little glycogen storage in the cultured rat liver cells was detected by the addition of crystalline insulin into the medium at the final concentration of 500μU/ml or by the medium containing glucose at high concentration of 1 per cent or by the medium supplemented with rat serum at different concentrations of 5, 10 and 20 per cent.

Phagocytosis of colloidal chondroitin sulfuric iron was rarely observed in 24 and 48 hours, i. e., in 0 and 0.3 per cent cells, respectively, but by the end of 72 hours of exposure 22 per cent cells had phagocytosed the colloidal iron and the cells containing iron colloid were not degenerative microscopically. The phagocytosing cells are shown in Fig. 4. Bovine serum albumin in culture fluid was incorporated into the cytoplasm of all the cells cultured in the medium with bovine serum supplement by immunofluorescent technique, but not in nuclei (Fig. 5).

The growth rate of the rat liver cells was investigated by the simplified replicate tissue culture method (18). When the rat liver cells were established as a strain, the population doubling time of the cells was about 40 hours in the medium consisting of 20 per cent bovine serum, 0.4 per cent lactalbumin hydrolysate, 0.08 per cent yeast extract and buffered saline.

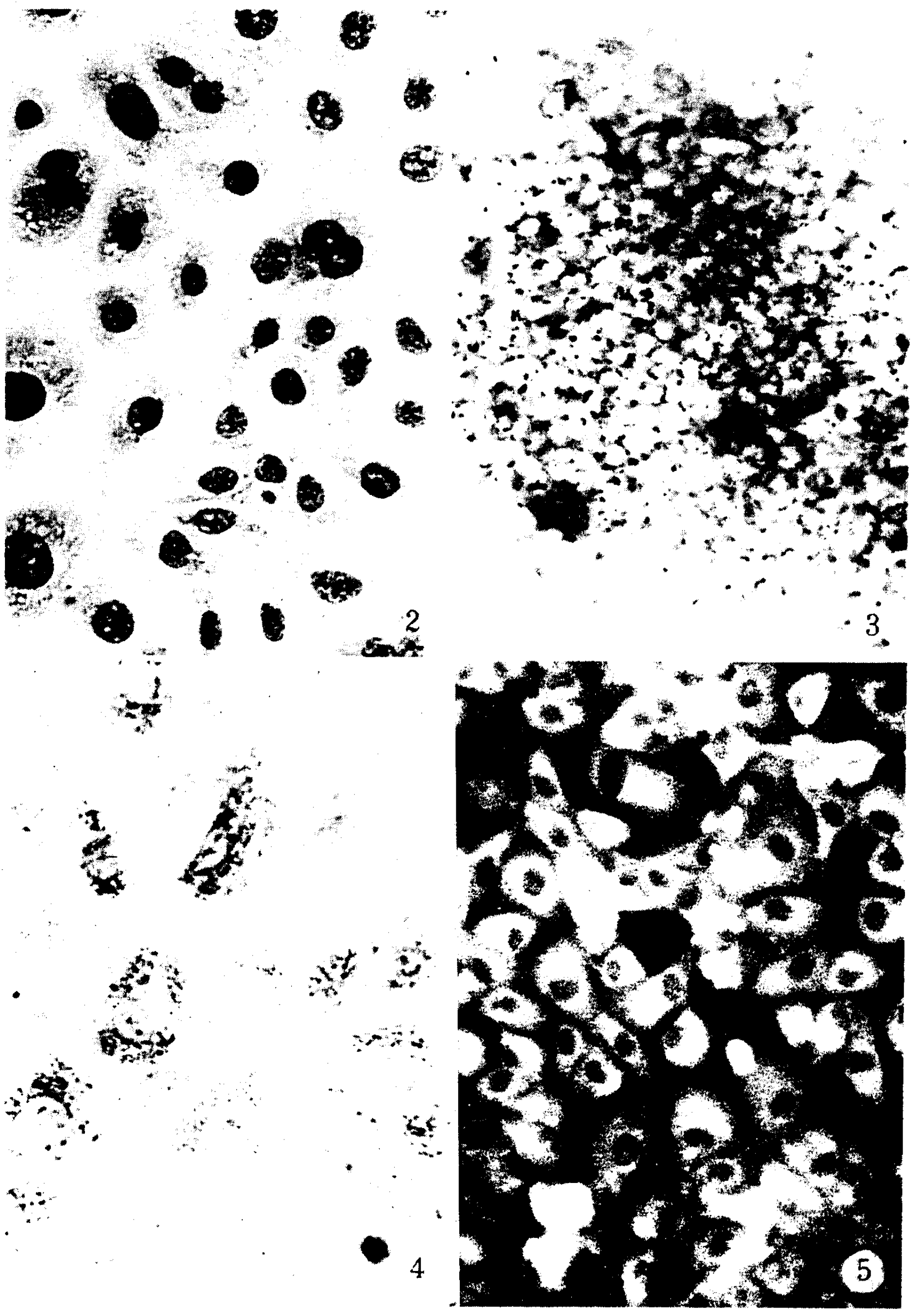
On 529th culture day the cells were trypsinised from glass bottles and 5×10^6 cells were injected intraperitoneally into newborn rats to test for

Figure 2. The cells of the cultured rat liver cells after 452 days of cultivation in total. Pavement-like sheet of epithelial cells is seen. The cells have abundant cytoplasm and their oval or round nuclei contain one to several nucleoli. The cells as well as the nuclei vary considerably in size, some being two to four times larger than others. Binuclear cells are observed. Giemsa staining.

Figure 3. Granules of glycogen are shown in the cytoplasm as black spots. PAS staining.

Figure 4. The cells phagocytosing colloidal chondroitin sulfuric iron by the end of 72 hours of the exposure. Iron colloids are seen in the cytoplasm as fine particles. Prussian blue staining.

Figure 5. Cultured rat liver cells in the medium containing bovine serum albumin react with rabbit anti-bovine serum albumin antiserum labelled with fluorescent isothiocyanate. Bovine serum albumin is incorporated in the cytoplasm of all cells, bnt does not in nuclei.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capacity to produce tumors by the method of our laboratory (19). No tumors were observed in the rats for at least 6 months. The same line cells treated with 4-nitroquinoline-N-oxide produced tumors, some of which were diagnosed as liver cell carcinoma.

DISCUSSION

Specific functions of liver cells, the production of serum albumin and α-globulin, were observed in the cultured rat liver cells established from rat liver tissues. The facts that glycogen, the enzymes investigated except acid phosphatase and adenosine triphosphatase, and albumin production were not present in all cells in the previous reports (1, 3) may depend upon mitotic cycle or dedifferentiation of some cells or culture containing different types of cells. If the pure cloning of parenchymal functional liver cells or synchronous culture is performed, these complicated problems will become clear, which will afford many advantages for many *in vitro* researches of carcinogenesis, viral and metabolic diseases of liver cells.

Histochemically, the enzyme activities of acid phosphatase, glucose-6-phosphate dehydrogenase, lactic dehydrogenase, succinic dehydrogenase and adenosine triphosphatase were consistent with the enzymes of parenchymal liver cells. The fact that little alkaline phosphatase was observed furnished the evidence that the cultured cells had originated from the cells other than the cells of blood capillary, but inactivity of this enzyme did not necessarily prove the cells to have originated from liver cells, because the proliferating cells in culture have general disposition to lose their specific characters and to dedifferentiate.

Though glycogen synthesis was reported in the cell lines established from liver tissue (1, 3, 20), the evidence of glycogen storage does not necessarily indicate the cells to have originated from parenchymal liver cells, because glycogen was also detected in other cultured cells, HeLa, Detroit-6, FL, HEp-2 and YOSHIDA sarcoma strain cells by us (21). Little glycogen was observed in the cells cultured in the growth medium consisting of 20 per cent bovine serum, 0.4 per cent lactalbumin hydrolysate, 0.08 per cent yeast extract and buffered saline. However, when the bovine serum concentration was reduced to 5 per cent, a considerable amount of glycogen was demonstrated in a few cells. The inhibition of cell growth by the decrease of bovine serum concentration seemed to be effective in the glycogen storage of cultured cells.

It is generally known that epithelial cells have no phagocytic activity *in vivo*. When the phagocytic behavior was tested in these cells by chon-

droitin sulfuric iron colloid, few cells were observed containing iron particles within 24 and 48 hours, but by the end of 72 hours phagocytosing cells increased to 22 per cent. It was difficult to explain the rapid incorporation of colloidal iron into the cells from 48 to 72 hours after the incubation from these experimental results. It remains to be clarified whether the colloidal iron was transported through intact cell membranes into living cells or not. The active phagocytosis and the digestion of cell homogenates by cultured rat liver cells were observed by cinemicrographic method (22). EVANS and her associates demonstrated also the phagocytic behavior of cultured mouse liver cells (2). As it is considered that many different types of cells are present in this cell strain, it is not precisely clear whether phagocytosing cells have truly originated from liver parenchymal cells. But the liver cells, especially their cell membranes may have been changed by culture conditions, to obtain the phagocytic ability for adapting themselves to culture enviroments. Increased phagocytic activity of cultured liver cells may have been induced by the structural differences of liver cells between *in vivo* and *in vitro* circumstances. These problems will be also dissolved by the successful cloning of liver cells.

The incorporation of bovine serum albumin in the culture medium was observed in all the cells. This incorporated albumin disappeared from the cells 3 days after the elimination of bovine serum from culture fluid. These facts suggested that proteins of about 50, 000 molecular weight might be easily taken in the cultured cells. Though the role of the albumin in the cells is still unknown, the albumin of the medium enters into cells and may be utilized as growth promoting nutrient elements. The rapid disappearance of albumin from the cells indicated that the albumin was quickly digested or destroyed in the cells.

No simple and accurate criteria of identification of the cultured liver cells have been established. The present method for the identification, i. e., the investigation of the production of serum proteins in the cultured liver cells, is relatively intricate and troublesome. As the morphological and functional transformations of the cells occur in long-term cultivation, the pure cloning of parenchymal liver cells is desired to be performed as soon as possible after the initiation of the culture.

SUMMARY

When cultured cells are used in experiments, it is very important to know from what kinds of cells the cultured cells are originated, and what characteristics the cultured cells maintain continuously *in vitro* Some

properties of rat liver cells in long-term cultivation were examined for the purpose of identifying the cultured cells with parenchymal liver cells by investigating their functions.

The production of rat serum albumin and α-globulin which is regarded as specific functions of liver parenchymal cells was detected in these cultured rat liver cells with the method of immunoelectrophoresis.

Histochemically, acid phosphatase, glucose-6-phosphate dehydrogenase, succinic dehydrogenase, lactic dehydrogenase, and adenosine triphosphatase were demonstrated in the cultured rat liver cells which were morphologically epithelial. Alkaline phosphatase showed little activity in these cells. Glycogen was recognized by the periodic acid-Schiff technique, when bovine serum concentration in the culture fluid was reduced to 5 per cent. These histochemical findings of cultured rat liver cells were identical with those of parenchymal liver cells *in vivo*.

These facts suggest that there is a possibility of the continuous cultivation of liver cells by the present methods and of the identification of the cultured cells with the parenchymal liver cells from their functions.

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