THE RESTORATION OF THE FUNCTIONS OF SERIALLY PASSAGED CALF HEPATOCYTES
BY SPHEROID FORMATION

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SUMMARY

A serial cultivation system of hepatocytes was established for the first time using calf liver as a cell source and, repeating passage of more than 30 cumulative population doublings (PDs), was obtained in the presence of long-acting ascorbic acid derivative (L-ascorbic acid 2-phosphate) and epidermal growth factor. The complete purification of hepatocytes was achieved by repeating ethylenediaminetetraacetic acid (EDTA) treatment, by which hepatocytes were easily detached from the culture dish, leaving most of the nonparenchymal cells on the dish. As the population cumulatively doubled, the cell density and albumin-synthesizing ability decreased gradually, and doubling time has exceeded 120 h at about 30 cumulative PDs. In serially passaged cells, the hepatocyte-specific histochemical and biochemical markers—including glucose-6-phosphatase, ornithine carbamoyltransferase, glutamate dehydrogenase, and ammonia-metabolizing activities—have been lost after 20 cumulative PDs. However, when these passaged cells were allowed to form spheroids, the morphologic and biochemical characteristics of hepatocytes have rapidly been restored to levels comparable to those in younger generations. Because no extrinsic factor was needed for this restoration, three-dimensional cell-cell interaction would be indispensable for the differentiation of the hepatocytes. The routine serial cultivation of hepatocytes and their redifferentiation by spheroid formation will be useful for studying metabolism, gene regulation, and transplantation of hepatocytes.

Key words: hepatocytes; serial cultivation; spheroid; redifferentiation; cell-cell interaction.

INTRODUCTION

Primary culture of hepatocytes, predominantly derived from murine liver, has now become a widely used research technique for studies of hepatic metabolism, regeneration, and carcinogenesis (2). However, serial cultivation of hepatocytes has been practically impossible because hepatocytes have poor proliferative activity and it is difficult to completely remove the nonparenchymal cells, including fibroblasts. In cultured human (13) and rat (12) fetal hepatocytes, the number of cumulative population doublings (PDs) has not exceeded four. Moreover, in many reports on hepatocyte growth factors, uptake of [3H]thymidine was used as a marker for the proliferation, but no data on the actual increase in the number of hepatocytes were given (3,11,14,21). In addition, the hepatocytes in primary culture rapidly lose their functions. Various attempts have been undertaken to extend the lifetime of differentiated functional hepatocytes, and recently spheroid formation has been proposed as the most promising method for the maintenance of hepatocyte function in primary culture (15,17,33).

Cultivation of bovine cells are easy in general. For example, cultivation of capillary endothelial cells has become possible for the first time using bovine adrenal cortex (5,34). When developing a cultivation system of endothelial cells from various tissues including liver (31,32), we noticed the active division of calf hepatocytes. Because the proliferation of hepatocytes was more marked in newborns than in adults, we have established a serial hepatocyte cultivation system using liver from newborn calves, and have obtained repeated passage of more than 30 cumulative PDs. Although the morphologic and biochemical characteristics of hepatocytes have rapidly been restored to levels comparable to those in younger generations, because no extrinsic factor was needed for this restoration, three-dimensional cell-cell interaction would be indispensable for the differentiation of the hepatocytes. The routine serial cultivation of hepatocytes and their redifferentiation by spheroid formation will be useful for studying metabolism, gene regulation, and transplantation of hepatocytes.

Materials and Methods

Materials

Human recombinant epidermal growth factor (EGF) was purchased from Wakanaga, Pharmaceutical Co. (Tokyo, Japan). Collagenase and L-ascorbic acid 2-phosphate were from Wako Chemicals (Tokyo, Japan). [35S]Methionine (29.6 TBq/mmol) was from New England Nuclear (Boston, MA). Rabbit antitoxic albumin IgG and bovine albumin were from Cappel (Westchester, PA). Newborn calf serum was from Mitsubishi Chemical Industries (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were from GIBCO Laboratories, Life Technologies (Grand Island, NY). Dishes for tissue and bacteriological culture were from Nunc, Inc. (Naperville, IL). Immunocytochemical detection kit for monitoring cell proliferation using 5-bromo-2'-deoxyuridine (BrdU) was from Amersham International (Amersham, UK). All other reagents were of the highest grade, unless specified otherwise.

Methods

Separation and serial cultivation of hepatocytes. The livers of newborn calves (1 to 7 d old) were removed, packed in ice-cold containers, and trans-
ported to the laboratory. According to the method of Soglen (1976), the hepatocytes were prepared immediately or within 24 h after the calves were killed. The liver was sterilized with 5% povidone-iodine and Ca^2+ and Mg^2+-free phosphate-buffered saline (PBS) solution. A part of the liver was dissected and, through the cut end of the portal vein, was infused with PBS, with 0.02% ethylenediaminetetraacetic acid (EDTA)-PBS, and finally with 0.05% collagenase-PBS solution. After 20 min of incubation at 37°C, aliquots of digested tissues (corresponding to about 5 g of original tissue) were filtered through 100-μm nylon mesh. The cell suspension was centrifuged at 50 × g for 5 min at 4°C. The cells were washed four times; suspended in DMEM supplemented with 10% newborn calf serum, 2 mg/ml of recombinant human EGF, 0.2 mM L-ascorbic acid 2-phosphate, 100 U/ml of penicillin, and 100 μg/ml of streptomycin; and cultured at 37°C under 5% CO₂ and 100% humidity atmosphere. The yield of separated cells was about 1 to 2 × 10⁶ cells. The viability assayed by trypan blue exclusion was high when the cells were separated immediately after sacrificed (87.5 ± 7.4%, n = 6), but the result varied in preserved livers from 90 to 30%. The medium was renewed at 48 h.

Forty-eight hours after seeding, the cultures were washed with PBS and treated with 0.02% EDTA-PBS for 5 min at 37°C. The hepatocytes were easily detached as clusters composed of several cells, leaving most of the nonparenchymal cells, including fibroblasts, on the dish. This procedure was carried out for 3 successive d in order to purify the hepatocytes perfectly. Usually, the yield in each treatment was about 80%. The passage was performed by EDTA treatment without any protease because the cells were extremely labile to protease.

Uptake of 5-bromo-2′-deoxyuridine (BrdU). About 48 h after seeding, primary cultures of calf hepatocytes were washed with the PBS and fed with medium containing BrdU (30 μg/ml) and 5-fluoro-2′-deoxyuridine (5 μg/ml). After 16 h incubation, the cells were fixed with acid-ethanol, and the immunocytochemical detection of incorporated BrdU using anti-BrdU monoclonal antibody was carried out by the method described in the protocol of the kit (6). The cells cultured without BrdU were used as controls.

Growth of hepatocytes. For the determination of the effect of EGF on the growth of calf hepatocytes, were washed with the PBS and fed with medium containing BrdU (30 μg/ml) and 5-fluoro-2′-deoxyuridine (5 μg/ml). After 16 h incubation, the cells were fixed with acid-ethanol, and the immunocytochemical detection of incorporated BrdU using anti-BrdU monoclonal antibody was carried out by the method described in the protocol of the kit (6). The cells cultured without BrdU were used as controls.

Growth of a colony of hepatocytes in primary culture. Fig. 1 shows the growth of a colony of hepatocytes seeded in low cell density after the separation of hepatocytes from livers. As shown in Fig. 1 A, colonies of hepatocytes rich in granules with apparent nucleoli were observed 24 h after seeding. In the colony presented in this figure, nonparenchymal cells including fibroblasts were not seen. These hepatocytes began to divide within 48 h, and formed a cordlike structure (Fig. 1 B). Calf hepatocytes proliferated actively, forming a large colony 14 d after the separation (Fig. 1 C–E), but the morphologic characteristics of the hepatocytes became less prominent with the division of the cells (Fig. 5 A). Glom growth from a single hepatocyte was not seen; only hepatocyte clusters comprising at least three cells were able to proliferate.

To demonstrate that the calf hepatocytes actively proliferate, the primary cultures (48 h after the separation) were incubated in the presence of BrdU for 16 h. As shown in Fig. 2 A, the morphologic characteristics of hepatocytes still remained in the treated cells. Almost all the hepatocytes took up BrdU, indicating that the duration of S phase in those cells was less than 16 h (Fig. 2 B). No labeling was observed in the control experiments without BrdU.

Growth-promoting effect of EGF and ascorbic acid on purified hepatocytes. When the primary cultures of hepatocytes were treated with 0.02% EDTA (see “Materials and Methods”), almost all hepatocytes were able to be removed, with most of the coexisting residual endothelial cells and fibroblasts still adhering to the dish. Two days after the separation, hepatocytes were purified by repeating this treatment for 3 successive d. Complete purification of hepatocytes was not possible by any density gradient centrifugation. The population of hepatocytes had cumulatively doubled four to five times when the purification by successive EDTA treatment was completed. Calf hepatocytes showed active proliferation in the presence of EGF and long-acting ascorbic acid; the addition of L-ascorbic acid 2-phosphate reduced the optimum concentration of EGF by 3.3% or lower, to as low as 1 ng/ml (Fig. 3 A). In the coexistence of EGF and L-ascorbic acid 2-phosphate, calf hepatocytes proliferated into about four times higher level than the control (no addition) 7 d after the seeding (Fig. 3 B). Further stimulation of proliferation was not observed when the culture dish was coated with fibronectin or type I collagen.

Restoration of the functions of serially passaged hepatocytes by spheroid formation. As shown in Fig. 4, as the population cumulatively doubled, the cell density of the hepatocytes decreased gradually but the albumin-synthesizing ability showed no marked decrease up to more than the 30th PD. Population doubling time was about 18 h at the 4th PD, and increased to about 48 h at the 10th PD and more than 120 h at the 30th PD. Histochemically, however, the activities of G6Pase (19) and OCT (40), the markers of hepato-