Acta Medica Okayama

Volume 32, *Issue* 1 1978 *Article* 2

APRIL 1978

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Abstract

Primary mass culture of isolated cells from adult rat livers by the trypsin-perfusion method uas carried out to investigate cytological and biochemical properties of primary cultured cells. Two main types of cells were found in the course of primary culture of isolated hepatic cells. One was a group of polygonal cells with granular cytoplasm, which appeared to arrange themselves in cords and clusters 3 to 4 days postinoculation and then gradually decreased in number. These cells seemed to be associated with expression of liver specific functions such as higher tyrosine aminotransferase levels and transient increases in albumin production. Another type of cells was relatively small in size and had clear cytoplasm, which grew out rapidly in place of the polygonal cells and proliferated also in subculture. alpha-Fetoprotein was detected in the cultured media of the proliferating clear cells. These results suggest that the polygonal cells and the clear cells may correspond to the mature hepatocytes and the hepatocytic stem cells in vivo, respectively.

KEYWORDS: primary culture, adult rat hepatic sells, tyrosine aminotransferase, albumin, ?-fetoprotein

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*PMID: 77631 [PubMed - indexed for MEDLINE]
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Acta Med. Okayama **32**, (1), 11—22 (1978)

PRIMARY CULTURE OF ADULT RAT LIVER CELLS. II. CYTOLOGICAL AND BIOCHEMICAL PROPERTIES OF PRIMARY CULTURED CELLS

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Received December 27, 1977

Abstract. Primary mass culture of isolated cells from adult rat livers by the trypsin-perfusion method was carried out to investigate cytological and biochemical properties of primary cultured cells. Two main types of cells were found in the course of primary culture of isolated hepatic cells. One was a group of polygonal cells with granular cytoplasm, which appeared to arrange themselves in cords and clusters 3 to 4 days postinoculation and then gradually decreased in number. These cells seemed to be associated with expression of liver specific functions such as higher tyrosine aminotransferase levels and transient increases in albumin production. Another type of cells was relatively small in size and had clear cytoplasm, which grew out rapidly in place of the polygonal cells and proliferated also in subculture. α-Fetoprotein was detected in the cultured media of the proliferating clear cells. These results suggest that the polygonal cells and the clear cells may correspond to the mature hepatocytes and the hepatocytic stem cells *in vivo*, respectively.

Key words: primary culture, adult rat hepatic cells, tyrosine aminotransferase, albumin, α-fetoprotein.

Several laboratories have been successful in establishing epithelial cell lines from juvenile (1, 2) and adult (3–5) rat livers. These cell lines, however, have displayed few phenotypic properties of the mature hepatocytes *in vivo* and their exact cell type also remains uncertain. Several cell lines have been reported to undergo spontaneous malignant transformation in culture (6, 7).

The methods of preparation of primary mass culture of liver cells have been greatly improved in recent years by the use of continuous liver perfusion (8, 9). Optimal conditions of primary culture for maintaining liver specific functions have not been obtained yet. It has been observed that several days postinoculation most of parenchymal cells in culture showed progressive degeneration and finally died (8, 9). However, several islands of small proliferating cells of epithelial appearance developed in culture (3, 9), and these cells could be subcultured and maintained as permanent lines (3). It would be of great interest to determine which cellular fractions in liver can adapt *in vitro* to the culture environment and

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to elucidate their cytological and biochemical properties and the *in vivo* origin of rapidly growing epithelial small cells.

In the present study, the various conditions in the primary culture of isolated hepatic cells were examined to try and solve the problems described above. Cytological and biochemical properties of primary-cultured hepatic cells were also investigated with respect to time in culture.

MATERIALS AND METHODS

Preparation of isolated hepatic cells. Three to five-month-old Donryu male rats inbred in this institute were used for preparing isolated hepatic cells. Isolated hepatic cells were obtained by perfusing livers with 0.1% trypsin (1 : 250, Difco) in Ca^{2+}-free Hanks' balanced salt solution (CF-HBSS) (10) at 37°C for 30 min (11). Magnesium sulfate was added to the Ca^{2+} and Mg^{2+}-free HBSS as reported previously (11), since Mg^{2+} does not prevent the dissociation of liver tissue. Freshly isolated hepatic cells were washed twice with CF-HBSS. Cell viability was judged from exclusion of a vital stain, trypan blue (12), and the viability of cells used for the primary culture was 65 to 71%. All procedures described above were carried out under sterile conditions.

Primary culture of isolated hepatic cells. Eagle's minimal essential medium (MEM ; Chiba Pref. Serum Inst.) (13), which was supplemented with 20% bovine serum (BS) inactivated at 56°C for 30 min, was used for the primary culture. Penicillin and streptmycin sulfate were added to the culture medium at final concentrations of 100 units and 100 μg per ml, respectively. This culture medium was employed only for the initial 2 days postinoculation. Hepatic cells suspended in CF-HBSS before inoculation were washed once with MEM supplemented with 20% BS and antibiotics. They were inoculated at a concentration of either 35×10^4 cells per ml (4 ml) into 60 mm Falcon plastic dishes or 105×10^4 cells per ml (1.4 ml) into 35 mm Falcon plastic dishes. The inoculated cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The culture medium was renewed every 2 days and was stored at −20°C for determining albumin and α-fetoprotein (AFP) by respective radioimmunoassays. The morphological findings and growth patterns of cultured cells were followed for 2 weeks of culture under a phase contrast microscope or a light microscope.

Assay of enzyme activities in cultured hepatic cells. Cultured hepatic cells were removed from the culture dishes with a rubber-policeman at a time scheduled and washed twice with a cold medium of 0.154 M KCl containing 0.32 mM $KHCO_3$ and 4 mM ethylenediaminetetraacetate (EDTA), pH 7.5. The activities of glucose 6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9, G6Pase) (14, 15) and tyrosine aminotransferase (L-tyrosine : 2-oxoglutarate aminotransferase, EC 2.6.1.5, TAT) (16) were measured as reported previously (11). Specific activities were expressed in milliunits (nmoles of product formed per min) per mg protein of supernatants for TAT and homogenates for G6Pase. Protein contents were determined by the method of Lowry *et al.* (17).

Radioimmunoassay of albumin and α-fetoprotein. Albumin was highly purified from rat albumin Fraction V (Miles Lab., Inc., Kankakee) by using Disc electrophoresis. The purifiied albumin had the same antigenicity as Fraction V. Detailed procedures of the purification of albumin and preparation of anti-albumin serum will be reported elsewhere (18). The purified albumin was labeled with ^{125}I (Radiochemical Centre) by the chloramine T method of Hunter (18, 19) and ^{125}I-labeled albumin was separated from the free radioactive iodine by gel filtration. Quantitative determinations of albumin content of used culture media were carried out by a double antibody method using the anti-rat-albumin rabbit serum and the anti-rabbit-γ-globulin goat serum (Dainabot Radioisotope Lab., Tokyo, Japan) (20). This method can accurately quantitate the content of albumin in culture media at a range of albumin concentrations from zero to 500 ng per ml of the medium. AFP concentrations in the culture media were measured by the method reported previously (21).

RESULTS

Freshly isolated hepatic cells grew better in MEM supplemented with 20% BS than fetal calf serum (FCS) or calf serum (CS), although differences in the cell attachment efficiency and cell growth *in vitro* were observed between two different lots of BS. BS was found to facilitate growth of epithelial-like cells rather than fibroblast-like cells. The cell numbers on dishes 8 days postinoculation indicated that growth of isolated hepatic cells in the primary culture was rapid with cell inoculum sizes of 33×10^4 to 100×10^4 cells per ml.

The growth patterns of isolated hepatic cells, which were inoculated at two different inoculum sizes, *i.e.*, 105×10^4 and 35×10^4 cells per ml, are illustrated in Fig. 1-A_1 and 1-B_1. The cell attachment efficiency 1 day after inoculation was 25%, a figure which is independent of cell inoculum sizes. Cultured cells at different inoculum sizes started growing after a lag period of 1 day. Rapid growth of cultured cells was observed up to 4 days postinoculation in the case of small inoculum size (Fig. 1-B_1). Cell growth became slow thereafter and finally reached a stationary phase 8 days after the initiation. Although the cultures inoculated at the high density grew slowly as compared with those at the low density, the former reached stationary phase 4 days faster than the latter (Fig. 1-A_1). The cell density at the confluence was 9×10^4 nuclei per cm^2, this result being the same for all inoculum sizes.

The inoculated hepatic cells, which were spherical at the time of isolation (11), began to stretch immediately after attaching to the plastic substratum (Photo. 1). The polygonal cells with granular cytoplasm (polygonal cells) arranged themselves in cords and clusters 3 to 4 days postinoculation (Photos. 2 and 3). A small number of cells with clear cytoplasm (clear cells) appeared among the islands formed by the polygonal cells at this stage (Photos. 2 and 3). The polygonal cells rapidly decreased in their numbers thereafter and the clear cells with

various shapes proliferated vigorously during 6 days following inoculation (Photo. 4). Giant cells appeared at the time of cultivation, probably resulting from cell-fusions. The cell population in the primary culture finally consisted of clear cells (Photos. 5, 6 and 7). Cultured cells were detached 14 days postinoculation with 0.1% trypsin in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline and 0.02% EDTA in the same buffer, transferred into the new Falcon plastic dishes at a split ratio of 1 : 2 and cultured in the same conditions as the primary culture. The transferred cells grew and the subcultures became confluent a week later (Photo. 8).

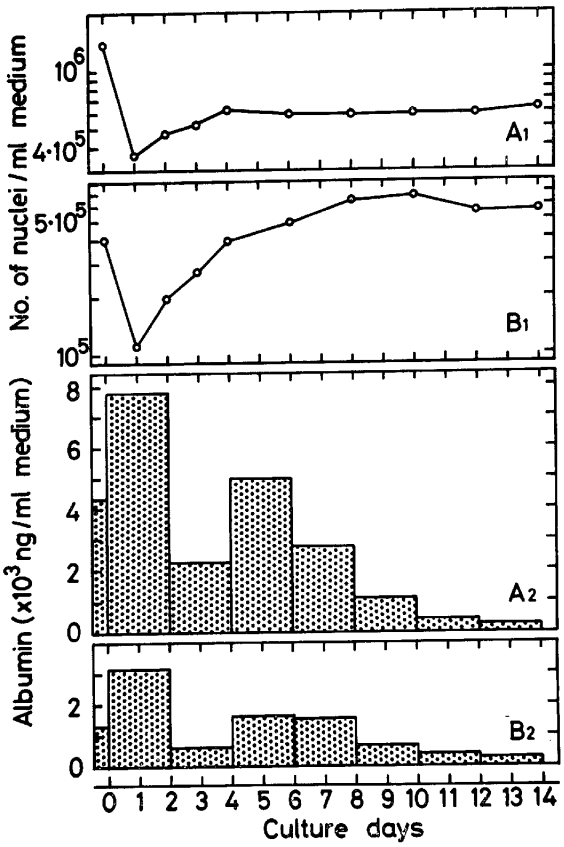

Fig. 1. Relationship between cell growth and albumin production of primary cultured cells with respect to time in culture. The numbers of nuclei were expressed as a mean of 3 cultures; A_1 and B_1. Albumin contents accumulated in the cultured media for 2 days were illustrated as a mean of 3 experiments; A_2 and B_2. Albumin content in the isolated cells at zero time is shown at the left side of histgrams. The intracellular albumin was extracted into inoculated-cell supernatants obtained by freezing and thawing homogenates 3 times with 0.1% triton X-100 in MEM supplemented with 20% BS. Supernatants were separated by centrifuging homogenates at 25000 g and 4°C for 60 min.

The time courses of G6Pase and TAT activities in primary-cultured hepatic cells are illustrated in Fig. 2. G6Pase activity in cultured cells rapidly decreased

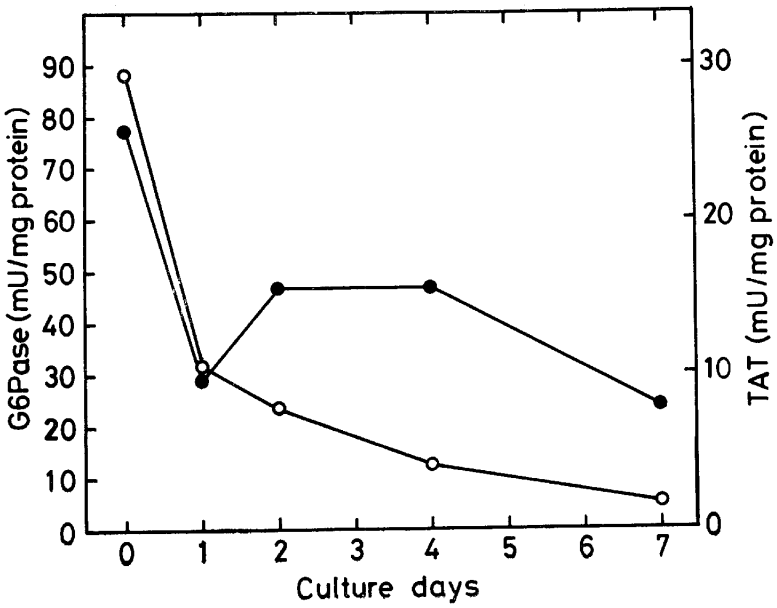

Fig. 2. Time courses of G6Pase (○-○) and TAT (●-●) activities in primary cultured cells. Isolated hepatic cells were inoculated at a concentration of 35×10^4 cells per ml. Cultured cells were pooled from 5 different dishes and homogenized. The enzyme activities were assayed as described in Materials and Methods.

with respect to time in culture. On the other hand, TAT activities in the cultured cells increased 1 day after inoculation and relatively higher activities were maintained for at least 4 days of culture. This finding seemed to correlate with the morphological change observed in the course of the primary culture. Albumin production by primary cultured cells was confirmed by detecting it in the culture media using the radioimmunoassay. The time courses of albumin production are compared with cell growth in Fig. 1-A_2 and 1-B_2. Albumin content of the culture media for the fiirst day (high inoculum size; 6132 ng per ml and low inoculum size; 1992 ng per ml) were higher than those in the inoculated cells at zero time (high inoculum size; 4496 ng per ml and low inoculum size; 1310 ng per ml). It seemed likely that the inoculated celles began to synthesize albumin 1 day postinoculation. Albumin contents in the culture media between the 2nd and 4th day of culture decreased to one fourth or one fifth of those for the initial 2 days. This may be caused by decreases of cell numbers 1 day after inoculation. However, about 2-fold increases of albumin content were detected

in the culture media between the 4th and 6th day of culture and thereafter decreased gradually, suggesting that the ability of the polygonal cells to produce albumin is transiently enhanced by an unknown mechanism(s). Albumin concentrations in the spent media of the cultures densely inoculated were always higher than those of the cultures sparsely inoculated. Differences in the time course of albumin production in cultured cells were not found in the two culture systems used. Albumin production in subcultured cells was observed to be very low as compared with that in primary cultured cells (Table 1).

TABLE 1. ALBUMIN CONTENT OF SPENT MEDIA FROM THE SUBCULTURES.

No. of passage	Incubation time (h)	Albumin (ng/ml)
2	0-48	26.2±4.2
	48-96	6.0±5.3
	96-144	3.0±3.0
3	0-48	2.1±2.7
	48-96	—
	96-144	3.2±2.7
4	0-48	3.2±2.7
	48-96	3.6±6.0
	96-144	4.0±3.0
	144-192	1.4±1.5

The primary culture was initiated by inoculating isolated hepatic cells at a concentration of 35×10^4 cells per ml. In the 14th day of the primary culture, cultured cells were detached from the culture dishes and subcultured at a split ratio of 1 : 2. The results are expressed as mean±standard deviation from 3 experiments.

A small amount of AFP was also detected in the spent media, and AFP content increased 4 days after inoculation (Table 2). This seems to be a very

TABLE 2. α-FETOPROTEIN CONCENTRATIONS IN THE SPENNT MEDIA FROM THE PRIMARY CULTURE OF ADULT RAT HEPATIC CELLS

Incubation time (h)	α-Fetoprotein (ng/ml)
0-48	12.7±8.2
48-96	5.7±1.5
96-144	7.5±6.1
144-192	13.2±8.0
192-240	14.2±9.7
240-288	19.2±7.8
288-336	6.5±5.0

Isolated hepatie cells were inoculated at a concentration of 35×10^4 cells per ml. The results are expressed as mean±standard deviation from 3 experiments.

interesting observation, but whether there is an association of increased AFP production with growth of clear cells remains to be seen.

DISCUSSION

It is very important to determine culture conditions (*i.e.*, inoculum cell number, nutrients, serum and hormones) that will permit the maintenance of functional hepatocytes *in vitro*. The maximal attachment of cells took place in Ham's F-10 medium supplemented with 20% FCS (3, 9). Williams (22) has already reported that cells inoculated into Williams' E (WE) medium with 10% FCS yielded the greatest attachment efficiency and also that WE medium provided good support for the growth of rat liver epithelial cells. However, MEM supplemented with BS, dexamethasone and insulin was more effective on primary cell attachment and the longevity of functional hepatocytes than WE medium with both FCS and hormones (unpublished data). Furthermore MEM with 20% BS, which has been used for establishing many cell lines from rat livers in this institute (2, 5), was much more suitable for the primary cell attachment and the epithelial-like cell growth than MEM with either FCS or CS. Inoculum sizes of 5×10^4 to 15×10^4 cells per cm^2 were much more effective for cell attachment and growth than higher cell densities, probably because of the relatively greater surface area available for attachment. This result agrees well with that of Bonney *et al.* (9), who have reported that approximately 7×10^4 cells inoculated per cm^2 on a 60 mm Petri dish allowed the maximal percentage attachment.

The cells prepared from regenerating liver by the use of collagenase-perfusion attached to the plate 4 to 6 h after inoculation and concomitantly exhibited morphological changes from spherical to cuboidal, coming into extensive contact with neighboring cells after 12 to 18 h of incubation (23). Isolated hepatic cells obtained by trypsin-perfusion seemed to take a relatively longer time to attach than those prepared with collagenase-perfusion. This may be due to injuries of hepatic cell membrane when they were dispersed by trypsin-perfusion (11). Therefore, it is not clear whether increases in cell numbers 2 days post-inoculation resulted from cell proliferation or increased attachment of cells.

Numbers of primary cultured cells have been reported in most cases to decrease with respect to time in culture (9, 24). Isolated hepatic cells described in the present report proliferated well, the difference may be due to a difference in the population of isolated hepatic cells and the culture conditions used. The trypsin-perfusion method seems to selectively yield nongranular cells with higher proliferating ability than other methods. Grisham *et al.* (25) have reported that epithelial clones developed only from small oval to spindle-shaped and nongranular cells which could be distinguished from hepatocytes. Since such cells represented only a small fraction (less than 1%) of the total cellular populations of

liver tissue, they appeared to have a very high cloning efficiency *in vitro* (25).

The polygonal cells with granular cytoplasm, which were found at the early stages of primary culture of isolated hepatic cells, seemed to be associated with expression of liver specific functions such as higher TAT levels and transient increases in albumin production. The findings described above suggested that these polygonal cells may correspond to the mature hepatocytes in the adult liver. It has been demonstrated that the hepatocytes could divide only once or twice in their cell cycles and soon stopped proliferating (26). On the other hand, the clear cells, which were few in number among isolated hepatic cells, proliferated vigorously in place of the polygonal cells. The cultures consisted mostly of these cells up to the 6th day of culture and they showed poor liver specific functions. A reason for the poor expression of specific functions in the clear cells may be their high mitotic activities (8). The cultured cells revealed increased AFP production 6 days postinoculation. This observation seems to be very interesting from the point of view that these clear cells may originate from the presumtive hepatocytic stem cells or the partially differentiated hepatic precursor cells in liver (25). Since these clear cells could also grow in subculture, the established cell lines may be derived from them (22).

It seems to be impossible to maintain functional hepatocytes during culture by raising the cell inoculum sizes alone to inhibit the cell growth at the early stage of primary culture. Changes in the cell population can take place even in the confluent cultures. The hepatocytic stem cells, however, had the ability to divide and then differentiate to hepatocytes when properly stimulated (25). These results suggest the possibility that the cultures of hepatocytes which retain more of their *in vivo* functional characteristics can be established under ideal conditions. Dexamethasone and hydrocortisone addition has the effect of maintaining albumin production in cultured cells at a relatively higher level for a longer time (27).

Primary mass culture from isolated hepatic cells would be useful in elucidating the *in vivo* origin of cultured cells. Further studies should be done in the future to elucidate what kinds of cell fractions in liver *in vivo* have the ability to adapt to the culture environment *in vitro*. This could be achieved by fractionating freshly isolated liver cells and separately culturing each fraction of cells thus prepared.

Acknowledgments. The author wishes to express sincere thanks to Professor J. Sato and Dr. A. Watanabe for many helpful suggestions and discussions and to Miss K. Miyano for her assistance.

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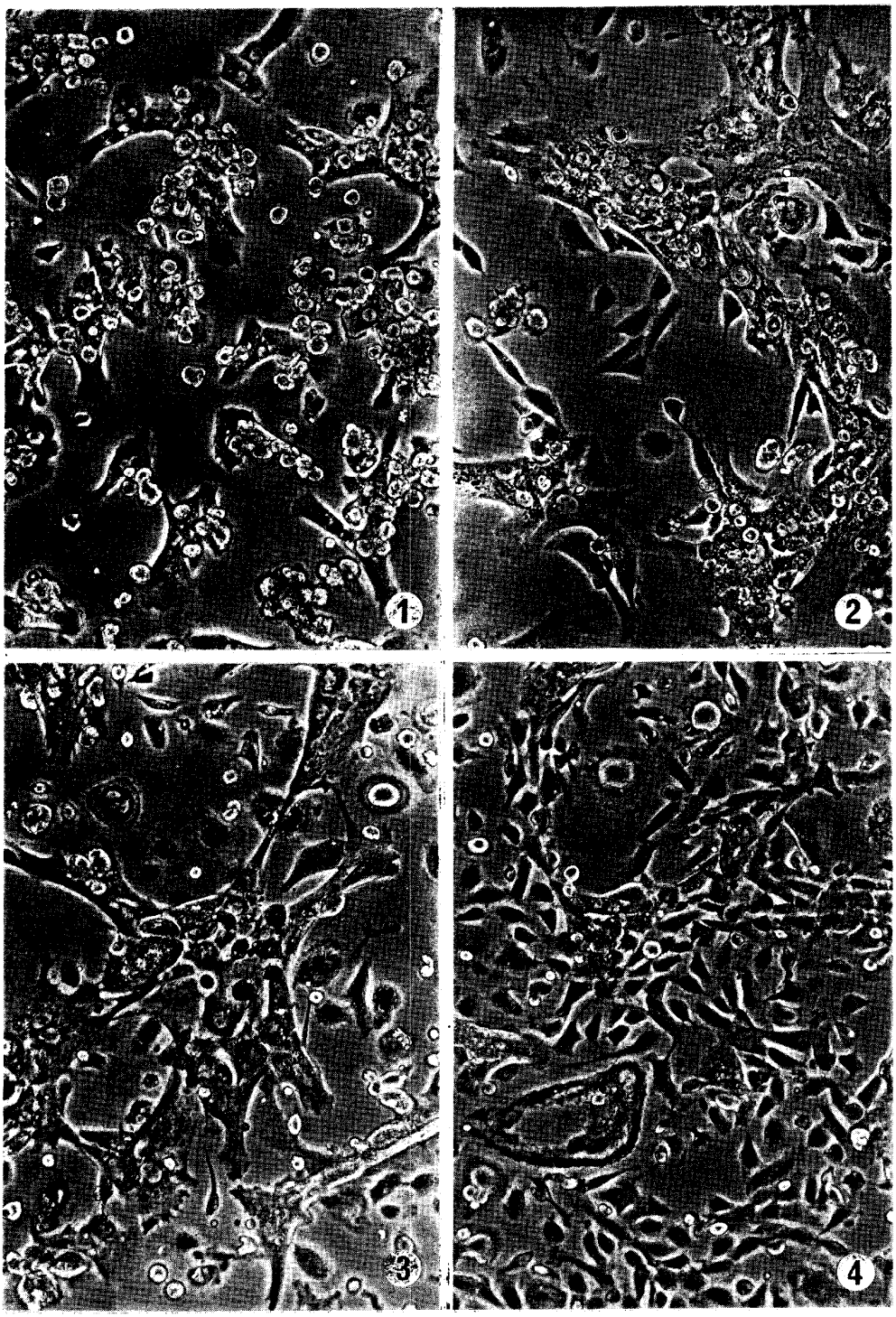
Legends to Photographs

Photographs. Isolated hepatic cells were inoculated at a concentration of 35×10^4 cells per ml. Cultured cells were observed under a phase contrast microscope at a magnification of 100 times. Photos. 1 to 7 show the morphology of primary cultured cells in the 2nd, 3rd, 4th, 6th, 8th, 10th and 14th day of culture, respectively. Photo. 8 illustrates secondary cultured cells in the 6th day.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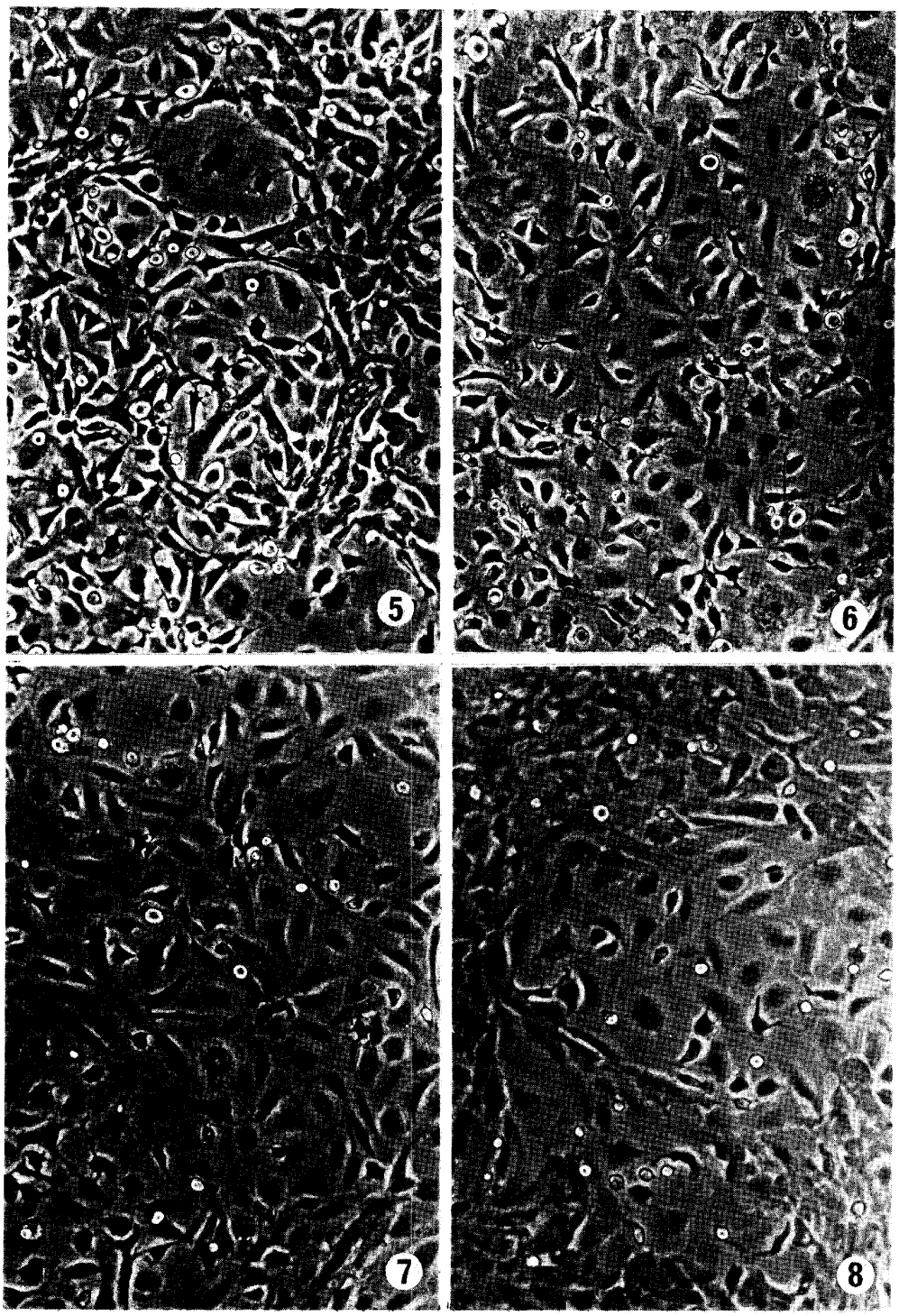
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