American Journal of Pathology, Vol. 148, No. 2, February 1996


# Short Communication

## Long-Term Cultivation of Adult Rat Hepatocytes That Undergo Multiple Cell Divisions and Express Normal Parenchymal Phenotypes

Chise Tateno* and Katsutoshi Yoshizato*†

*From the Yoshizato MorphoMatrix Project,* ERATO, Research Development Corporation of Japan, and Department of Biological Science,† Faculty of Science, Hiroshima University, Hiroshima, Japan*

***The present study succeeded in cultivating normal adult rat hepatocytes for at least 85 days without losing their replicative potential and differentiation capacity. Small pieces of hepatocyte aggregates (clusters) were prepared from the primary culture of hepatocytes and used as starting material for the growth experiment. Some of the hepatocytes started to proliferate at 3 days when the clusters were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 10 ng/ml epidermal growth factor, 10 mmol/L nicotinamide, 0.2 mmol/L L-ascorbic acid 2-phosphate, and 1% dimethylsulfoxide. Clusters continued to grow and formed colonies. All the cells covering colonies expressed normal hepatocyte-specific proteins. The number of albumin-expressing cells in the most replicative colonies increased sixfold during 32 days. Most of the cells were mononucleate and small in size and some of them expressed immature hepatocyte markers such as α-fetoprotein. Electron microscopy of cells in colonies revealed the presence of peroxisomes in the cytoplasm and desmosomes, tight junctions, and bile canaliculus-like structures between the cells. Depletion of one of the additives inhibited the growth of hepatocytes. The culture medium used also supported the growth of stellate cells (Ito cells) that had contaminated the original preparation in small numbers and seems to cooperatively stimulate a proliferative population of hepatocytes. (Am J Pathol 1996, 148:383–392)***

It is known that adult mouse hepatocytes show higher growth ability *in vitro* than adult rat hepatocytes.[1] Actually, it was shown that the former can divide repeatedly and survives for a long period when an appropriate culture condition is provided.[2] Many attempts have been made to increase the proliferative capacity of adult rat hepatocytes in primary culture.[3–9] However, there have been few studies that succeeded in inducing multiple cell divisions of the cells *in vitro*.[7] The present study was undertaken to seek an appropriate culture condition in which adult rat hepatocytes can continuously proliferate and express their normal differentiation phenotypes for a longer period of time.

Intracellular nicotinamide adenine dinucleotide of rat hepatocytes drops to an unphysiological level during culture.[10] Nicotinamide was shown to stimulate the cell division of cultured hepatocytes and increased cell number 2.7-fold on day 5, probably by acting as a precursor for nicotinamide adenine dinucleotide synthesis.[11] Mitaka et al[7] also showed that adult rat hepatocytes in primary culture can replicate several times within a week in the medium containing nicotinamide and epidermal growth factor (EGF). As a result, hepatocytes

Accepted for publication November 1, 1995.

Address reprint requests to Dr. K. Yoshizato, Yoshizato MorphoMatrix Project, ERATO, Research Development Corporation of Japan, Hiroshima Techno-Plaza, 242–37, Misonou, Higashihiroshima, Hiroshima 739, Japan.

A part of this study was presented at the 27th annual meeting of the Japanese Society for Developmental Biologists.

were increased 1.8-fold in number.[7] Wu et al[2] showed that adult mouse hepatocytes can replicate repeatedly in serum-free medium containing nicotinamide and EGF and survive for more than 2 months in primary cultures. These studies indicate the importance of nicotinamide for hepatocyte replications.

Rat hepatocytes produce ascorbic acid *in vivo*, which, however, is lost from the cells during isolation.[12] Senoo et al[13] showed that the addition of L-ascorbic acid 2-phosphate (Asc-2P), a long-acting vitamin C derivative, improved the life span of cultured adult rat hepatocytes and made it possible to maintain their functional activity for a long term. It has been well established that EGF is a potent mitogen of hepatocytes.[3,14,15] Dimethylsulfoxide (DMSO) increases the life span of hepatocytes *in vitro* and helps them to maintain a high level of albumin synthesis[16] and a basal activity of tyrosine aminotransferase.[17] Recently, Mitaka et al[18] showed that DMSO suppresses growth of nonparenchymal cells.

In addition to ingredients to be included in the culture medium, we tried to improve the cell preparation for studying growth potential of hepatocytes. Conventionally, freshly prepared hepatocytes have been used for this purpose as primary culture. However, the primary culture is thought to contain heterogeneous populations of hepatocytes with regard to their growth potential; some are highly proliferative and others are terminally differentiated and cannot replicate any more. We tried to use cells in colonies formed in the primary culture of hepatocytes as a starting material for investigating growth and differentiation potential, assuming that the colonies contain a population of hepatocytes with relatively high growth potential at a higher concentration than a fresh preparation of hepatocytes.

Considering the possible importance of the ingredients and the hepatocyte preparation, the present study developed the culture method of adult rat hepatocytes by which the cells can be induced to make multiple cell divisions and maintained for a long period, expressing their normal phenotypes. In addition, stellate cells (Ito cells) were found to actively grow and secrete collagens in the culture, suggesting their contributions to the successful long-term culture of hepatocytes. The cells cultured with this method appear to be useful as experimental materials for investigating liver-specific processes in both physiological and pathological conditions.

## Materials and Methods

### Isolation and Primary Culture of Hepatocytes

Hepatocytes were isolated from 8-week-old male Fisher 344 rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) by the two-step perfusion method of Seglen.[19] The cells were obtained as precipitates by Percoll isodensity centrifugation.[20] Their viability was approximately 95% according to the trypan blue exclusion test. The cells were suspended in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 20 mmol/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, 30 μg/ml L-proline, 0.5 μg/ml insulin, $10^{-7}$ mol/L dexamethasone, and 44 mmol/L $NaHCO_3$. Routine medium contained antibiotics of 100 IU/ml penicillin G and 100 μg/ml streptomycin. Cells were plated at $6.7 \times 10^4$ cells/$cm^2$ in 35-mm Falcon dishes (Becton Dickinson Labware, Franklin, NJ) or 13.5-mm Sumilon Celldesks (Sumitomo Bakelite Co., Tokyo, Japan) and were incubated in a 5% $CO_2$/95% air atmosphere at 37°C. At 2 to 3 hours after plating, the medium was replaced with fresh Dulbecco's modified Eagle's medium that contained 10 ng/ml EGF, 10 mmol/L nicotinamide, and 0.2 mmol/L Asc-2P in addition to the above ingredients. Cells were cultured for 4 days with a medium change at 2 days.

### Preparation of Hepatocytes for Growth and Differentiation Studies

The primary hepatocytes cultured for 4 days as above were immersed in phosphate-buffered saline containing 0.02% ethylenediamine tetraacetic acid (EDTA). The cells were detached as fragments of cell sheets in irregular forms and sizes, which were then gently pipetted twice to get a homogeneous suspension of small pieces of hepatocyte aggregates (hepatocyte clusters). Typical clusters contained approximately 100 hepatocytes and had diameters of approximately 300 μm. It was found later, as described below, that clusters were contaminated with small numbers of stellate cells (less than 1%). The viability of hepatocytes in clusters was more than 95%. Hepatocyte clusters were replated in new dishes containing medium for the primary culture described above, and 1% DMSO was added to the medium to suppress proliferations of nonparenchymal cells.[18]

### Determination of Number of Hepatocytes

A scratch was made on day 1 by a syringe needle on the back of a culture dish. A field of growth surface of

the dishes was fixed for observing cell growth through a phase-contrast microscope utilizing the scratch as a marker. Hepatocyte clusters in the field were photographed periodically by a Nikon Diaphot phase-contrast microscope (Nikon, Tokyo, Japan). The area of clusters on the microphotographs was determined by a Macintosh computer using NIH Image 1.52 software. Cells were periodically fixed in ethanol at −30°C and were stained with hematoxylin and eosin. The number of hepatocytes in a unit area was counted during the culture for different cell preparations under a Vanox AHBS light microscope (Olympus, Tokyo, Japan). As shown in Figure 2A, the size of hepatocytes in colonies did not change significantly during the culture for 89 days and among the different cell preparations; a value of $3.2 \times 10^5$ hepatocytes per $cm^2$ of clusters was obtained for the hepatocyte density by averaging them. Therefore, the growth rate was expressed as changes in colony area instead of hepatocyte number.

### *Determination of Bromodeoxyuridine (BrdU) Labeling Index*

Hepatocyte clusters were obtained, cultured in a 13.5-mm Celldesk as above for 89 days, and labeled for 48 hours at appropriate times during culture with 1 mmol/L BrdU (Amersham International, Buckinghamshire, United Kingdom). BrdU-positive cells were detected as described below and were counted. BrdU labeling index was determined in two independent cultures.

### *Immunocytochemistry and Enzyme Cytochemistry*

Cells were fixed in ethanol at −30°C and subjected to immunocytochemistry. Primary antibodies used were as follows: rabbit anti-rat albumin antibody (Cappel, Durham, NC), rabbit anti-rat transferrin (Tf) antiserum (Cappel), rabbit anti-human $\alpha_1$-antitrypsin ($\alpha_1$-AT) antibody (Dakopatts A/S, Glostrup, Denmark), mouse anti-BrdU monoclonal antibody (Dakopatts A/S), rabbit anti-desmin polyclonal antibody (Monosan, Uden, Holland), rabbit anti-rat $\alpha$-fetoprotein (AFP) antiserum (a generous gift from Dr. T. Mitaka), rabbit anti-bovine type I collagen antibody (LSL Co., Tokyo, Japan), mouse anti-human cytokeratin 14 (CK14) monoclonal antibody (Bimeda Corp.), mouse anti-cytokeratin 8 (CK8) monoclonal antibody (Amersham), mouse anti-cytokeratin 18 (CK18) monoclonal antibody (Amersham), mouse anti-cytokeratin 7 (CK7) monoclonal antibody (Amersham), and mouse anti-human cytokeratin 19 (CK19) monoclonal antibody (Amersham). The antibodies were visualized by Vectastain ABC kit or Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA) using diaminobenzidine or Vector Red (Vector) as substrates. Nonspecific esterase and catalase of cells were stained according to Li et al[21] and Goldfischer et al,[22] respectively.

### *Transmission Electron Microscopic Examination*

Cells on 13.5-mm Celldesks were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, postfixed in 0.2% osmium tetroxide in the same buffer, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with lead citrate and uranyl acetate and examined in a JEM-100S transmission electron microscope (JEOL, Tokyo, Japan).

## *Results*

### *Growth of Hepatocytes*

Primary culture was performed for 4 days by inoculating hepatocytes at $6.7 \times 10^4$ cells/$cm^2$, which allowed the cells to multiply 1.3-fold. Then, the hepatocyte clusters were obtained from dishes with EDTA and used for growth and differentiation studies. Most clusters were adhered to dishes (Figure 1A), and hepatocytes began to proliferate at 3 days and continued to grow slowly (Figure 1B). Some hepatocytes were detached on day 8 and died (Figure 1C). Hepatocytes regained their growth around day 10 and then kept up slow but steady multiplications for at least 49 days, resulting in the increase in the size of the colonies (Figure 1D). It was noticed that a few nonparenchymal cells appeared around the hepatocyte clusters at 2 days (Figure 1A) and then gradually increased their numbers (Figure 1, B, C, and D). At 49 days, hepatocytes in some parts of colonies piled up in two to three layers (Figure 1D). This is not a result of transformation but their normal characteristic, because it has been known that cells including hepatocytes constitute a three-dimensional tissue-like structure in the presence of vitamin C.[13] Most hepatocytes in the colonies were mononucleate and small in size, having a mean diameter of approximately 20 $\mu$m.

It was found that the rate of growth of hepatocytes varied among clusters in a marked area. The growth curve of hepatocytes was obtained from five different cultures (Figure 2A), which shows the average

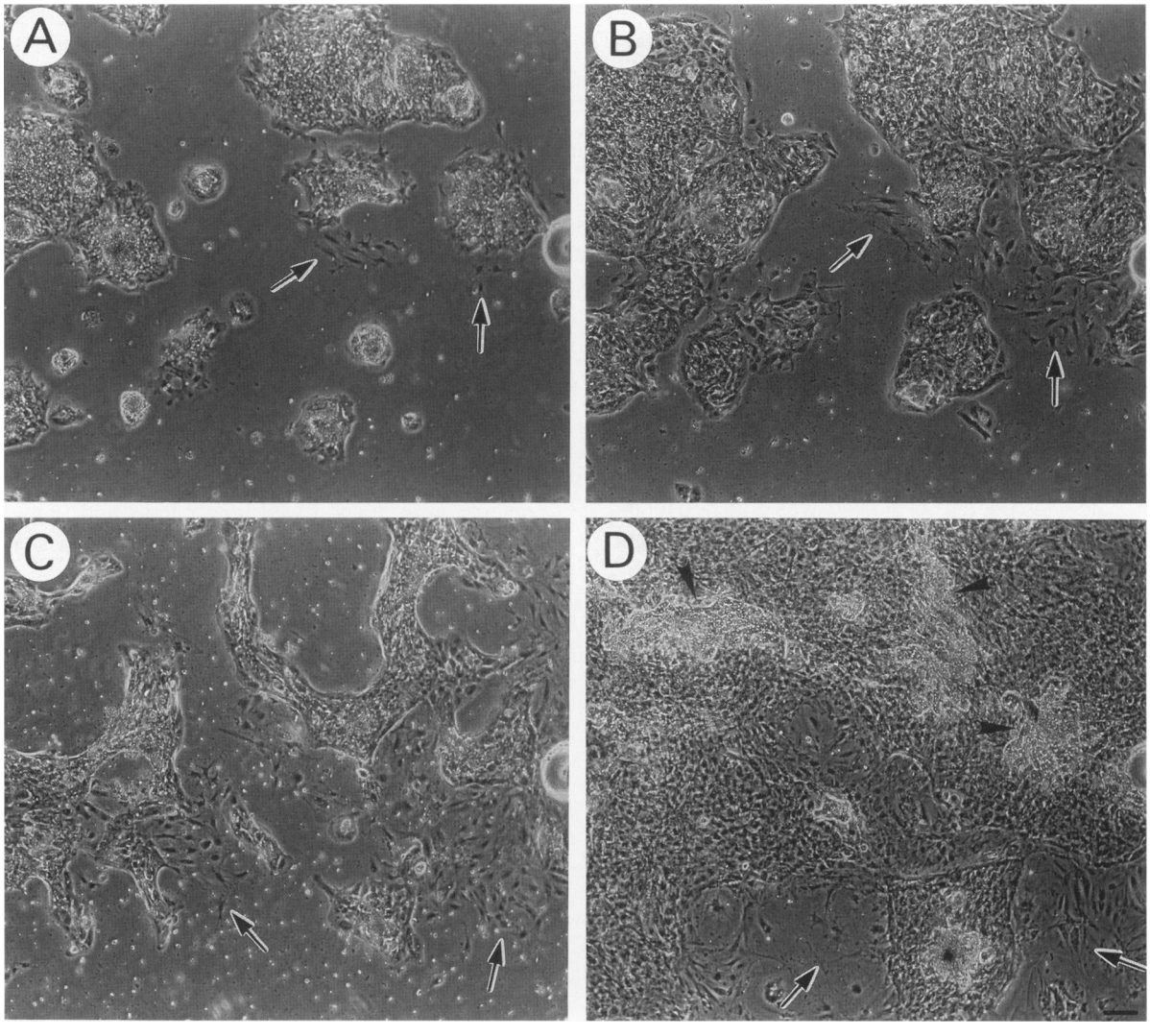



**Figure 1.** *Phase-contrast microscopic observations of hepatocyte clusters. Hepatocytes of primary cultures were detached at 4 days with the aid of EDTA and obtained as clusters. They were re-plated in new dishes in Dulbecco's modified Eagle's medium containing 10% FBS, 10 ng/ml EGF, 10 mmol/L nicotinamide, and 0.2 mmol/L Asc-2P. DMSO was added on day 4 of culture. The same field of a dish was observed through 49 days of culture.* **A**, *day 2;* **B**, *day 5;* **C**, *day 8;* **D**, *day 49.* **Arrows** *indicate nonparenchymal cells.* **Arrowheads** *in* **D** *represent hepatocytes that piled up in two to three layers. Bar, 100 μm.*

growth rate of hepatocytes in the clusters. The curves were similar among five determinations and showed that the number of hepatocytes increased approximately 3.4-fold on average during 32 days (from day 9 to day 41). The most replicative culture showed a 6-fold increase during this period. Hepatocytes in three dishes of the five cultures were allowed to grow up to 85 days. They increased approximately 4-fold in number. The increase in cell number was ascribed to the multiplication of hepatocytes but not to that of nonparenchymal cells, because all of the cells covering colonies were positive for albumin, $\alpha_1$-AT, and Tf as described below.

BrdU-labeling indexes were determined for two independent cultures. Hepatocytes showed high indexes in early days up to approximately 20 days (34, 48, 45, and 42% at days 5, 10, 16, and 20, respectively). The labeling then dropped to 9% at 30 days. Steady labeling at this level continued thereafter up to 63 days (6%). The index became 2% at 89 days. In accordance with changes of BrdU-labeling index, cells with mitotic figures were also often found in the early days of the culture up to 20 days (0.2%) and then decreased.

## *Characterization of Cells*

Hepatocytes at 4 days in the primary culture expressed hepatocytic phenotypes such as albumin, Tf, CK8, and CK18 but not $\alpha_1$-AT (data not shown).

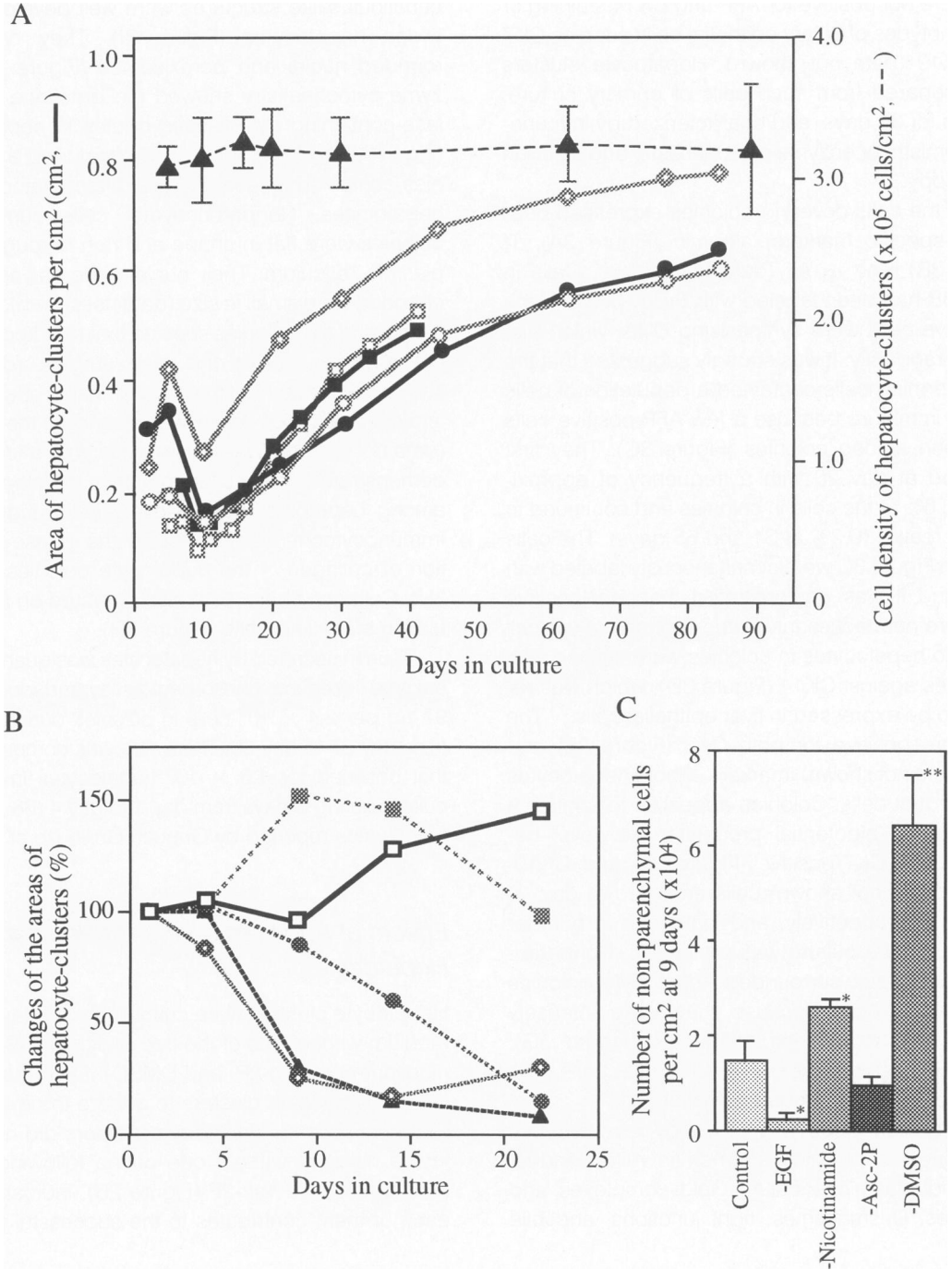



**Figure 2. A:** *Growth of hepatocytes. Cultures were carried out as in Figure 1. Hepatocyte clusters in the marked field of five independent dishes were photographed periodically through 41 days or 85 days by a phase-contrast microscope. Growth of hepatocytes in clusters is expressed as changes in area of the clusters, because the hepatocyte density (▲) did not change significantly during culture up to 89 days. Cell density was determined as detailed in Materials and Methods. Vertical bars represent standard errors of the mean of 10 determinations.* **B:** *Effects of additives on the growth of hepatocyte clusters. Hepatocyte clusters were cultured as in Figure 1 in the medium without one of the four additives (EGF, nicotinamide, Asp-2P, and DMSO). Each experiment contained three dishes in which the areas of the hepatocyte clusters were determined as a measure of hepatocyte growth as in* **A**. *Areas of the hepatocyte clusters were quantified on phase-contrast photomicrographs taken at days indicated. Areas of the clusters were obtained for each experiment by summing up those of three dishes. We noticed that the removal of each of the four additives did not affect significantly the size of hepatocytes. Relative values are shown making the area on day 1 100%.* □, *in the presence of all additives;* ▲, *without EGF;* ●, *without nicotinamide;* ◇, *without Asp-2P;* ▩, *without DMSO.* **C:** *Nonparenchymal cells were counted at 9 days in cultures shown in* **B**. *Vertical lines represent standard errors of the mean of three determinations. Significantly different from control by Student's t test:* $^{**}P < 0.01$; $^{*}P < 0.05$.

They were not positive for AFP and CK14 staining or for phenotypes of biliary epithelial cells such as CK7 and CK19 (data not shown). Hepatocyte clusters were prepared from such cells of primary culture, cultured for 30 days, and characterized by immunocytochemistry or enzyme cytochemistry and electron microscopy.

All of the cells covering colonies expressed hepatocyte-specific markers, albumin (Figure 3A), Tf (Figure 3B), and $\alpha_1$-AT (data not shown). Cells in Figure 3B had been labeled with BrdU. Some of the Tf-positive cells were synthesizing DNA, which distributed randomly. It was strongly suggested that the colony-forming cells contained a population of cells that are immature because a few AFP-positive cells were seen among colonies (Figure 3C). They first appeared at day 20 with a frequency of approximately 0.6% of the cells in colonies and continued to exist thereafter (0.2% at 31 and 65 days). The cells shown in Figure 3C were simultaneously labeled with BrdU, and it was demonstrated that AFP-positive cells were not necessarily in the S phase of the cell cycle. No hepatocytes in colonies were stained with antibodies against CK14 (Figure 3D), which was reported to be expressed in liver epithelial cells.[23] The cells were positive for both CK8 (Figure 3E) and CK18 (data not shown), markers of both hepatocytes and bile duct cells. Colonies appeared to contain a population of bipotential progenitor-like cells, because a few CK7-positive (Figure 3F) and CK19-positive (data not shown) cells emerged at day 10 and day 5, respectively, and increased in number gradually as the culture was prolonged. Nonparenchymal cells that surrounded hepatocyte colonies were stellate cells because they were intensely stained with anti-desmin antibodies (Figure 3G). Nonspecific esterase, a marker of Kupffer cells, was not detected in the nonparenchymal cells.

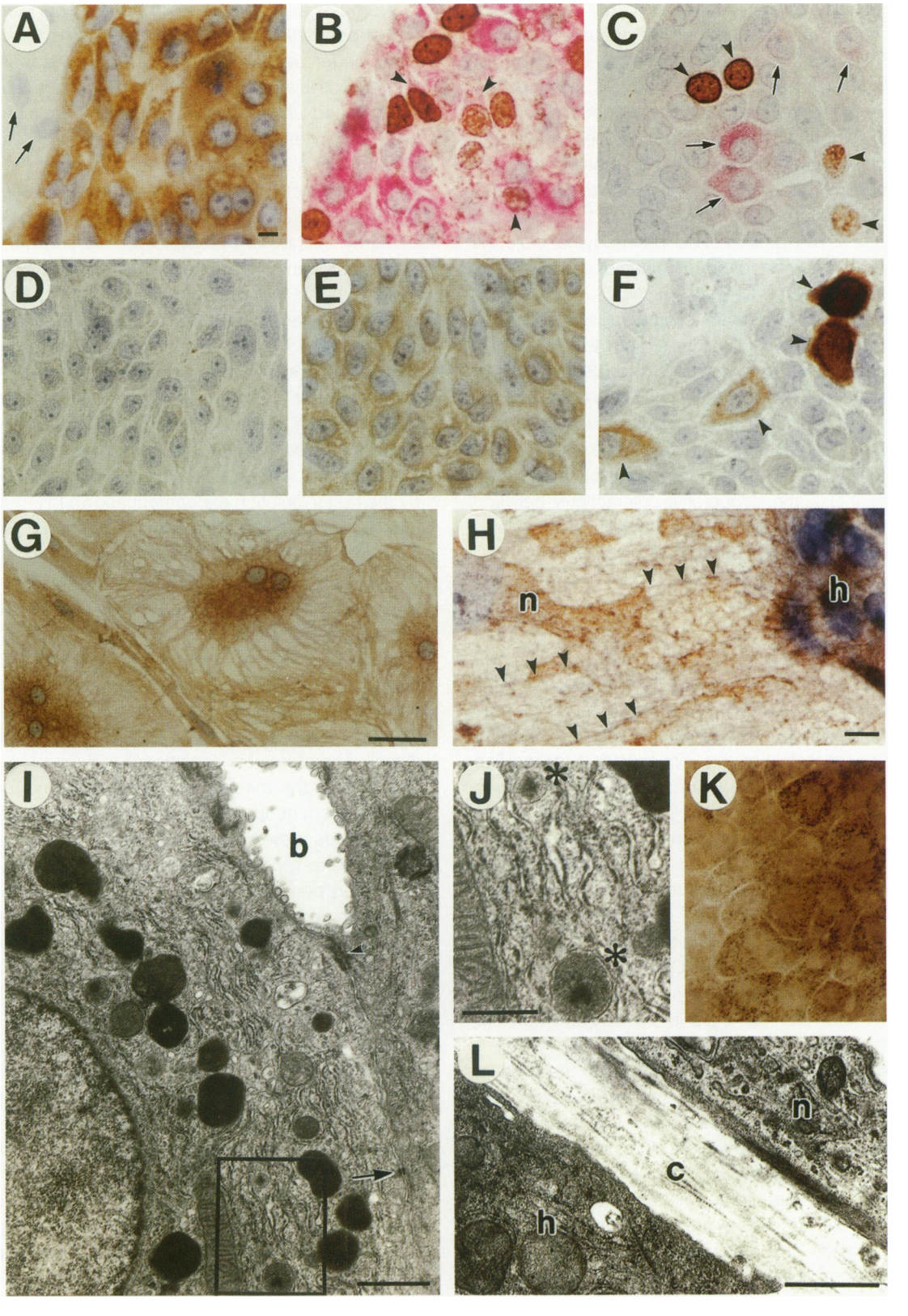
Transmission electron microscopy revealed that hepatocytes in colonies are rich in mitochondria, rough endoplasmic reticulum, Golgi complexes, and lysosomes. Desmosomes, tight junctions, and bile canaliculus-like structures were well developed between hepatocytes (Figure 3I). They contained rounded nuclei and peroxisomes (Figure 3J). Enzyme cytochemistry showed the presence of catalase-containing cytoplasmic bodies in hepatocytes (Figure 3K). Therefore, it was concluded that colonies contained ultrastructurally differentiated normal hepatocytes. Nonparenchymal cells surrounding colonies were flat in shape and rich in rough endoplasmic reticulum. Their nuclei were flat and mitochondria were small in size (data not shown). Vertical sections of the colonies showed that hepatocytes did not directly contact the dish surface (data not shown). Nonparenchymal cells (most probably stellate cells) and cell debris were found on the surface (data not shown). Transmission electron microscopy demonstrated that collagen fibers developed well among hepatocytes and stellate cells (Figure 3L). Immunocytochemistry revealed the dense deposition of collagen in the hepatocyte colonies (Figure 3H). Collagen fibers were also detected on the population of stellate cells (Figure 3H).

Albumin secreted by hepatocytes was quantified by enzyme-linked immunosorbent assay and found to be 97 $\mu$g per $2.4 \times 10^6$ cells in colonies during 3 days from day 27 to day 30. This value was comparable to that obtained for $1.5 \times 10^6$ hepatocytes in primary culture during 3 days from day 1 to day 4 (88 $\mu$g) and to the value reported by Guguen-Guillouzo et al.[24]

## *Effects of Additives on the Proliferation of Hepatocytes*

Hepatocyte clusters were cultured for 22 days in the medium without one of the five additives (FBS, EGF, nicotinamide, Asc-2P, and DMSO). FBS was essential for hepatocyte clusters to adhere to new dishes (data not shown). Hepatocyte clusters did not grow in the medium without one of the following: EGF, nicotinamide, or Asc-2P (Figure 2B), indicating that each of them contributes to the successful prolifer-

**Figure 3.** *Characterization of cells in colonies by immunocytochemistry, enzyme cytochemistry, transmission electron microscopy. Cells were cultured for 30 days as in Figure 1.* **A:** *Albumin immunostaining. Cells with brown cytoplasm are positive cells. Cells indicated by* **arrows** *are nonparenchymal cells. Bar, 10 μm.* **B:** *Double immunostaining of BrdU and Tf. BrdU was incorporated for 48 hours before fixation. Cells with brown nuclei and with red cytoplasm are positive for BrdU and Tf, respectively. Some hepatocytes are doubly stained, representatives of which are indicated by* **arrowheads**. **C:** *Double immunostaining of BrdU and AFP. AFP-containing cells are those with red cytoplasm and are scattered among colonies (***arrows***) and are not necessarily labeled with BrdU (brown nuclei,* **arrowheads**). **D:** *CK14 immunostaining. No positive cells are seen.* **E:** *CK8 immunostaining. All of the cells in the colonies express CK8.* **F:** *CK7 immunostaining. A few cells in the colonies are positive (***arrowheads**). **G:** *Desmin immunostaining. Nonparenchymal cells surrounding colonies are positive for desmin, which is shown as brown fibers in the cytoplasm. Bar, 50 μm.* **H:** *Type I collagen immunostaining. Both hepatocytes (h) and nonparenchymal cells (n) are positively stained. Some of the prominent extracellular collagen fibers are indicated by* **arrowheads**. *Bar, 10 μm.* **I:** *Electron microscopic observation of hepatocyte clusters. Bile canaliculus-like structures (b) often develop between hepatocytes. Tight junctions marked by* **arrowheads** *and desmosomes marked by* **arrows** *contribute to the formation of such structures. Bar, 1 μm.* **J:** *High magnification of* **I**. *The portion marked with a* **rectangle** *in* **I** *is enlarged. Hepatocytes contain peroxisomes (*•*). Bar, 0.5 μm.* **K:** *Catalase staining. More than 80% of cells in the colonies contain catalase-positive cytoplasmic bodies.* **L:** *Electron microscopic observation of collagen fibers. Collagen fibers (c) are deposited between hepatocytes (h) and nonparenchymal cells (n). Bar, 1 μm. Magnification of* **B** *to* **F** *and* **K** *is identical to that of* **A**.


A
B
C
D
E
F
G
H
n
h
I
b
J
K
L
n
c
h

ation of hepatocytes. Deprivation of EGF and Asc-2P was more effective than that of nicotinamide, which might have some connection to the effects of removal of these additives on changes in nonparenchymal cells (stellate cells) as described below. On the other hand, hepatocytes in the medium without DMSO increased much more than those in the control medium at 9 and 14 days. However, colonies began to detach from dishes around 15 days probably due to overgrowth of nonparenchymal cells. As a result, the number of hepatocytes decreased at 22 days (Figure 2B).

The number of nonparenchymal cells was also determined at 9 days of culture (Figure 2C). Growth of nonparenchymal cells decreased in EGF or Asc-2P-deprived medium as compared with the complete medium. On the contrary, medium lacking nicotinamide or DMSO increased the growth. Taking the results shown in Figure 2, B and C, into consideration it was suggested that some cooperative interactions between hepatocytes and nonparenchymal cells (stellate cells) support the growth of hepatocyte clusters.

## *Discussion*

We attempted to develop a culture method for adult rat hepatocytes that allows the cells to undergo multiple cell divisions without losing normal differentiation phenotypes. For this aim we tried to improve both the preparation of hepatocytes as a starting material for cultivation and the ingredients of the culture medium. Conventionally, hepatocytes in primary culture have been used for growth experiments. The present study tried to obtain a hepatocyte population that is of relatively high growth potential from the primary culture to use as a starting material for growth study. Colony-forming hepatocytes were recovered as hepatocyte clusters. Actually, we showed that cells in the clusters can undergo multiple cell divisions.

The hepatocyte culture described in the present study contains stellate cells. Their presence and growth did not affect growth curves of hepatocytes shown in Figure 2, A and B. The growth rate was determined by measuring the area of hepatocyte colonies that were fully covered with albumin-expressing cells. Most stellate cells were present and grew outside the colonies. Electron microscopy showed the presence of stellate cells at the bottom layer of piled regions of colonies. However, the number of stellate cells in the colonies was much fewer as compared with the majority of cells (hepatocytes) and did not affect the size of the colony area at all.

We have also succeeded in specifying medium that supports the continuous growth of hepatocytes for a longer period. The medium contains four key substances in addition to FBS, ie, EGF, nicotinamide, Asc-2P, and DMSO. Potent mitogenic effects of EGF on hepatocytes have been well known.[3,14,15] Nicotinamide has also been reported to prolong the viability and function of differentiated rat hepatocytes and to stimulate their replication for a long term, partly by maintaining physiological intracellular levels of the coenzyme nicotinamide adenine dinucleotide.[11] Asc-2P stimulates the synthesis of collagens of hepatocytes[13] and stellate cells.[25] We also showed that collagen fibers are well developed over stellate cells and hepatocytes, which leads us to speculate that stellate cells together with the extracellular matrix activate proliferative hepatocytes to keep their growth potential and express parenchymal functions. This speculation is further supported by the factor-deletion experiment shown in Figure 2, B and C, which suggests a good correlation between the deprivation-induced changes in number of hepatocytes and those of stellate cells. Loréal al et al[26] also observed the active accumulation of the extracellular matrix in the mixed culture of hepatocytes and stellate cells. The present study showed that the depletion of DMSO enhances the proliferation of nonparenchymal cells, which leads to the detachment of some hepatocyte colonies from culture dishes. Therefore, it is considered that the presence of DMSO in the medium is necessary to suppress overgrowth of nonparenchymal cells and to maintain hepatocytes growing for a long period. Mouse hepatocytes were reported to replicate repeatedly for a prolonged period in serum-free medium containing nicotinamide and EGF, but not vitamin C,[2] which differs from the medium we employed. This apparent discrepancy cannot be reasoned at present but might be attributed to the species difference.[1]

We have obtained a few lines of evidence that support the idea that the surviving cells contain a special population of cells, ie, committed progenitor cells. These cells are expected to be proliferative and further differentiate into maturity. As shown in the present study, the surviving cells can keep growing, continuously producing cells in the S phase of the cell cycle. They express characteristics of differentiation such as albumin, $\alpha_1$-AT, and Tf. The cells possess catalase-positive cytoplasmic bodies (peroxisomes) and form desmosomes, tight junctions, and bile canaliculus-like structures between them, indicating that they are ultrastructurally differentiated as well. Some of the surviving cells express AFP, a marker of immature hepatocytes. These cells are

scattered among hepatocyte colonies. At present, it is not clear that the cells expressing AFP represent a population of the committed progenitor cells. Hepatocyte colonies were also shown to contain a few cells that begin to express bile duct cell phenotypes such as CK19 and CK7 at approximately days 5 to 10. These cells gradually increased in number afterward. Therefore, it is suggested that the hepatocyte clusters contain a population of bipotent progenitor-like cells.

Hepatocytes that continuously proliferate for a long period did not show any characteristics of transformation. They appear quite normal both morphologically and in phenotype expressions. The expression of AFP is not an indication of transformation but an indication of the presence of a population of immature hepatocytes, because AFP-expressing cells were few and distributed randomly among colonies.

## *Acknowledgments*

The authors thank Dr. T. Mitaka and Dr. T. Karikusa for their helpful advice on the primary culture technique and Dr. Y. Shigenaka for kindly giving us the chance to use an electron microscope in his laboratory. The authors are grateful to Ms. M. Hiromitsu and Y. Terao for their excellent assistance in preparing the manuscript.

## *References*

1. Sawada N, Lee G-H, Mochizuki Y, Ishikawa T: Active proliferation of mouse hepatocytes in primary culture under defined conditions as compared to rat hepatocytes. Jpn J Cancer Res 1988, 79:983–988
2. Wu JC, Merlino G, Cveklova K, Mosinger B, Fausto N: Autonomous growth in serum-free medium and production of hepatocellular carcinomas by differentiated hepatocyte lines that overexpress transforming growth factor $\alpha$. Cancer Res 1994, 54:5964–5973
3. McGowan JA, Strain AJ, Bucher NLR: DNA synthesis in primary cultures of adult rat hepatocytes in a defined medium: effects of epidermal growth factor, insulin, glucagon, and cyclic-AMP. J Cell Physiol 1981, 108:353–363
4. Nakamura T, Nawa K, Ichihara A: Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem Biophys Res Commun 1984, 122:1450–1459
5. Nakamura T, Teramoto H, Tomita Y, Ichihara A: L-Proline is an essential amino acid for hepatocyte growth in culture. Biochem Biophys Res Commun 1984, 122: 884–891
6. Houck KA, Zarnegar R, Muga SJ, Michalopoulos GK: Acidic fibroblast growth factor (HBGF-1) stimulates DNA synthesis in primary rat hepatocyte cultures. J Cell Physiol 1990, 143:129–132
7. Mitaka T, Sattler CA, Sattler GL, Sargent LM, Pitot HC: Multiple cell cycles occur in rat hepatocytes cultured in the presence of nicotinamide and epidermal growth factor. Hepatology 1991, 13:21–30
8. Yamamoto N, Imazato K, Masumoto A: Growth stimulation of adult rat hepatocytes in a primary culture by soluble factor(s) secreted from nonparenchymal liver cells. Cell Struct Funct 1989, 14:217–219
9. Fujinaga Y, Morozumi N, Sato K, Tokumitsu Y, Fujinaga K, Kondo Y, Ui M, Okajima F: A pertussis toxin-sensitive GTP-binding protein plays a role in the G0-G1 transition of rat hepatocytes following establishment in primary culture. FEBS Lett 1989, 245:117–121
10. Paine AJ, Hockin LJ, Legg RF: Relationship between the ability of nicotinamide to maintain nicotinamide-adenine dinucleotide in rat liver cell culture and its effect on cytochrome P-450. Biochem J 1979, 184:461–463
11. Inoue C, Yamamoto H, Nakamura, T, Ichihara T, Okamoto H: Nicotinamide prolongs survival of primary cultured hepatocytes without involving loss of hepatocyte-specific functions. J Biol Chem 1989, 264:4747–4750
12. Bissell DM, Guzelian PS: Ascorbic acid deficiency and cytochrome P-450 in adult rat hepatocytes in primary monolayer culture. Arch Biochem Biophys 1979, 192: 569–576
13. Senoo H, Tsukada Y, Sato T, Hata R: Co-culture of fibroblasts and hepatic parenchymal cells induces metabolic changes and formation of a three-dimensional structure. Cell Biol Int Rep 1989, 13:197–206
14. Bucher NLR, Patel U, Cohen S: Hormonal factors and liver growth. Adv Enzyme Regul 1978, 16:205–213.
15. Eckl PM, Whitcomb WR, Michalopoulos G, Jirtle RL: Effects of EGF and calcium on adult parenchymal hepatocyte proliferation. J Cell Physiol 1987, 132:363–366
16. Isom HC, Secott T, Georgoff I, Woodworth C, Mummaw J: Maintenance of differentiated rat hepatocytes in primary culture. Proc Natl Acad Sci USA 1985, 82:3252–3256
17. Staecker JL, Sattler CA, Pitot HC: Sodium butyrate preserves aspects of the differentiated phenotype of normal adult rat hepatocytes in culture. J Cellular Physiol 1988, 135:367–376
18. Mitaka T, Sattler GL, Pitot HC, Mochizuki Y: Characteristics of small cell colonies developing in primary cultures of adult rat hepatocytes. Virchows Arch B Cell Pathol 1992, 62:329–335
19. Seglen PO: Preparation of isolated rat liver cells. Methods Cell Biol 1976, 13:29–83
20. Kreamer BL, Staecker JL, Sawada N, Sattler GL, Hsia MTS, Pitot HC: Use of a low-speed isodensity Percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. In Vitro 1986, 22:201–211
21. Li CY, Lam KW, Yam LT: Esterases in human leukocytes. J Histochem Cytochem 1973, 21:1–12
22. Goldfischer S, Essner E: Peroxidase activity in peroxisomes (microbodies) of acatalasemic mice. J Histochem Cytochem 1970, 18:482–489

23. Bisgaard HC, Nagy P, Ton PT, Hu Z, Thorgeirsson SS: Modulation of keratin 14 and $\alpha$-fetoprotein expression during hepatic oval cell proliferation and liver regeneration. J Cell Physiol 1994, 159:475–484
24. Guguen-Guillouzo C, Clément B, Baffet G, Beaumont C, Morel-Chany E, Glaise D, Guillouzo A: Maintenance and reversibility of active albumin secretion by adult rat hepatocytes co-cultured with another liver epithelial cell type. Exp Cell Res 1983, 143:47–54
25. Senoo H, Hata R: Extracellular matrix regulates and L-ascorbic acid 2-phosphate further modulates morphology, proliferation, and collagen synthesis of perisinusoidal stellate cells. Biochem Biophys Res Commun 1994, 200:999–1006
26. Loréal O, Levavasseur F, Fromaget C, Gros D, Guillouzo A, Clément B: Cooperation of Ito cells and hepatocytes in the deposition of an extracellular matrix *in vitro*. Am J Pathol 1993, 143:538–544