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A Long-Term Culture of Human Hepatocytes Which Show a High Growth Potential and Express Their Differentiated Phenotypes

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The present study succeeded for the first time in cultivating for more than 2 months human normal hepatocytes which showed a high growth potential and expressed their differentiated phenotypes. Constituents of culture medium were critical for this culture, and the medium optimized for their growth contained fresh human serum, fetal bovine serum, Swiss 3T3-cell conditioned medium, L-ascorbic acid 2-phosphate, epidermal growth factor, nicotinamide, and dimethyl sulfoxide. Hepatocytes steadily replicated and formed colonies which continued to increase in size up to around 35 days. The number of hepatocytes in the most replicative colonies increased 17-fold during 31 days. Cells in colonies expressed normal differentiated hepatocytic phenotypes for as long as 35 days. These hepatocytes retained normal liver functions at least for 70 days such as to secrete albumin, and to metabolize lidocaine and D-galactose.

Up to date few studies succeeded in a long-term culture of primary human hepatocytes (1–3), which usually can survive for 2 to 3 weeks, but lose differentiated phenotypes in a week. Co-culturing of hepatocytes with epithelial cells was shown to increase the culture period of hepatocytes. Albumin-secreting human hepatocytes were maintained for more than two months in the presence of rat liver epithelial cells (4). Gripon et al. developed a long-term culture of human hepatocytes by incorporating rat liver epithelial cells and dimethyl sulfoxide (DMSO) (1). Three-dimensional culture has been another type of long term culture of human hepatocytes. Li et al. first tried to culture human hepatocytes in multicellular spheroids and showed their survival up to one month (2). Ryan et al. cultured hepatocytes in three-dimensional gels of collagen in which the cells could be maintained for two months exhibiting normal morphology and sustaining capability of albumin secretion (3). However, there have been no successful attempts to culture human hepatocytes which are highly replicative, form clonogenic colonies, and express normal phenotypes for a long term in conventional monolayers without intentionally incorporating other types of cells.

We devised a new culture method by which adult rat hepatocytes can be maintained up to at least 2 months, repeatedly dividing and showing a bipotential differentiation capacity (5, 6). There were two important improvements in our method. The cells cultured were not hepatocytes in the conventional parenchymal fraction but hepatocytes recovered in the nonparenchymal cell fraction (the small hepatocyte fraction). In addition, a new culture medium (HCGM, hepatocyte clonal growth medium) was developed by adding five additives, epidermal growth factor (EGF), nicotinamide, L-ascorbic acid 2-phosphate (Asc-2P), DMSO, and fetal bovine serum (FBS). Hepatocytes in this medium grow

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Abbreviations used: α1-AT, α1-antitrypsin; AFP, α-fetoprotein; Asc-2P, L-ascorbic acid 2-phosphate; BrdU, bromodeoxyuridine; CK, cytokeratin, CM, conditioned medium; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; FBS, fetal bovine serum; HGF, hepatocyte growth factor; HS, human serum; MEGX, monoethylglycine-xylidine; Tf, transferrin.

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clonally. They undergo multiple divisions and form typical hepatocyte-colonies. The cells in the colony express lineage-specific proteins of both hepatocytes and biliary epithelial cells.

Recently, we showed that rat parenchymal hepatocytes could proliferate continuously when they were co-cultured with Swiss 3T3 cells (7). The conditioned medium (CM) derived from Swiss 3T3 cells also stimulated the growth of hepatocytes (7). The CM was shown to contain at least two potent mitogens for parenchymal hepatocytes, one of which was identified as pleiotrophin (7).

Such a progress in culturing replicative hepatocytes of adult rats led us to apply this method to the primary culture of adult human hepatocytes. The small hepatocyte fraction was obtained from normal tissues of human liver, and hepatocytes in this fraction were cultured in HCGM. A direct application of HCGM did not result in a noticeable enhancement of replicative potential of the cells. After several trials we found that the incorporation of human serum (HS) and the 3T3 cell-CM into HCGM greatly improves the growth of human hepatocytes. These hepatocytes multiply repeatedly to form clonogenic colonies without losing their normal differentiated phenotypes. The present study details the growth medium optimized for human hepatocytes and characterizes the colony-forming hepatocytes grown in this medium. This culture method should be useful for pharmacological studies of human hepatocytes. The proliferative human hepatocytes can be used as carrier cells of transgenes of the gene therapy of diseased liver.

MATERIALS AND METHODS

Isolation of small hepatocytes. Normal liver tissues were obtained from 14 patients (9 males and 5 females) at the Hiroshima University Hospital after receiving their consent before operations according to the 1975 Helsinki declaration. The range of age was 23 to 78 years. The hepatocytes prepared from 5 patients by the method detailed below were separately cultured in HCGM which had been previously optimized for rat hepatocytes (5, 6). However, hepatocytes failed to grow in these trials.

After optimizing the culture condition for human hepatocytes, we made 9 separate trials to culture hepatocytes from 9 patients. The optimized medium contained 10% fresh HS prepared from a healthy 28 to 57-year-old volunteers, 5% FBS (Hyclone Laboratories Inc., Utah), and 50% 3T3 cell-CM. These trials were all successful in growing hepatocytes and their colonies. The cells obtained from younger persons (23 to 56-year-old) formed hepatocyte colonies more frequently than those from older ones (63 to 78-year-old).

The present report describes in detail a case obtained from a 44-year-old male patient with a restricted bile duct tumor for studies of the growth and the phenotype expression. The hepatocytes obtained from this patient were used for the characterization of proliferation, colony formation, and expression of lineage-specific phenotypes. A liver tissue (1 g wet weight) of a non-diseased region was excised from the liver. The tissue looked normal without fibrosis, although it was tinted dark brown due to the obstructive jaundice. Hepatocytes for testing liver functions were obtained from a normal liver tissue (2 g wet weight) of a 23-year-old male patient who had mesenchymal hamaltoma with massive coagulative necrosis. The liver tissues were perfused with ice-cold University of Wisconsin Solution (Du Pont, Newark, DE) for 10 min, dipped in the same solution, placed in an ice box, and transferred to a laboratory clean bench. Appropriate parts were removed from their periphery and excised in a V-shape at the two margins because this shape was suitable for an effective perfusion. The tissues were kept covered by the hepatic capsule except at the two cut surfaces.

The liver specimens were infused with 37°C-warmed Hanks' solution containing 0.5 mM EGTA (Sigma Chemical Co., St. Louis, MO) for 10 min through vascular cut ends using a syringe, avoiding the injection of air bubbles. The tissues were then subjected to the two-step enzyme digestion by similarly infusing first with a 37°C-warmed solution containing 0.05% collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1,000 IU/ml dispase (Godo shusei, Tokyo, Japan) for 3 min, and then with a solution containing 0.05% collagenase alone for 15 min. The tissues became swollen by these treatments. They were then deprived of the liver capsule by a pair of forceps and were gently pipetted with a solution containing 10% bovine serum albumin (Sigma) and 0.4 IU/ml DNase to disperse them into single cells. The cell suspension was passed through double layers of sterile cotton gauze to remove undigested tissues and was filtered through a single 75-μm mesh layer of nylon cloth (NBC Industries Co., Tokyo, Japan). The filtrate was centrifuged at 50 $\times$ g for 1 min to remove large hepatocytes. The supernatant was further centrifuged at 150 $\times$ g for 5 min to obtain a fraction of small hepatocytes and nonparenchymal cells as pellets, which were suspended and centrifuged as above to "purify" the fraction (6). This procedure was repeated once. The fraction thus obtained was enriched with small hepatocytes (6) and was dubbed the small hepatocyte fraction in the present study. Viability of cells was determined by the trypan blue exclusion test. Cells were counted with a hemocytometer (Becton Dickinson and Company, Parsippany, NJ).

Preparation of 3T3 cell-conditioned medium. 3T3 cell-CM was prepared as previously described in detail (7). Briefly, Swiss 3T3 cells were obtained from American Type Culture Collection (Rockville, MD), cultured, and passaged in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Life Technologies Inc., Rockville, MD) containing 10% FBS according to the standard procedure (8). The cells were plated at a density of 8.6×10^4 cells/cm^2 in 10-cm dishes containing 10 ml of DMEM-10% FBS and were cultured for 3 days. The medium was removed, passed through a 0.45 μm Millipore filter, and used as CM.

Culture of small hepatocytes. The small hepatocyte fraction was suspended at a density of 5×10^5 cells/ml in DMEM containing 20 mM Hepes (Gibco BRL), 15 μg/ml L-proline, 0.25 μg/ml insulin (Sigma), 5×10^{-8} M dexamethasone (Sigma), 44 mM $NaHCO_3$, 10 mM nicotinamide, 5 ng/ml EGF, 0.1 mM Asc-2P, 100 IU/ml penicillin G (Gibco BRL), 100 μg/ml streptomycin (Gibco BRL), 10 μg/ml gentamicin (Gibco BRL), 5% FBS, 10% freshly prepared normal HS, and 50% 3T3 cell-CM. The cells were seeded at 10^5 cells/cm^2 in 35-mm diameter dishes (Becton Dickinson Labware, Lincoln Park, NJ) containing 2 ml of medium or 13.5-mm Sumilon Celldesks (Sumitomo Bakelite, Tokyo) in 24-well plates (Corning Incorporated, Corning, NY) containing 0.4 ml of medium, and cultured at 37°C in a 5% CO_2-incubator. They were allowed to attach themselves to the dishes for 24 h and the first medium change was carried out. Medium was refilled every 3 days during culture. DMSO (Sigma) was added to the medium from day 4 at the concentration of 1% (5, 6). The dishes had been coated with acid soluble type I collagen (Cellgen 1-AC, Koken, Tokyo, Japan) according to Nakamura et al. (9).

Observation of hepatocyte colonies. A scratch was made on day 1 by a syringe needle on the surface of 35-mm dishes to mark the location of specific small hepatocytes. Cells in a fixed field of growth surface of the dishes were selected and were observed for their

growth through a Nikon Diaphot phase contrast microscope (Nikon, Tokyo, Japan) utilizing the scratch as a marker. Hepatocyte colonies in the field were photographed periodically. Cells were immunocytochemically stained with antibodies against albumin as described below. Clusters containing more than 8 albumin-positive cells were defined as hepatocyte colonies, small and large colonies being colonies containing 8 to 29 hepatocytes and more than 30 hepatocytes, respectively. The area of colonies on microphotographs was determined by a Macintosh computer using NIH Image 1.52 software. The number of hepatocytes in a unit area was counted under a Vanox AHBS light microscope (Olympus, Tokyo, Japan) and was found not to change significantly during the culture. A value of $9.8 \times 10^4 \pm 1.5 \times 10^4$ (the average $\pm$ S.E.) hepatocytes/cm^2 of colonies was obtained as the average density of hepatocytes in the colonies. The number of hepatocytes in the colonies was calculated by multiplying the area of the colonies by the average density of the hepatocytes.

Determination of total number of hepatocytes. Hepatocytes cultured on 13.5-mm Celldesks (24-well plates) were periodically fixed with ethanol during culture and immunocytochemically stained with antibodies against albumin or cytokeratin 18 (CK18) as described below. Albumin- or CK18-positive cells were counted in 10 different fields under a light microscope, then the number of hepatocytes per unit growth area and total number of hepatocytes in the well were calculated.

Bromodeoxyuridine (BrdU) labeling indexes. Hepatocytes were cultured on 13.5-mm Celldesks up to 35 days and were labeled for 48 h at appropriate times during the culture with 1 mM BrdU (Amersham Pharmacia Biotech, Uppsala, Sweden). BrdU-positive cells were detected as described below and were counted in 5 colonies containing more than 8 hepatocytes under a microscope or on microphotographs.

Immunocytochemistry. Cells were fixed in ethanol at −30°C and subjected to immunocytochemistry. Primary antibodies used were as follows: rabbit anti-human albumin antibody (Dakopatts, Glostrup, Denmark), rabbit anti-rat transferrin (Tf) antiserum (Cappel, Durham, NC), rabbit anti-human α1-antitrypsin (α1-AT) antibody (Dakopatts), mouse anti-BrdU monoclonal antibody (Dakopatts), rabbit anti-desmin polyclonal antibody (Monosan, Uden, The Netherlands), rabbit anti-rat α-fetoprotein (AFP) antiserum (a gift from Dr. T. Mitaka), mouse anti-human CK14 monoclonal antibody (Biomeda Corp, Foster City, CA), and mouse monoclonal antibodies against CK8, CK18, CK7, and CK19 (Amersham Pharmacia Biotech). The antibodies were visualized by a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) using diaminobenzidine as a substrate. Chemicals not specified above were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

Functional tests of human hepatocytes. The cells in the fraction were seeded at 10^5 cells/cm^2 in 13.5-mm Celldesks containing 0.4 ml of media and cultured for 72 days. During the culture functions of hepatocytes were assessed by measuring the ability to secrete albumin, and to metabolize lidocaine and galactose.

For measurement of albumin secretion at day 4, 44, 59, and 72, cells were washed with PBS three times to remove human albumin derived from HS, and cultured in 0.4 ml of the media without HS for 3 days. The media were clarified by centrifugation, and stored at −30°C until use. Human albumin in the media was quantified by the Latex agglutination photometric immunoassay with LX-6000 (A & T Co. Ltd., Tokyo, Japan), using a LX-Alb II reagent (Eiken Chemical Co. Ltd., Tokyo, Japan). The kit contained anti-human albumin mouse serum IgG conjugated to latex beads, which crossreacted with human albumin but not with bovine albumin. Albumin secretion at each time point was calculated as the amount of albumin per ml secreted during 3 days per culture.

To measure P450 enzyme activity, lidocaine metabolism was determined according to Flendrig et al. (10) with some modifications. At day 45, 59, and 70, the cells were incubated for 24 h in 0.4 ml of the media containing lidocaine hydrochloride at a concentration of 500 μg/ml. The media were collected and analyzed for lidocaine and its metabolite, monoethylglycinexylidine (MEGX), by HPLC, Smart System (Amersham Pharmacia Biotech). Lidocaine hydrochloride was obtained from Sigma Chemical Co. and MEGX was generously gifted by Astra Japan Ltd. (Tokyo, Japan), both of which were used to construct standard curves for the quantification of the corresponding substances in culture medium.

D-galactose elimination was determined according to Flendrig et al. (10) with some modifications. At day 24, 36, 48, 59, and 70, the cells were incubated for 24 h in 0.4 ml of the media containing 1 mg/ml of D-galactose (Sigma). Galactose was determined spectrophotometrically at 340 nm with a spectrophotometer H-3000 (Hitachi Ltd., Tokyo, Japan) using an enzymatic test kit (Boehringer Mannheim, Wiesbaden, Germany).

RESULTS AND DISCUSSIONS

Approximately 3.9×10^7 cells were obtained from 1 g of liver tissues of a 44-year-old male patient as the cells in the small hepatocyte fraction, the viability being 65.4%. Hepatocytes in this fraction were characterized for their growth, colony formation, and expression of lineage-specific proteins.

The cells in the fraction were cultured in HCGM which had been originally optimized for the proliferation of rat hepatocytes (5, 6). Albumin-positive cells attached well to dishes and could be maintained as single cells in a normal appearance in this medium for 3 to 4 days. However, they did not divide and were detached from dishes as nonparenchymal cells increased in number. Some single hepatocytes remained attached for a longer period, but their shapes became flat and extended. They finally lost stainability for albumin immunocytochemistry. We made several trials to optimize HCGM for the growth and the maintenance of hepatocytes by changing the concentration of FBS and incorporating HS at various concentration, and found that the mixture of 5% FBS and 10% HS much improved the growth of hepatocytes. A further improvement was accomplished by supplying 50% 3T3 cell-CM. Compared to the absence of CM, an approximately 5-fold higher efficiency of large colony formation was obtained in its presence. From these trials we formulated the culture medium which supports most effectively the growth of human hepatocytes as HCGM containing 5% FBS, 10% HS, and 50% 3T3-cell CM. Hepatocytes in this medium grew well and formed 49 colonies in a 13.5-mm Celldesk at 35 days of culture, which was an example of successful cultures in our experiences.

HS has been reported to support the growth of human hepatocytes (11). The serum utilized by these workers was prepared from fulminant hepatitis patients and increased both the amount of DNA by 75%–100% and the number of cells by 50%. However, normal HS was not effective in producing proliferating human hepatocytes in their study, which was quite different

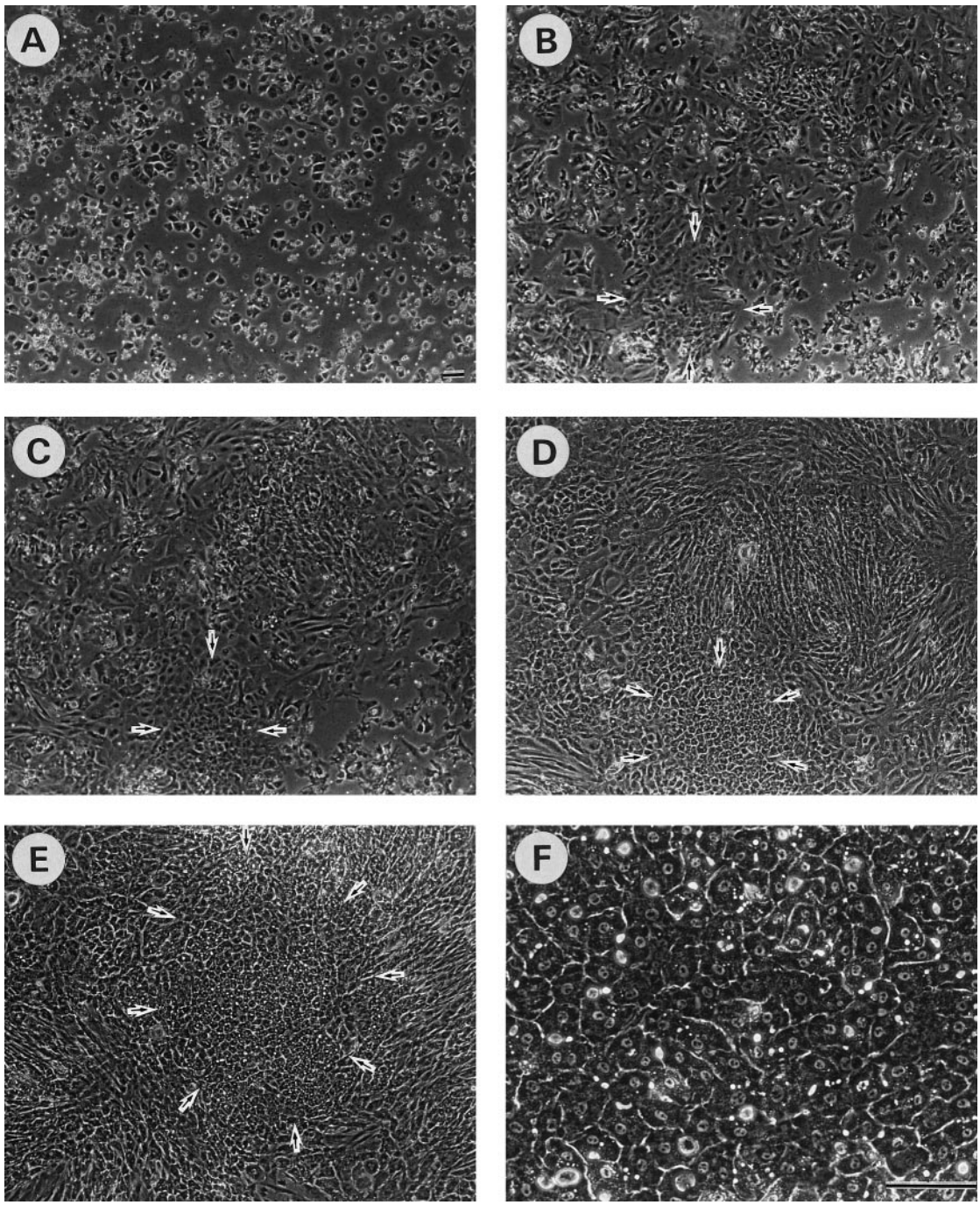

FIG. 1. Hepatocyte-colony formation observed through a phase contrast microscope. Cells of the small hepatocyte fraction were seeded at a density of 10^5 cells/cm^2 and cultured for 35 days in 35-mm dishes. (A) Day 2; (B) Day 8; (C) Day 15; (D) Day 28; (E and F) Day 35. The arrow in (B) through (E) points to the same colony of hepatocytes. Hepatocytes before the formation of colony could hardly be distinguished from nonparenchymal cells in their morphological appearances as shown in (A). The cells in a colony in (D) and (E) took on typical forms of hepatocytes. (F) is a high magnification of the colony in (E). Bar, A, 100 μm; F, 100 μm. Magnifications of B, C, D, and E are the same as A.

from our study. We clearly showed that normal fresh HS promotes the growth of human hepatocytes. This discrepancy might be ascribed to the difference in the type of hepatocytes cultured between the two studies and the absence of nonparenchymal cells from the culture utilized by the cited researchers.

Two possibilities may explain the growth stimulatory effect of the 3T3 cell-CM on hepatocytes. CM may

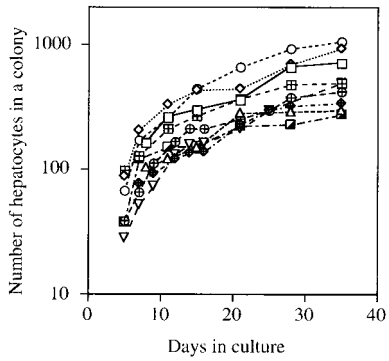

FIG. 2. Growth curves of colony-forming hepatocytes. Cells of the small hepatocyte fraction were cultured as in Figure 1. Hepatocyte colonies in the field were photographed periodically up to 35 days. Nine curves were obtained by tracing the growth of 9 colonies on phase contrast microphotographs. Similar growth curves were obtained for each of them. The growth rate decreased with days in culture. Each symbol represents one colony traced.

contain growth promoting factor (s) for hepatocytes or growth inhibitory factor(s) for nonparenchymal cells. Recently, we reported that co-cultivation with Swiss 3T3 cells or CM derived from Swiss 3T3 cells promoted growth of rat parenchymal hepatocytes (7). We purified a hepatocyte growth-promoting factor from 3T3 cell-CM and identified it as pleiotrophin. We suggested that pleiotrophin is an important factor in the development and regeneration of the liver, because the expression of pleiotrophin was increased in the fetal liver and the regenerating liver either after two-third hepatectomy or D-galactosamine treatment (7). In the present study, CM promoted colony formation of human hepatocytes. Pleiotrophin seems to be effective for not only rat hepatocytes but also for human hepatocytes. We observed rapid growth of nonparenchymal cells in early days of culture in the absence of CM. The overgrowth of nonparenchymal cells appears to inhibit the growth of small hepatocytes. Presence of CM apparently suppressed this nonparenchymal cell overgrowth. At present, there are no experimental data available which suggest the identity of the possible growth suppressor. As suggested in the previous studies (6), nonparenchymal cells in the small hepatocyte fraction, most probably stellate cells, play an important role for supporting the growth of hepatocytes. Therefore, there should be an appropriate growth of nonparenchymal cells, neither overgrowth nor under-

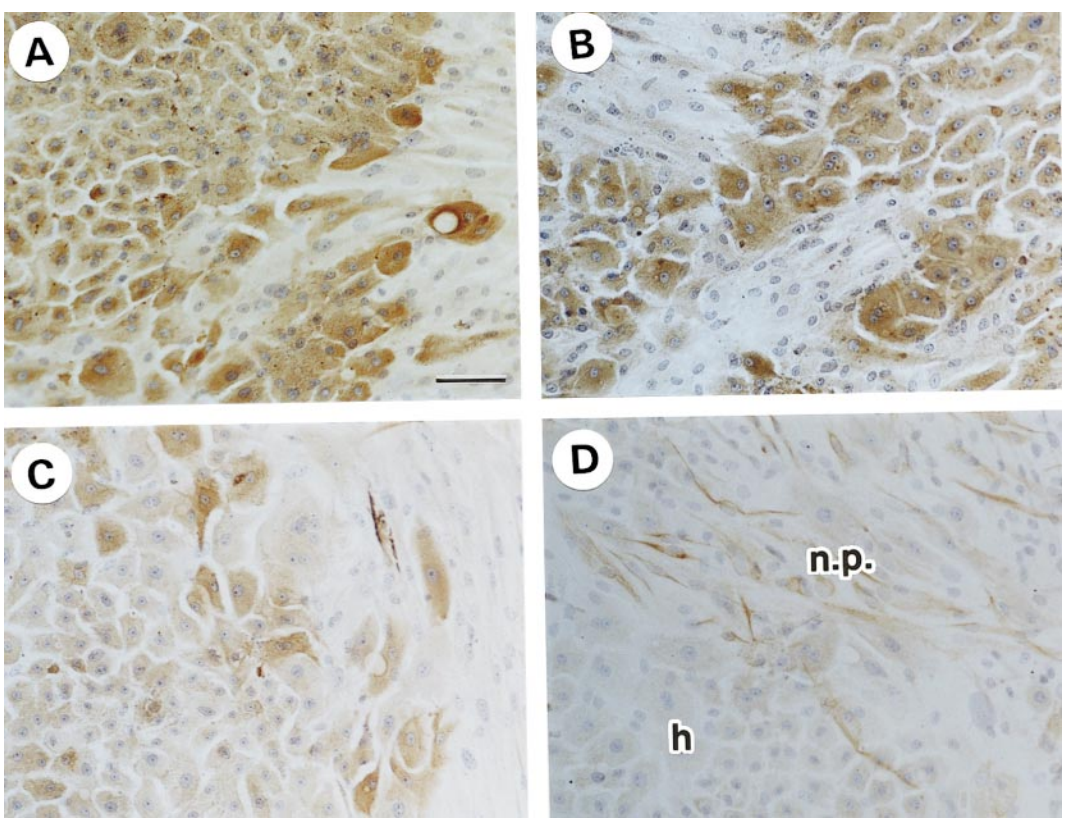

FIG. 3. Characterization of cells in colonies by immunocytochemistry. Cells of the small hepatocyte fraction were seeded at 10^5 cells/cm^2 on 13.5-mm Celldesks and cultured for 35 days. They were fixed at −30°C in ethanol and subjected to immunocytochemistry. Cells with brown cytoplasm are positive cells. (A) Albumin and (B) α1-AT. All the cells in colonies expressed these antigens. (C) CK7. Some cells in marginal regions of colonies were stained with the anti-CK7 antibody. (D) Desmin. Nonparenchymal cells (n.p.) that surrounded hepatocyte colonies (h) were positively stained with anti-desmin antibodies. Bar, 100 μm.

growth, which allows an optimal growth of hepatocytes. FBS alone is deleterious for hepatocyte growth in the present medium, but in a combination with HS and CM it might allow such an appropriate growth of nonparenchymal cells.

The growth of human hepatocytes was observed through a phase contrast microscope (Figure 1). Before the formation of colonies, hepatocytes could hardly be distinguished from nonparenchymal cells in their morphological appearances (Figure 1A) in contrast to rat hepatocytes which were morphologically distinguishable from nonparenchymal cells when attached to dishes (6). Large hepatocytes which had been recovered in the small hepatocyte fraction did not divide and detached from dishes. Instead the small hepatocytes began to proliferate at day 4 to 5 and formed small colonies around a week (Figure 1B). The nonparenchymal cells increased in number, changing shapes to spindle-like forms (Figure 1D). The cells in colonies took on typical morphologic appearances of hepatocytes after around 4 weeks in culture (Figure 1D–F). The hepatocytes colonies continued to increase their sizes (Figure 1B–E).

Growth of hepatocytes was evaluated by measuring the number of hepatocytes in each of 9 colonies up to 35 days (Figure 2). Similar growth curves were obtained for each of them and showed that the number of hepatocytes increased approximately 4.2- and 9.3-fold on average during 10 and 31 days, respectively. The most replicative colony showed a 5.6- and a 17.3-fold increase during 10 and 31 days, respectively. The growth rate decreased with days in culture: the average doubling time was 48 $\pm$ 6 h for 5 to 7 days, 190 $\pm$ 42 h for 7 to 15 days, 456 $\pm$ 127 h for 15 to 21 days, 1389 $\pm$ 831 h for 21 to 28 days, and 1671 $\pm$ 499 h for 28 to 35 days (mean $\pm$ S.E.). Total number of hepatocytes increased 2.8-fold from 1 to 35 days. The growth of hepatocytes was also examined for the cells obtained from the 23-year-old male patient. A similar hepatocytic colony formation was observed as in the case of the 44-year-old male patient described above. The total number of hepatocytes increased 3.6-fold from 4 to 70 days (Figure 4A).

BrdU labeling indexes were determined every 7 days for 35 days. The index was high at early days in the culture and decreased sharply with the time of cultivation: 46.6% at 7 days, 24.4% at 14 days, 9.8% at 21 days, 4.5% at 28 days, and 1.1% at 35 days.

The present study clearly demonstrates that the human liver contains a population of highly proliferative hepatocytes in the small hepatocyte fraction, as in the rat liver. The colony forming efficiency of rat hepatocytes was about 16% at day 10 of culture in HCGM (6). The colony-forming efficiency of human hepatocytes was about 0.8% at the day 35 of culture. Direct comparison of these calculations between rat and human hepatocytes might not be appropriate, because the culture media were different between the two. In spite of this limitation, it can be said that the number of proliferative hepatocytes per small hepatocyte fraction appears to be much lower in humans than that in rats.

The cells in the colonies were characterized by their expression of hepatocyte-specific markers. All the cells in the colonies at 35 days expressed albumin (Figure 3A) (12), Tf (data not shown) (13), and α1-AT (Figure 3B) (14). Only a few cells were weakly positive for AFP, a marker for immature or neoplastic hepatocytes (data not shown) (12). All the colony-forming cells expressed both CK8 (data not shown) and CK18 (data not shown), markers of both hepatocytes and bile duct cells (15). No cells in colonies expressed CK19 (data not shown), a biliary epithelial cell-marker (15), but the antibody against CK7, another such marker (15), stained some cells in marginal areas of colonies (Figure 3C). Few cells were positive for CK14, a liver epithelial cell marker (data not shown) (16). The nonparenchymal cells that surrounded the hepatocyte colonies were identified as stellate cells (Ito cells), because they were stained with anti-desmin antibodies (Figure 3D).

The function of hepatocytes which had been cultured by the method developed in the present study was assayed for their albumin secretion, and activities to metabolize lidocaine and D-galactose. Number of hepatocytes in these assays was increased gradually at least until 70 days (Figure 4A). Hepatocytes secreted albumin whose level continued to increase during the culture and reached to 22 μg/1.7 $\times$ 10^5 hepatocytes/3 days at 72 days (Figure 4B). Albumin secreted by hepatocytes was found to be 13.5 μg per 1.45 $\times$ 10^5 cells during 3 days from day 56 to day 59. This value was comparable to that obtained for coculture of primary human adult hepatocytes (1.5 $\times$ 10^6) and rat liver epithelial cells per day (approximately 24 μg) at 40 days reported by Clement et al. (17). The levels of albumin secretion for 3 days per cell were calculated as 0.05, 0.11, and 13.5 ng, at 44, 59, and 72 days, respectively. It appears that the decrease in proliferation paralleled as increase in differentiation phenotype.

The cytochrome P450 activity of the hepatocytes was assessed by determining the rate of conversion of lidocaine to MEGX at day 45, 59, and 70. Lidocaine was applied to the hepatocytes at a concentration of 500 μg/ml for 24 h at these days. The hepatocytes were able to metabolize lidocaine even at 70 days in culture, although the ability decreased gradually there after: lidocaine eliminated at day 45, 77 μg/8 $\times$ 10^4 hepatocytes/24 h; 32 μg/1.5 $\times$ 10^5 hepatocytes/24 h at day 59; and 44 μg/1.7 $\times$ 10^5 hepatocytes/24 h at day 70 (Figure 4C). MEGX was detected at these days: 2.1

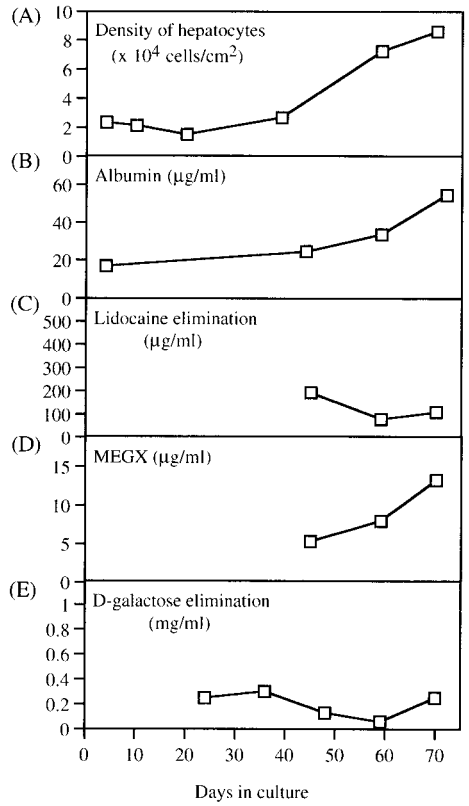

FIG. 4. Functional tests of human hepatocytes. Cells of the small hepatocyte fraction were seeded at 10^5 cells/cm^2 on 13.5-mm Celldesks were cultured for 72 days. Each point represents the mean of double cultures. (A) Density of hepatocytes, (B) Albumin secretion, (C) Lidocaine elimination, (D) MEGX formation, and (E) Galactose elimination.

μg/8 $\times$ 10^4 hepatocytes/24 h at day 45; 3.2 μg/1.5 $\times$ 10^5 hepatocytes/24 h at day 59; and 5.3 μg/1.7 $\times$ 10^5 hepatocytes/24 h at day 70 (Figure 4D). The concentration of the metabolite (MEGX) rather increased during the period of assessment, irrespective of the fact that the ability to eliminate lidocaine decreased. This might be due to the gradual loss of enzymatic activities involved in the further metabolism of MEGX. The similar phenomenon was also reported in cultures of hepatocytes by Flerdrig et al. (10). Hepatocytes were loaded with 1 mg/ml galactose at day 24, 36, 48, 59, and 70. The cells metabolized it through 70 days, although its extent was variable: 0.10 μg/4 $\times$ 10^4 hepatocytes/24 h at day 24; 0.12 μg/5 $\times$ 10^4 hepatocytes/24 h at day 36; 0.05 μg/1.0 $\times$ 10^5 hepatocytes/24 h at 48 days; 0.02 μg/1.5 $\times$ 10^5 hepatocytes at day 59; and 0.10 μg/1.7 $\times$ 10^5 hepatocytes at 70 day (Figure 4E).

The porcine hepatocytes (2.2 $\times$ 10^8 cells) could metabolize about 7.5 mg of lidocaine (3.4 $\times$ 10^{-2} ng/cell/hr) and produced about 600 μg of MEGX (2.7 $\times$ 10^{-3} ng/cell/hr) for 1 hr when 500 μg/ml lidocaine was added into the medium, and eliminated about 10.5 mg of D-galactose (1.6 $\times$ 10^{-2} ng/cell/hr) for 3 h at day 3 in the bioartificial liver they devised (10). Human hepatocytes (approximately 1.7 $\times$ 10^5 cells) in the present study could metabolize about 44 μg of lidocaine (1.1 $\times$ 10^{-2} ng/cell/hr) and produced about 5.3 μg of MEGX (1.3 $\times$ 10^{-3} ng/cell/hr) for 24 h and eliminated about 0.1 mg of D-galactose (2.5 $\times$ 10^{-2} ng/cell/hr) for 24 h at 70 days. These calculations clearly show that human hepatocytes in our cultures can retain their functions for more than 2 months at levels comparable to those of short-term cultures of porcine hepatocytes.

Recently, several types of bioartificial livers have been devised to treat patients with liver failures (10, 18, 19). Demetriou et al. (18) and Susman et al. (19) have developed bioartificial livers that are clinically applicable as a bridge to transplants. These investigators have utilized porcine hepatocytes or a transformed human liver cell line (HepG3), which makes the use of these devices limited to transient treatments. Further improvements of our method to grow human hepatocytes should make it possible to obtain proliferative normal hepatocytes more efficiently which can be used to reconstruct bioartificial livers. These cells are also useful for pharmacological studies, and can be used as carrier cells of transgenes for the gene therapy of diseased liver.

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