TABLE I. Efficiency of the Recovery and the Ratio of the Attachment of Isolated SH Colonies

	Average ± SD (%)
The efficiency of the recovery	81.93 ± 5.78
The ratio of the attached colonies	78.41 ± 2.43

Cells were cultured in DMEM supplemented with 10% FBS, 10 mM nicotinamide, 1 mM Asc2P, and 10 ng/ml EGF. One percent of DMSO was added to the medium from day 4. When SHs proliferated and formed colonies consisting of about 10–40 cells (8–12 days after plating), the number of colonies per viewing area was counted under a light microscope (10 × 10). One hour after replating, the number of colonies per viewing area was counted under a microscope (10 × 10). The efficiency of the recovery of the colonies was calculated and expressed as a percentage of the total number of replated colonies at 1 h per that of recovered colonies. One day after replating, the number of attached colonies per viewing area was counted again. The ratio of the attached colonies was expressed as a percentage of the total number of colonies at 1 h per that of attached colonies at day 1. More than 30 fields per dish were counted, three dishes were examined per experiment were performed, and three independent experiments were carried out.

Nuclei of large and rising/piled-up cells in the colonies were positive for HNF6 (Fig. 1K,L) and C/EBPα (Fig. 1N,O).

Replating of SH Colonies

To enrich SHs, we collected SH colonies from dishes and replated them on new ones. Table I shows the ratios of the recovery and the attachment on dishes of SH colonies. About 82% of SH colonies were recovered from the dishes and, 1 day after replating, about 78% of the colonies attached on the new dishes. Most cells in the colonies attached on the dish could proliferate and were maintained in a monolayer. As shown in Figure 2, the average area of SH colonies and the number of the cells per colony were about three and five times larger at day 9 after replating than at day 1, respectively.

Effect of Matrigel Overlay on SH Colonies

In the dishes treated with Matrigel SH colonies dramatically changed shape within several days (Fig. 3D–F). SHs in the colony became large and rose on the colony, and BC-like structures were observed between the cells (Fig. 3E,F). Although the colonies covered with Matrigel did not rapidly expand, piled-up cells gradually formed plate-like structures and slowly extended to the gels. However, in the region that Matrigel did not cover them, SHs continued to proliferate. Parts of colonies covered with Matrigel sometimes formed spheroid-like structures (Fig. 7I). Perpendicular sections of the colony treated with Matrigel are shown in Figure 3G,H. The photos show the moment that a flattened leading edge of a cell may extend upward along Matrigel. The colony covered with Matrigel consisted of large, tall cells and had a multilayered structure. Examination of the cells by TEM showed that they were cuboidal and/or rectangular and appeared to be MHs that possessed many mitochondria, peroxisomes with a crystalline nucleoid, and glycogen granules (Fig. 3I). In addition, BC structures were well developed between the cells.

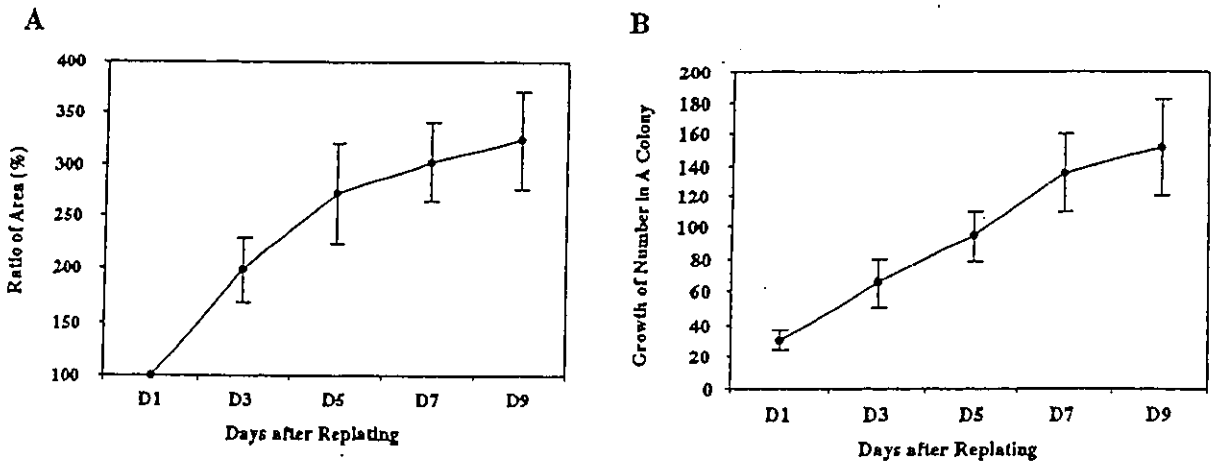
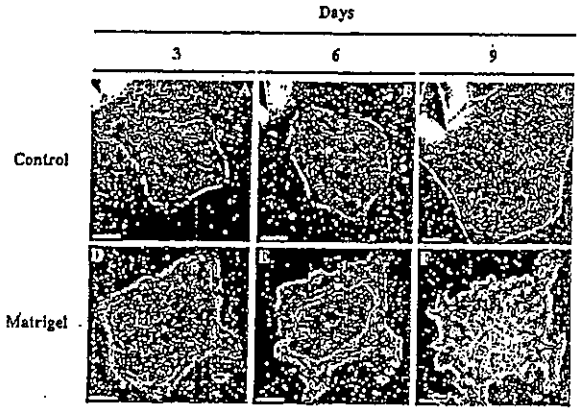


Fig. 2. Growth capacity of replated colonies. Growth of colonies replated on the dishes (A) and the numbers of the cells in colonies (B). The area of the colonies digitally recorded was measured and the number of cells in the colony was counted. More than 20 colonies per dish and three dishes per experiment were analyzed. The area of the colonies at day 1 after replating is shown as 100%. Bars show the average ± SD of three independent experiments.



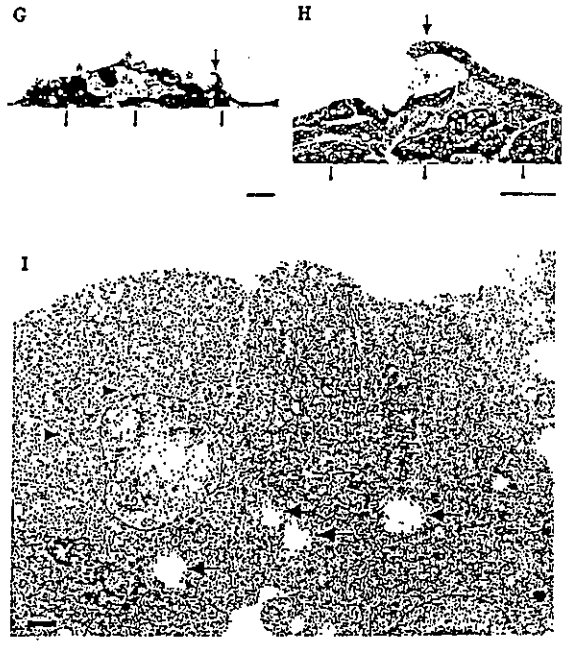


Fig. 3. Morphological changes of the colonies treated with Matrigel. Isolated colonies were plated on dishes and cultured in the medium without 10% FBS. Eleven days after replating, the cells were treated with 500 μg/ml of Matrigel (D–F). Control, no treatment (A–C). Arrows in (E) and (F) show BCs reformed between the cells. All photos are of the same magnification. Scale bar, 50 μm. Perpendicular views of the colony 4 days after Matrigel treatment. The semithin sections were stained with toluidin blue (G,H). Arrowheads show the dish surface. Amorphous materials (asterisks) are Matrigel. The flattened leading edge of a cell extends upward along the Matrigel (arrow). A colony covered with Matrigel consists of mutilayered large cells. Scale bars, 20 μm. The electron micrograph of a perpendicular section of a colony 8 days after Matrigel treatment (I). The cells possess many mitochondria, rough endoplasmic reticulum, peroxisomes with a crystalline nucleoid (arrowheads), and glycogen granules (white stars). Well developed BCs are observed between the cells (arrows). Scale bar, 200 nm.

Production of Serum Proteins by Matrigel Overlay

We examined the production of several hepatic proteins in the cells treated with or without Matrigel. As shown in Figure 4A, albumin secretion into culture medium dramatically increased after the cells were treated with Matrigel. The secretion of albumin by the cells treated with Matrigel reached about 2 μg/ml/h at day 23 and the rate was about six times larger than that in the control. Western blot analysis of the medium (Fig. 4B) and the cells (Fig. 4C) showed that the production of albumin, Tf, α_1-AT, and fibrinogen increased in the cells treated with Matrigel.

Expression of LETFs in the SH Colonies Treated with Matrigel

To examine the expression of HNF1α, HNF3α, HNF4α, HNF6, C/EBPα, and C/EBPβ proteins, we separated nuclei from the cells and the proteins extracted from the nuclei were analyzed by Western blotting (Fig. 4D). When cells were treated with Matrigel, the expression of HNF4α, HNF6, C/EBPα, and C/EBPβ proteins gradually increased, whereas the expression of HNF1α was reduced. In spite of the existence of Matrigel, HNF3α expression gradually increased with time in culture. On the other hand, without Matrigel, HNF1α was strongly expressed in the cells, whereas HNF3α and HNF4α proteins were faintly expressed. Expression of HNF6 and C/EBPα was also detected in the late phase of the culture. However, the amounts of the expression were quite small compared to those in the cells with Matrigel. The expression of HNF4α and C/EBPβ proteins did not change with time in culture.

TO and SDH Expression in SHs Treated with Matrigel

As shown in Figure 5A, the production of TO protein gradually increased in the cells with Matrigel and its synthesis was not clearly enhanced by the addition of both glucagon and dexamethasone. On the other hand, SDH protein was not detected in the cells throughout the

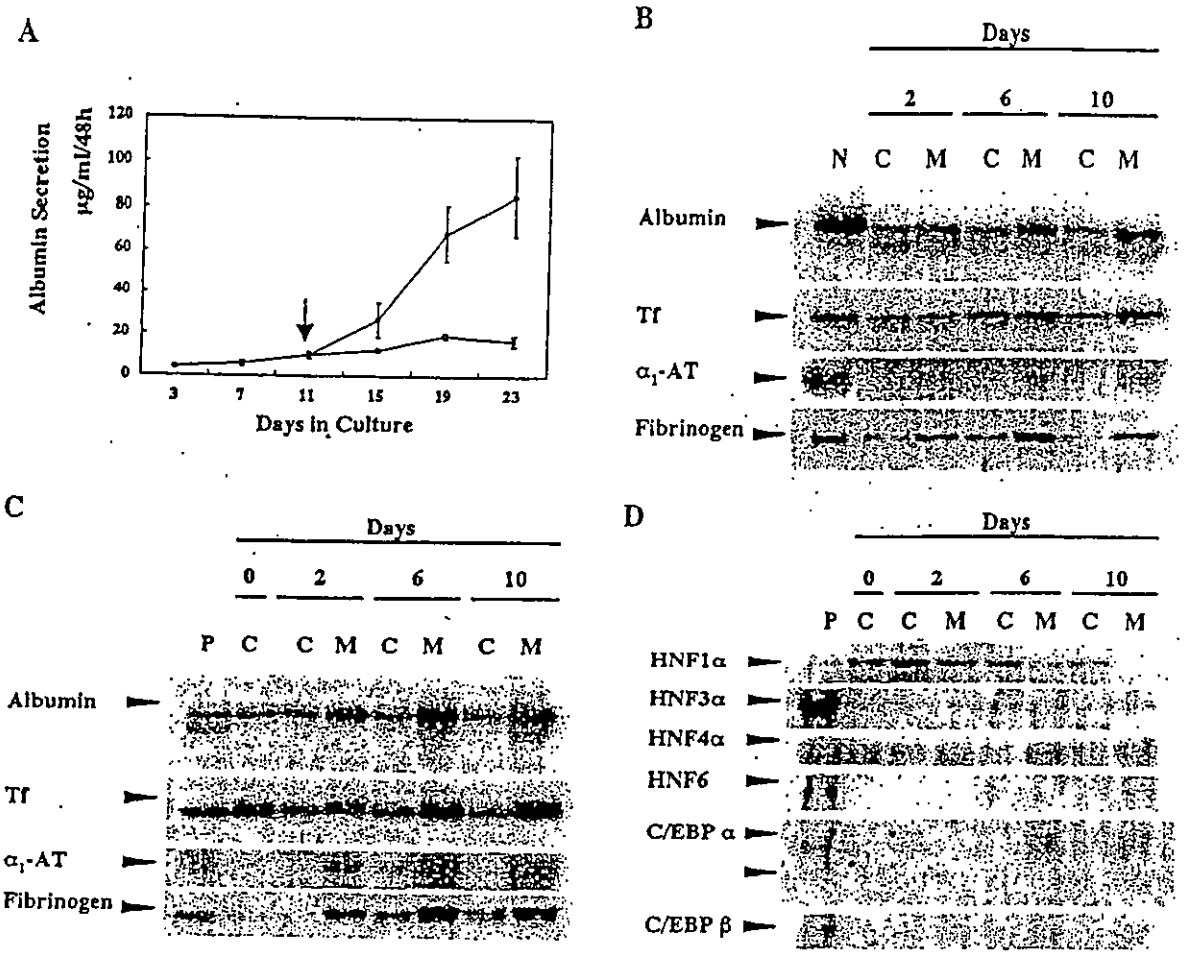


Fig. 4. (A) Albumin secretion of the cells treated with Matrigel. After replating, the cells were cultured in the medium without 10% FBS for 11 days and treated with (●) or without 500 μg/ml Matrigel (○). An arrow shows the time of the treatment. Albumin concentration in the medium was measured by ELISA. The points show the average ± SD. Western blot analysis for albumin, Tf, α_1-AT, and fibrinogen of the medium (B) and cells (C) treated with Matrigel. Isolated SH colonies were plated on collagen-coated dishes and cultured in the medium without 10% FBS. Eleven days after replating, the cells were treated with (M) or without (C) Matrigel (500 μg/ml). Samples (cells 20 μg/lane; medium, 1 μl/lane) were separated by 7.5% or 10% SDS–PAGE. P, primary MHs at 3 h after plating; N, normal serum or plasma (× 1,000 dilution with PBS). (D) Western blot analysis for HNF1α, HNF3α, HNF4α, HNF6, C/EBPα, and C/EBPβ proteins in the cells treated with Matrigel. Isolated SH colonies were plated on collagen-coated dishes cultured in the medium without 10% FBS. Eleven days after replating, the cells were treated with (M) or without (C) Matrigel (500 μg/ml). Samples (20 μg/lane) were separated by 10% SDS–PAGE. P, primary MHs at 3 h after plating.

culture, in spite of the treatment with Matrigel. However, dramatic induction was observed in the cells treated with Matrigel when the hormones were added to the culture medium. As shown in Figure 5B, a small amount of TO mRNA was expressed in the control during the culture, whereas the expression clearly increased in the cells treated with Matrigel. Addition of hormones could not induce the TO mRNA in the cells with or without Matrigel. On the other hand, no expression of SDH mRNA was observed in the cells with or without Matrigel. However, the addition of both glucagon and dexamethasone dramatically induced its expression, especially in the cells treated with Matrigel.

Effects of Growth Factors and ECM on SHs Colonies

We examined whether similar morphological changes induced by the overlay of Matrigel occurred in the SH colonies when each component of Matrigel was added to the medium. Growth factors such as NGF (Fig. 6j), PDGF (Fig. 6k), and bFGF (Fig. 6l) could not induce alteration of SHs. However, 1 ng/ml TGF-β completely suppressed the growth of SH colonies

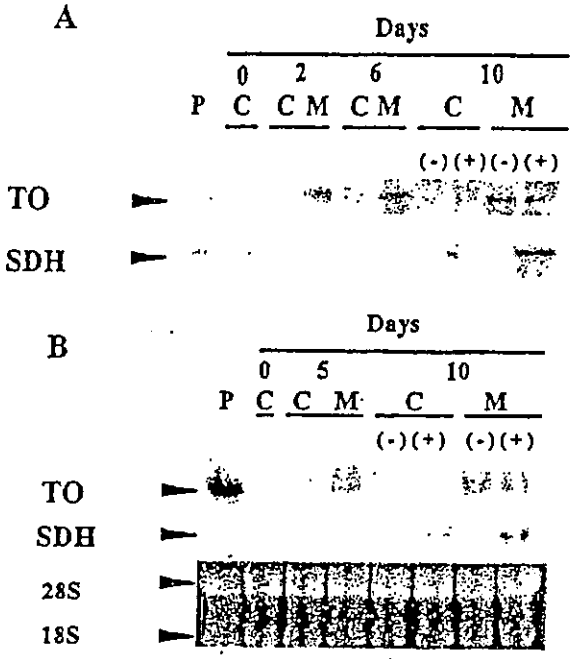


Fig. 5. Western blot (A) and Northern blot analyses (B) for TO and SDH in the cells treated with Matrigel. Isolated SH colonies were plated on collagen-coated dishes cultured in the medium without 10% FBS. Eleven days after replating, the cells were treated with (M) or without (C) Matrigel (500 μg/ml). Twelve hours before harvest, the cells were treated with (+) or without (−) both 10^{-5} M dexamethasone and 10^{-7} M glucagon. Proteins (20 μg/lane) were separated by 10% SDS–PAGE and total RNA (20 μg/lane) was loaded on 1% agarose gel. P, Primary MHs at 3 h after plating.

and many cells were detached from dishes (Fig. 6g–i). Although 0.1 ng/ml TGF-β partially suppressed the growth of SH colonies (Fig. 6d–f), less than 0.01 ng/ml TGF-β did not suppress the growth of SH colonies and no cells were detached from dishes (Fig. 6a–c). Addition of TGF-β did not make the cells pile-up and cells remained flat and small. In the dishes treated with laminin (Fig. 7a–c), type IV collagen (Fig. 7d–f), fibronectin (data not shown), the mixture of laminin and type IV collagen (Fig. 7g–i), or CG (Fig. 7j,k), SH colonies did not show any apparent morphological changes either. To examine the effects of ECM and growth factors on hepatic differentiated functions, immunoblotting for TO protein was carried out. As shown in Figure 6B, no apparent difference of TO expression was observed among the cells treated with various growth factors. Although the expression of TO protein was induced in the cells treated with laminin, type IV collagen, the mixture, or CG better than in the control cells, that of TO was much less than the cells treated with Matrigel (Fig. 7B).

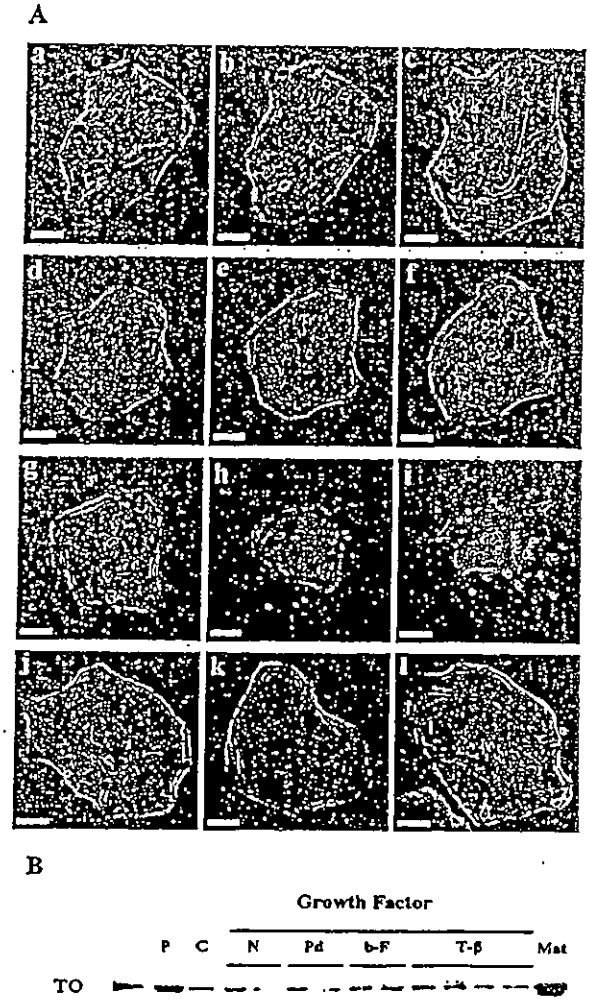


Fig. 6. (A) Phase-contrast photographs of the colonies treated with growth factors. Isolated colonies were plated on dishes and were cultured in the medium without FBS. Eleven days after replating, the cells were treated with 0.01 ng/ml TGF-β (a–c), 0.1 ng/ml TGF-β (d–f), 1 ng/ml TGF-β (g–i), 0.01 ng/ml NGF (j), 0.6 pg/ml PDGF (k), or 0.05 pg/ml bFGF (l). Fresh growth factors were added to the medium at the time of medium change. The same colony was followed and the images were recorded; at day 3 (a, d, g), day 6 (b, e, h), and day 9 (c, f, i, j, k, l) after the treatment with growth factors. All photos are of the same magnification. Scale bar, 50 μm. (B) The expression of TO protein in the cells treated with NGF, PDGF, bFGF, and TGF-β. SHs colonies were replated and cultured in the medium without FBS. Eleven days after replating, the cells were treated with growth factors. Ten days after the treatment, the cells were harvested. Proteins (20 μg/lane) were separated by 10% SDS–PAGE. Lanes show from left: primary hepatocytes at 3 h after plating (P), no treatment (control, c), 0.01 and 0.1 ng/ml NGF (N), 0.25 and 2.5 pg/ml PDGF (P), 0.005 and 0.05 pg/ml bFGF (b-F), and 0.001, 0.01, 0.1, 1 ng/ml TGF-β (T-β), and 500 μg/ml Matrigel (Mat).

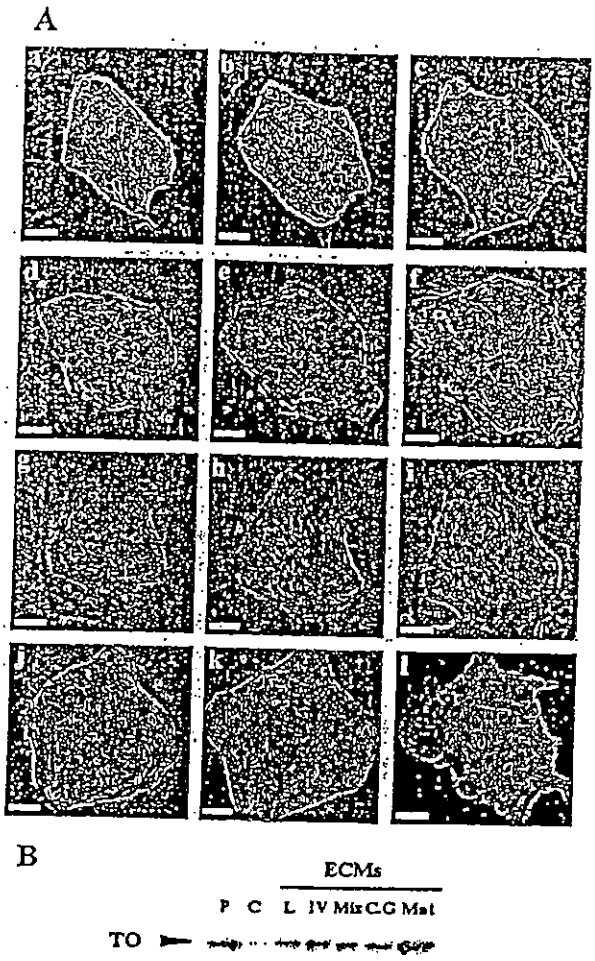


Fig. 7. (A) Phase-contrast photographs of the colonies treated with laminin, type IV collagen, a mixture of laminin and type IV collagen, and CG. Isolated colonies were plated and cultured in the medium without FBS. Eleven days after replating, the cells were treated with 300 μg/ml laminin (a–c), 150 μg/ml type IV collagen (d–f), a mixture of 300 μg/ml laminin and 150 μg/ml type IV collagen (g–i), CG (j, k), and 500 μg /ml Matrigel (l). The same colony was followed and the images were recorded; at day 3 (a, d, g), day 6 (b, e, h, j), and day 9 (c, f, i, k, l) after the treatment with ECM. All photos are of the same magnification. Scale bar, 50 μm. (B) The expression of TO protein in the cells treated with laminin, type IV collagen, a mixture of laminin and type IV collagen, CG, and Matrigel. SHs colonies were replated and cultured in medium without FBS. Eleven days after replating, the cells were treated with 300 μg/ml laminin (L), 150 μg/ml typeIV collagen (IV), a mixture of 300 μg/ml laminin and 150 μg/ml type IV collagen (Mix), CG, and 500 μg/ml Matrigel (M). Ten days after the treatment, the cells were harvested. Proteins (10 μg/lane) were separated by 10% SDS–PAGE. P, primary hepatocytes at 3 h after plating. C, control.

Growth Activity of SHs Treated with Matrigel

To examine growth activity of SHs treated with Matrigel, immunostaining, and Western blot analysis for PCNA were performed. As shown in Figure 8a, many replated SHs were positive for PCNA. This result meant that many SHs in monolayer colony actively proliferated. However, the ratio of PCNA-positive nuclei to PCNA-negative ones in a colony decreased with the expansion of the colony at the time when some cells became large hepatocytes (Fig. 8b). On the other hand, when the cells were covered with Matrigel, the number of PCNA-positive cells clearly decreased (Fig. 8d). However, the nuclei of the cells in the process of changing their shapes showed the PCNA-positivity,

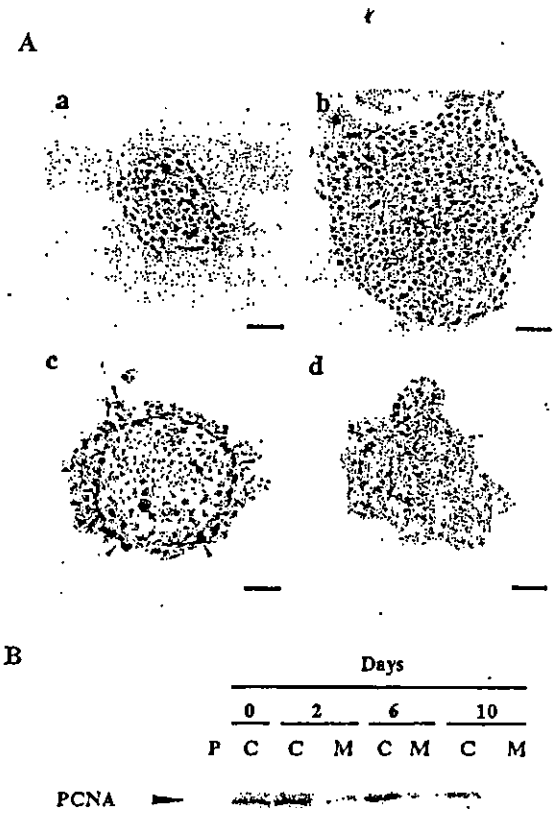


Fig. 8. (A) Immunocytochemistry for PCNA of the cells in colonies. The colonies were treated with (a, b) and without Matrigel (c, d). (a) At day 3 and (b) at day 8 after replating, (c) at day 3, and (d) at day 9 after the treatment with 500 μg of Matrigel. Darkened nuclei are positive for PCNA. The cells were counter-stained with hematoxylin. Arrows in (c) show dead cells and arrowheads show the cells that are rising and positive for PCNA. All photographs show the same magnification. Scale bars, 100 μm. (B) Western blot analysis for PCNA proteins in the colonies treated with Matrigel. Nuclear proteins (20 μg/lane) were separated by 10% SDS–PAGE. Isolated SH colonies were plated on collagen-coated dishes cultured in the medium without 10% FBS. Eleven days after replating, the cells were treated with (M) or without (C) Matrigel (500 μg/ml). P, primary hepatocytes at 3 h after plating.

whereas most piled-up cells were not stained (Fig. 8c). As shown in Figure 8B, the expression of PCNA protein was remarkably inhibited in the nuclei of the cells treated with Matrigel. In addition, with time after the treatment, the amount of the protein decreased and the expression was scarcely detected at 10 days after the treatment. This result was similar to that of the immunostaining.

DISCUSSION

Morphological Changes and Maturation of SHs

We previously showed that SHs in colonies sometimes changed shape [Mitaka et al., 1999]. In such cases NPCs invaded under the colony and the formation of BM-like structures, which might be reconstituted with ECM produced by NPCs, was observed. On the other hand, many genes of liver-specific proteins are known to be mainly regulated by LETFs and their expression seems to be correlated with hepatic maturation [Birkenmeir et al., 1989; Kuo et al., 1990; Cereghini, 1996; Uzma and Costa, 1996]. Therefore, to investigate whether the alteration of cell shape was correlated with the maturation of SHs and whether BM-like structures could induce the morphological changes of SHs, we first immunocytochemically examined the expressions of LETFs in the primary cultured cells showing various morphologies and, second, examined whether Matrigel could induce the similar morphological changes of SHs. As shown in Figure 1, the sequential expression pattern of LETFs accompanying morphological changes of SHs was observed. When SHs were maintained in a flat monolayer, staining for HNF6, C/EBPα and C/EBPβ proteins was negative in their nuclei, TO expression was quite low, and SDH expression was not induced. On the other hand, when SHs were covered with Matrigel, the cells rapidly changed shape and increased the secretion of serum proteins such as albumin, Tf, α_1-AT, and fibrinogen. In addition, the expression of LETFs could be recovered in the cells and the amounts of HNF4α, HNF6, C/EBPα, and C/EBPβ proteins at day 10 after Matrigel addition were near those of proteins in MHs, although the expression of HNF1α and HNF3α was not affected with or without Matrigel. Furthermore, TO was well expressed and SDH could be induced by hormones. Therefore, not only morphological changes from small to large/piled-up but also the synergistic induction of LETFs like HNF4, HNF6, C/EBPα, and C/EBPβ expressions may be necessary for SHs to differentiate into MHs.

Effects of ECM Components and Growth Factors in Matrigel

We showed that accumulation of ECM could result in morphological changes and the maturation of SHs [Mitaka et al., 1999]. In this experiment Matrigel could induce a change of shape of SHs. To investigate whether induction of those morphological changes was dependent on the individual components of Matrigel or mechanical stress resulting from the overlay of a gel-formed material, we examined the effects of major ECM components, a CG, and growth factors on SH colonies. These individual components did not influence the shape of the colonies shown with Matrigel. Although TGFβ (more than 0.1 ng/ml) had the ability to suppress the growth of SHs as was the case of Matrigel, no differentiation of the cells was induced. On the contrary, the higher concentration of TGFβ resulted in cell death. TGFβ in Matrigel may be not a soluble form but a form binding to ECM. Such different forms may affect the growth and death of SHs. On the other hand, although a large amount of each ECM might possibly induce the morphological changes of SH colonies, the concentration used in the present experiment did not affect the cell shapes. However, ECM could enhance the synthesis of TO proteins compared to the control. Some hepatic differentiated functions may be induced by each ECM, though the degree of the expression was lower than in the cells treated with Matrigel. Furthermore, to exclude the possibility that the morphological changes of SHs resulted from the mechanical stress caused by the overlay of gel-formed materials such as Matrigel, the colonies were covered with a thick CG. Although TO expression was enhanced in the cells, no alteration of the cell shape was observed. However, after the cells were cultured for more than 1 month, they gradually formed cystic structures under the gel (data not shown). Although we have no clear data, the gradual proliferation of NPCs bound to SH colonies and the production of ECM might have caused the cellular changes. These findings suggested that the individual components were insufficient and the combination and/or complex of ECM and growth factors might be required for morphological changes of SHs. The ideal form may be natural

BM. Further experiments will be required to form a compatible bed for the cells similar to BM.

Formation of Plate-like Structures in Matrigel

It is of interest that SHs and SH-derived large hepatocytes could proliferate and migrate in the Matrigel. As shown in Figure 8, the number of PCNA-positive cells decreased in those colonies and the speed of the colony expansion became slow. However, plate-like structures slowly elongated in Matrigel. Hepatocytes, as is the case with many other cell types, can maintain differentiated functions but do not proliferate in Matrigel [Bissell et al., 1987; Rana et al., 1994]. However, Michalopoulos et al. [1999] showed that MHs isolated from an adult rat liver could form plate-like structures within Matrigel after they were cultured on collagen-coated polystyrene beads in roller bottle for about 2 weeks. They reported that the epithelial cells on beads had characteristics of small MHs and that, after the cells on beads were implanted in Matrigel, the elongation of liver plate-like protrusions was observed. The protrusions consisted of hepatocytes showing cytoplasmic differentiation compared to the cells in the roller bottle. Those phenomena were quite similar to our observations in this experiment. Block et al. [1996] and Michalopoulos et al. [1999] suggested that MHs once lost their differentiated functions to become epithelial cells (they called them "hepatoblasts") and then could redifferentiate to MHs. However, considering our present results, it may be feasible to think that their "hepatoblasts" may be SHs because about 2% of hepatocytes isolated from the adult rat liver are estimated to be SHs [Mitaka et al., 1993].

In summary, although it is unclear how Matrigel can induce the maturation of SHs, our present data suggest that a complex gel-form of ECM like Matrigel and the formation of BM-like structures may result in the morphological changes of SHs that can induce specific LETFs such as HNF4α, HNF6, C/EBPα, and C/EBPβ. These LETFs may synergistically work for the expression of hepatic differentiated functions. Although we used growth factor-reduced Matrigel, and investigated the effects of the individual major components included in Matrigel, the effects of other possible contaminants can not be ignored. Further experiments will be necessary to examine the exact mechanisms of differentiation/maturation of SHs.

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Long-Term Culture of Primary Human Hepatocytes with Preservation of Proliferative Capacity and Differentiated Functions[1]

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Background. The aim of this study was to develop a suitable method for the prolonged culture and maintenance of human hepatocytes with preservation of both proliferative capacity and differentiated functions.

Materials and methods. Primary human hepatocytes were isolated from small pieces of liver tissue obtained from 15 patients who underwent hepatic resection. Hepatocytes were cultured in keratinocyte-stimulating factor medium supplemented with 10% human serum, 10 mM nicotinamide, 10 ng/ml epidermal growth factor, 0.5 μg/ml insulin, 10^{-7} M dexamethasone, and antibiotics. Hepatic differentiation and function were analyzed by immunocytochemistry, Western blot, ELISA, lidocaine metabolism, and urea synthesis. Ultrastructural analysis of cultured hepatocytes was performed by electron microscopy.

Results. Many primary hepatocytes were maintained for more than 56 days. Hepatocytes proliferated during the initial 14 days, and bromodeoxyuridine labeling indices were 15.2, 12.2, and 6.2% at days 5, 10, and 15, respectively. Electron micrographs of the hepatocytes at day 28 demonstrated numerous mitochondria, rough endoplasmic reticulum, large peroxisomes, and glycogen granules. Albumin secretion increased for the first 14 days and then gradually decreased thereafter but was maintained at levels greater than 2 μg/ml/h until day 56. α_1-Antitrypsin, α_1-antichymotrypsin, and ceruloplasmin production was also observed at day 56, while lidocaine metabolism and urea synthesis were maintained for a long time.

Conclusion. This hepatocyte culture method facilitates the prolonged culture of primary human hepatocytes with preservation of hepatocyte differentiation, function, and proliferative capacity.

Key Words: primary human hepatocyte; differentiation; proliferation; keratinocyte stimulating factor medium.

INTRODUCTION

The demand for the clinical use of hepatocytes is increasing. Many patients suffering from either end-stage liver disease or congenital hepatic disorders are awaiting liver transplantation but a donor organ shortage is a serious problem. It is envisaged that hepatocyte transplantation and extracorporeal liver support systems may be a future alternative therapy to orthotopic liver transplantation for such patients [1, 2]. In such therapies large quantities of healthy differentiated hepatocytes are required, with porcine hepatocytes having been utilized in earlier devices [3]. Prior to use in the clinical arena, the potential risk of disease transmission from porcine cells to humans should be stringently evaluated [4] and it is therefore important to try to provide a supply of human hepatocytes for such patients. However, it is very difficult to obtain large numbers of fresh human hepatocytes which both proliferate and maintain functions characteristic of hepatic differentiation. Although hepatocytes are well documented to exhibit a great replicative capacity *in vivo*, prolonged *in vitro* culture of primary hepatocytes [5, 6], especially human hepatocytes, with both proliferation and maintenance of liver-specific functions has not yet been established [7, 8].

Another important application of cultured human hepatocytes is as a resource for investigating drug metabolism. Most investigators analyze the induction and activity of the cytochrome P450 enzyme to evaluate the

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TABLE 1

Patient Profile

Sample	Age (years)	Sex	Disease	Weight (g)	Viability (%)
1	56	Male	Metastatic liver cancer	3.5	86
2	29	Female	Hemangioma	5.3	88
3	58	Female	Gallbladder cancer	5.8	90
4	55	Male	Metastatic liver cancer	3.4	89
5	62	Male	Metastatic liver cancer	23.8	86
6	63	Male	Metastatic liver cancer	15.8	82
7	36	Male	Hemangioma	5.5	92
8	72	Male	Gallbladder cancer	12.4	78
9	54	Female	Metastatic liver cancer	5.5	88
10	17	Male	Focal nodular hyperplasia	3.0	94
11	52	Male	Metastatic liver cancer	2.8	85
12	61	Female	Gallbladder cancer	3.9	87
13	42	Female	Metastatic liver cancer	8.6	91
14	50	Male	Metastatic liver cancer	4.2	90
15	59	Male	Metastatic liver cancer	3.3	86

function of hepatocytes [9–12] and the majority of pharmacological and toxicological studies have been performed using rodent hepatocytes. These studies were performed on short-term cultures of hepatocytes, mostly within a week, since the response of the cells to the agents rapidly decreased over time. However, the existence of important interspecies differences in all aspects of hepatic function has recently been well recognized [13–15]. Therefore, human hepatocytes are more suitable than other mammalian cells for such pharmacological and toxicological studies.

The aim of this study was to develop a suitable method for the prolonged culture of human mature hepatocytes with preservation of maintenance of proliferative capacity and liver-specific functions. In the present study we demonstrate that primary hepatocytes isolated from a small amount of liver tissue could proliferate and survive *in vitro* with maintenance of differentiated functions for about 56 days.

MATERIALS AND METHODS

Liver specimens from patients. Normal liver tissue was obtained from 15 patients who underwent hepatic resections at Kyoto University Hospital. Informed consent was obtained and the study met with the approval of the Kyoto University Ethics Committee. Nine patients had liver metastasis of colon cancer, 3 patients had cancer of the bilary system and gallbladder, 2 patients had hepatic hemangioma, and 1 patient had focal nodular hyperplasia of the liver. All patients had no evidence of concurrent hepatic viral infections. The mean age of the patients was 51.1 ± 13.9 years while the mean wet weight of the liver tissue obtained was 7.4 ± 5.8 g (Table 1).

Isolation of human hepatocytes. Hepatocytes were isolated from liver tissues by the injection method using a 10-ml disposable injector with a 27-gauge needle. Small hepatic vessels within the liver tissue were identified and Hanks' balanced salt solution (Gibco BRL, Grand Island, NY) supplemented with EGTA (Dojindo Chemical Lab, Kumamoto, Japan), 0.05% collagenase (Wako Pure Chemicals, Osaka, Japan), and 0.5% dispase (Godo Shyusei, Tokyo, Japan) was injected into these vessels. This procedure was repeated multiple times and the swollen tissue was soaked in the enzyme solution during the procedure. The isolated cells were collected into centrifuge tubes and the cell suspension was centrifuged at 50*g* for 1 min with the cell pellet being resuspended in William's E medium (WEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS; ICN Biomedicals, Inc., Aurora, IL), 0.5 μg/ml insulin (Wako Pure Chemicals), 10^{-7} M dexamethasone (Wako Pure Chemicals), and 10^5 U/L penicillin G and 100 mg/L streptomycin (Meiji Seika Co., Tokyo, Japan). The centrifugation was repeated four times. The viability of the cells was determined by trypan blue exclusion and was 87.5 ± 3.9%. Viable cells (2×10^5/ml) were plated on tissue culture plastic.

Media for human hepatocyte culture. We compared the effects of three kinds of tissue culture media upon hepatocyte survival and maintenance of hepatic function. About 24 h after plating, the medium was changed to WEM supplemented with 10% FBS (Group 1), keratinocyte-stimulating factor medium (KSFM; Gibco BRL) supplemented with 10% FBS (Group 2), or KSFM supplemented with 10% human serum (HS) (Group 3). All media contained 10 mM nicotinamide (Wako Pure Chemicals), 30 mg/L proline (Gibco BRL), 1 mM ascorbic acid 2-phosphate (Wako Pure Chemicals), 0.5 μg/ml insulin, 10^{-7} M dexamethasone, 10 ng/ml epidermal growth factor (EGF; Gibco BRL), 10^5 U/L penicillin G, and 100 mg/L streptomycin. Human serum was obtained from several healthy volunteers. The medium was changed every other day and 1% dimethyl sulfoxide (DMSO; Wako Pure Chemicals) was added to the medium from 4 days after plating.

Albumin synthesis of cultured human hepatocytes. Albumin secretion was measured by an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Albuwell II; ExoCell, Inc., Philadelphia, PA). The cultured cells were washed twice with medium without serum and then cultured for 2 h after which the tissue culture supernatant was aspirated. Samples were collected every 7 days from the same three cell culture dishes and stored at −80°C until analyzed. Five independent experiments were performed using hepatocytes from patients ages 56, 29, 58, 55, and 17 years. It is important to note that the albumin present in FBS did not cross-react with the anti-human albumin antibody utilized in the ELISA.

Labeling indices and double-fluorescence immunocytochemistry for albumin and 5-bromo-2'-deoxyuridine (BrdU). Human hepatocytes were incubated with 40 μM BrdU (Sigma Chemical Co., St. Louis, MO). After 24 h the cells were fixed with cold absolute ethanol and kept at −20°C until use. Following a rinse with PBS, the dishes were incubated at room temperature for 30 min with 2 N HCl and then

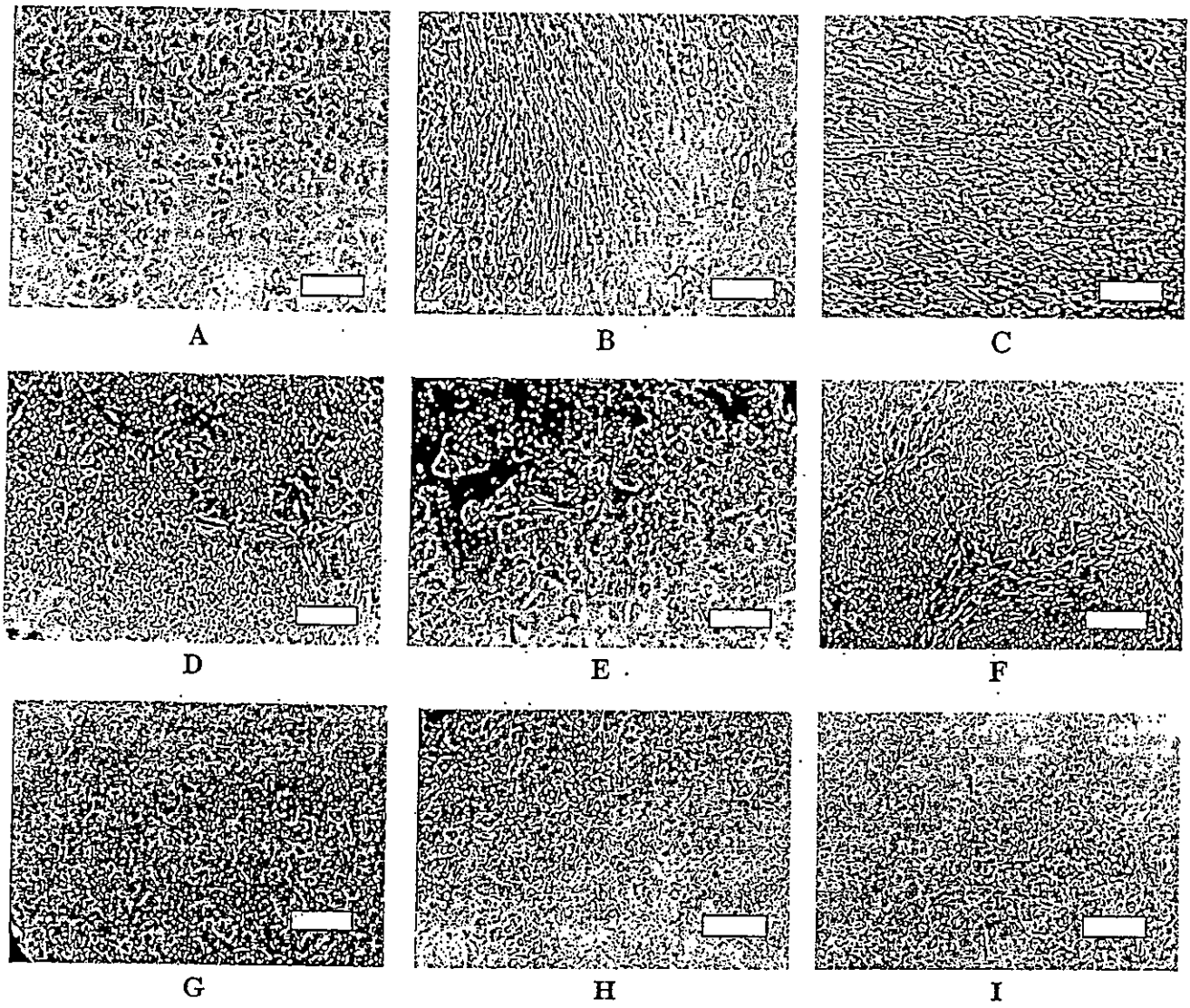

FIG. 1. Phase-contrast photographs of cultured hepatocytes. Morphological observations were performed consecutively. (A–C) Group 1: (A) day 5, (B) day 10, (C) day 15. (D–F) Group 2: (D) day 7, (E) day 28, (F) day 56. (G–I) Group 3: (G) day 7, (H) day 28, (I) day 56. All photos are the same magnification. Scale bars, 100 μm.

treated with Block Ace (Dainippon Pham. Ltd., Tokyo, Japan) for 1 h. A mouse anti-BrdU antibody (DAKO, Copenhagen, Denmark) was used as the primary antibody followed by the ABC method (Vectastain ABC Elite kit; Vector Laboratories, Inc., Burlingame, CA) with 3,3'-diaminobenzidine (DAB; Tokyo Kasei Industries, Tokyo, Japan) used as a substrate. For double immunostaining for BrdU and albumin, the cells were incubated with Block Ace and then with the anti-BrdU antibody for 1 h. After being rinsed with PBS three times, cells were incubated with Alexa^{488}-conjugated anti-mouse IgG antibody (Molecular Probes, Eugene, OR) and the dish was covered with aluminum foil for 30 min. After being washed with PBS three times, the cells were incubated with a rabbit anti-human albumin antibody (DAKO) for 1 h followed by an Alexa^{594}-conjugated anti-rabbit IgG antibody (Molecular Probes) for 30 min. The dishes were mounted with 90% glycerol containing 1 mg/ml *p*-phenylenediamine. Digital images of the fluorescence distribution in the cells were obtained using a Zeiss Axioskop 20 microscope equipped for epifluorescence (40× lens). Images were analyzed with the CELLscan System (Scanalytics, Billerica, MA). Labeled cells with BrdU-positive nuclei were counted by microscopy (magnification ×200) with 20 fields per dish being analyzed. More than 1000 cells were counted per dish and three dishes were examined per experiment. The labeling index (LI) was determined by dividing the total number of BrdU-positive cells which also exhibited positive cytoplasmic staining with albumin by the total number of albumin-positive cells. Three independent experiments were performed using hepatocytes derived from 36-, 52-, and 72-year-old patients.

Ultrastructure of cultured hepatocytes. Cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 30 min, postfixed in 2% osmium tetroxide in buffer, dehydrated by graded ethanols, and embedded *in situ* in Epon 812. Ultrathin sections were cut using a Sorvall Ultramicrotome MT-5000 (DuPont Co., Newtown, CT). The sections were stained with uranyl acetate followed by lead citrate and examined at 60 kV with a JEM transmission electron microscope (JEOL, Tokyo, Japan). Some dishes were enzyme-cytochemically stained with DAB. DAB-positive organelles were considered to be indicative of peroxisome structures.

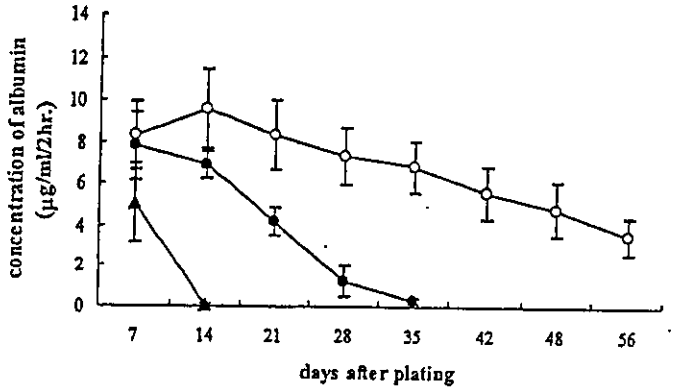

FIG. 2. Albumin concentration in hepatocyte conditioned medium (2 h) was measured by the ELISA method. Symbols: ▲, Group 1; ●, Group 2; O, Group 3. The points show the mean ± SD. Five independent experiments were performed.

Western blotting analysis. Immunoelectrophoretic blot analysis was performed by the method of Towbin *et al.* [16]. The culture medium was collected every 48 h at the time of medium replacement and centrifuged at 1×10^4 rpm for 10 min. The supernatant was kept at −80°C until analysis. After heat denaturation at 95°C for 5 min, the samples (1 μl/lane) were subjected to SDS–polyacrylamide gel electrophoresis and transferred electrophoretically to an Immobilon-P membrane (Millipore Corp., Bedford, MA). The membranes were treated with 50% Block Ace/PBS supplemented with 0.1% Tween 20 for 1 h at 37°C. Rabbit anti-α_1-antitrypsin, anti-α_1-antichymotrypsin, and anti-ceruloplasmin antibodies (Cappel, Costa Mesa, CA) were applied overnight at 4°C. After vigorous washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit Ig antibody (DAKO) as secondary antibody for 1 h. Immunoreactive bands were visualized on SuperSignal West Dura Extended Duration substrate (Pierce Chemical Co., Rockford, IL). In this experiment, cultured hepatocytes from a 50-year-old patient were used.

Urea synthesis and lidocaine metabolization. The cells were treated with 1 mM NH_4Cl (Wako Pure Chemicals) for 4 h and the urea produced by hepatocytes was measured by the dehydrogenase assay. In the present study, three independent experiments were performed using hepatocytes cultured from 36-, 52-, and 72-year-old patients. The capacity of the cells to metabolize lidocaine is an estimate of cytochrome P450 activity. One milligram per milliliter lidocaine (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was added to the culture medium and the medium was collected 4 h later. The amount of lidocaine remaining in the medium was measured by ELISA using a TDX analyzer (Dainabot, Tokyo, Japan). In the present study, three independent experiments were performed using hepatocytes derived from 42-, 50-, and 59-year-old patients.

RESULTS

Morphological Changes and Albumin Secretion by Cultured Hepatocytes

We compared the effect of two different tissue culture media upon human hepatocyte survival and function. WEM is generally used in many laboratories for the culture of primary hepatocytes while KSFM was originally used for the culture of keratinocytes and is known to inhibit the growth of fibroblastic cells.

In Group 1 (WEM), the cells maintained their polygonal shape until day 5 (Fig. 1A). However, fibroblastic cells rapidly proliferated (Fig. 1B), with a concomitant decrease in the number of hepatic cells. Eventually, the culture was dominated by fibroblastic cells (Fig. 1C). On the other hand, in Group 2 (KFSM) fibroblastic cells were scarcely observed at day 7 (Fig. 1D) and the hepatocytes maintained their polygonal shape and remained tightly adherent to each other for 28 days (Fig. 1E). The number of hepatocytes was maintained until day 14 and gradually decreased thereafter (data not shown). At day 56, the residual hepatocytes exhibited a

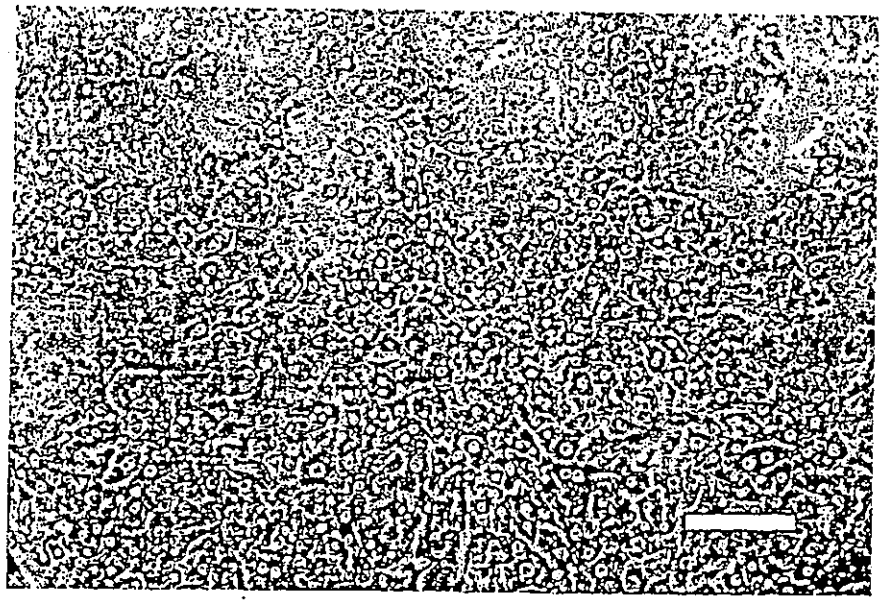

FIG. 3. Appearance of small hepatocyte-like cells. Small hepatocyte-like cells could be observed in the areas left following mature hepatocyte detachment at day 28. However, proliferation of these cells was not observed. Scale bar, 100 μm.

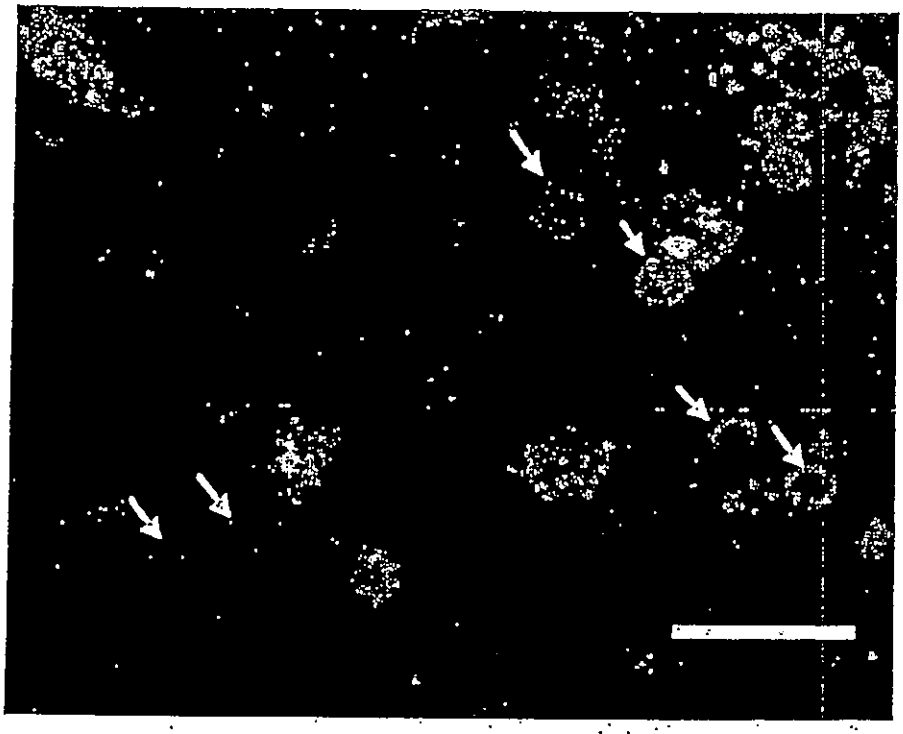

FIG. 4. Double immunocytochemical staining of bromodeoxyuridine (BrdU) and albumin. Several BrdU-positive cells (white arrows) exhibited cytoplasmic staining with albumin. This day 10 sample was cultured from a 36-year-old male patient. Scale bar, 50 μm.

colony-like appearance which comprised fibroblastic cells surrounding the hepatocytes (Fig. 1F).

We serially examined the secretion of albumin into the culture medium by hepatocytes. As shown in Fig. 2, there was a rapid decrease in albumin secretion in Group 1 by day 14. On the other hand, the hepatocytes in Group 2 maintained albumin secretion until day 28 and we therefore decided to use KSFM for further experiments.

We next examined the effect of HS upon the maintenance of hepatocyte function. In Group 3 (KSFM with 10% HS) the cells exhibited a tight adherent polygonal pattern and became a subconfluent monolayer after several days. The subconfluent monolayer was maintained for 28 days (Fig. 1H). Thereafter, cells gradually detached from the dish although many hepatocytes maintained the polygonal shape at day 56 (Fig. 1I). In addition, the growth of nonparenchymal cells (NPCs) was adequately suppressed at day 56. Albumin secretion by the hepatocytes increased until day 14 (Fig. 2) and gradually decreased thereafter although a secretion rate of more than 3 μg/ml/h was maintained for about 56 days. Although albumin secretion was dependent upon the concentration of HS and increased with the amount of serum, we found that 10% HS was maximal (data not shown).

Appearance of Small Hepatocyte-like Cells

When human hepatocytes were cultured in KSFM supplemented with 10% HS for more than 28 days, we observed cells that were morphologically similar to but smaller than mature hepatocytes. These small hepatocyte-like cells were surrounded by large hepatocytes and formed a cluster (Fig. 3). The appearance was very similar to the small hepatocyte colonies which are observed in the culture of rat hepatocytes [17].

Labeling Indices and Double-fluorescence Immunocytochemistry Staining for Albumin and BrdU

We determined the proliferation index of cultured KSFM supplemented with HS. Cells were incubated

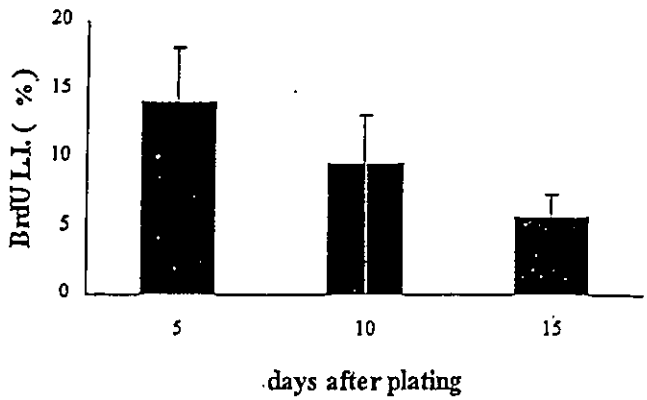

FIG. 5. Bromodeoxyuridine labeling indices (BrdU L.I.) were calculated from 1000 cultured hepatocytes from three patients: a 36-year-old male patient, a 52-year-old female patient, and a 61-year-old male patient. The points depict the means ± SD.

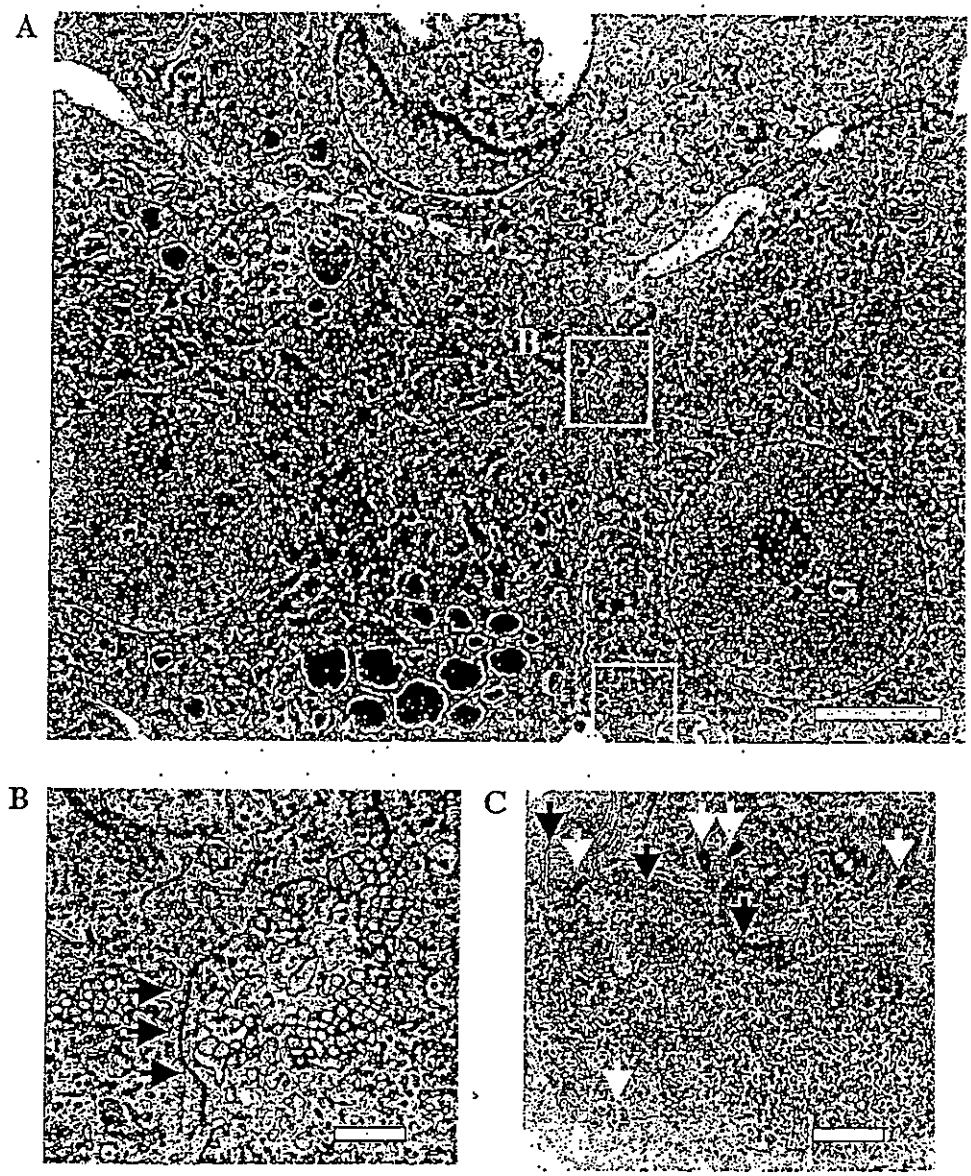

FIG. 6. Transmission electron microscopic observation of hepatocytes at day 28. The sample was taken from a 62-year-old male patient. (A) Three cells can be observed. (B) Close-up of inset B. Bile canaliculi formation is evident. The black arrows indicate a tight junction. (C) Close-up of inset C. The white asterisks indicate glycogen granules while the black arrows indicate mitochondria. The white arrows indicate peroxysome granules, which indicate that this cell is a hepatocyte. Scale bars: A, 5 μm; B and C, 500 nm.

with 40 μM BrdU for 24 h. Double immunostaining for BrdU and albumin was performed in order to demonstrate that BrdU-positive cells were hepatocytes. Most hepatocytes were strongly positive for albumin at day 10 with some albumin-positive cells staining with the anti-BrdU antibody (Fig. 4). NPCs were often BrdU positive, indicative of significant proliferation.

The LI of cultured hepatocytes was determined. As shown in Fig. 5, the mean LI were 15.2, 12.2, and 6.2% at days 5, 10, and 15, respectively. The highest LI was in cells isolated from a 36-year-old patient's liver and were 21.5, 15.3, and 7.4% at days 5, 10, and 15, respectively (data not shown).

Transmission Electron Microscopic (TEM) Observation

The ultrastructure of cultured hepatocytes was investigated by TEM. As shown in Fig. 6, the cells at day 28 exhibited numerous mitochondria, rough endoplasmic reticulum, DAB-positive peroxysomes, and glycogen granules. Bile canaliculi formation and tight junctions between hepatocytes were also evident between hepatocytes. The morphology of the cells at day 56 was comparable with that at day 28 (data not shown).

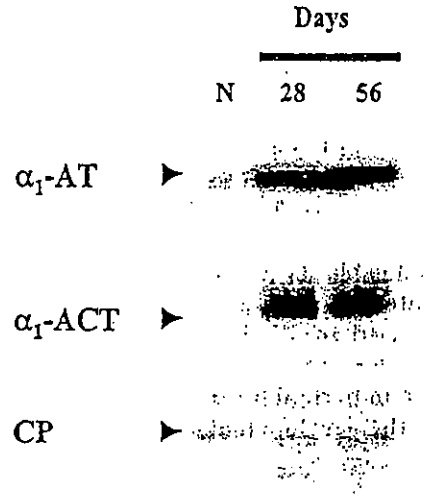

FIG. 7. Western blotting of serum proteins secreted by cultured hepatocytes. This sample was the serum-free culture medium of day 28 and day 56 hepatocytes derived from a 61-year-old patient. N, normal human serum diluted 1 to 1000; 28, supernatant at day 28; 56, supernatant at day 56; α_1-AT, α_1-antitrypsin; α_1-ACT, α_1-antichymotrypsin; CP, ceruloplasmin.

Hepatic Differentiated Functions of Cultured Hepatocytes

We examined the production of liver-derived serum proteins including α_1-antitrypsin, α_1-antichymotrypsin, and ceruloplasmin by the cultured hepatocytes. One microliter of medium underwent SDS–PAGE, and Western blot analysis was carried out. As shown in Fig. 7, the three proteins were produced and the amounts of proteins produced over a 48-h period were similar between day 28 and day 56 although hepatocyte numbers at day 56 might have been less than that at day 28.

Lidocaine metabolism (Fig. 8A) and urea synthesis (Fig. 8B) of hepatocytes cultured in the modified KSFM with 10% HS were examined. Both functions were maintained for about 1 week and then gradually decreased. However, the lidocaine metabolizing ratio was more than 15% even at day 28 while the synthesis of urea was greater than 0.5 μg/h until day 28.

DISCUSSION

Primary cultures of human hepatocytes play an important role in the investigation of liver-specific functions and the biology of viral pathogens or parasites as well as drug metabolism in phase I and II studies. Most studies have been performed in short-term cultures of about a week. However, the long-term maintenance of human hepatocyte cultures for several weeks has been infrequently described in a few reports. In order to maintain hepatocyte function, various attempts have been made using different substrates, culture media, and hormonal conditions. Clement *et al.* reported that human cells survived 2 to 3 weeks in a coculture with a rat liver epithelial cell [18]. Gripon *et al.* and Kojima *et al.* suggested that DMSO could improve *in vitro* human liver cell survival and functional stability [19, 20]. DMSO is supposed to prevent the growth of contaminating NPCs but we did not observe this in our study. Recently, the use of human serum was found to prolong hepatocyte survival *in vitro* [21]. Furthermore, Moshage *et al.* noted prolongation of survival of cryopreserved human hepatocytes with ECM derived from human liver [22] while cells cultured in a collagen gel sandwich were also reported to be maintained for a long time [23]. Although these experiments successfully prolonged the survival of human hepatocytes, the cells did not proliferate.

In the present study we used KSFM supplemented with human serum, nicotinamide, ascorbic acid 2-phosphate, EGF, proline, insulin, dexamethasone, DMSO, and antibiotics as the basic medium. This medium supported the survival of human hepatocytes for about 56 days. Importantly the cells could produce albumin, α_1-antitrypsin, α_1-antichymotrypsin, and ceruloplasmin following 56 days in culture [24]. In addition, albumin secretion increased with time in culture until

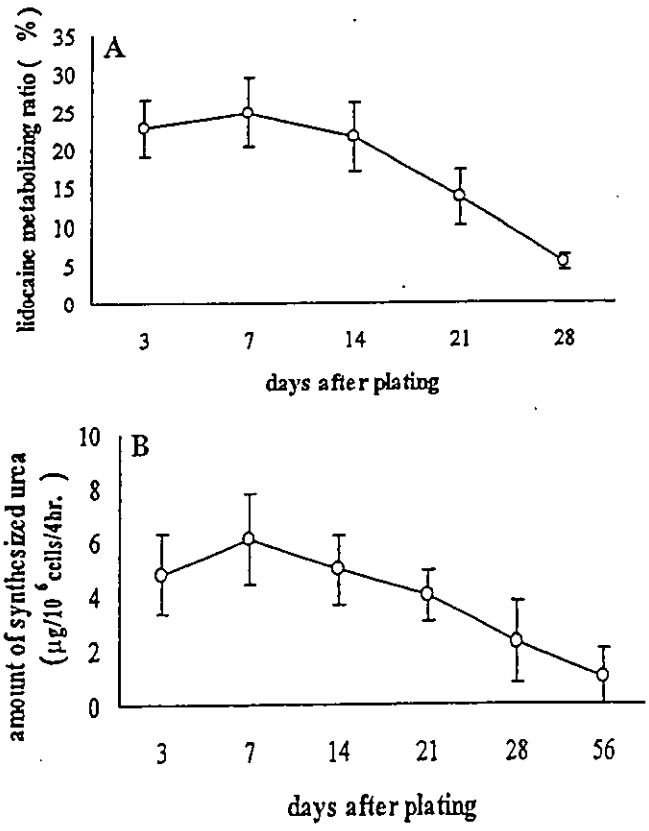

FIG. 8. Function of the long-term-cultured hepatocytes. (A) Lidocaine metabolizing ratio for 4 h. (B) Urea synthesis over 4 h. The points show the means ± SD. Three independent experiments were performed.

day 14. Primary human hepatocytes are generally known to exhibit diminishing albumin secretion with time in culture. Therefore, the early increase in albumin secretion suggests either that the number of cells initially increases during the culture period or that the hepatocytes may recover aspects of their original function following the isolation procedure.

KSFM was originally produced for the culture of primary keratinocytes. Among the specific features of KSFM is a low concentration of calcium ions (0.09 mM). This characteristic of the medium appears to be advantageous to the culture and proliferation of human hepatocytes as well as the maintenance of differentiated functions. It was reported that DNA synthesis of primary rat hepatocytes could be enhanced by culture in the presence of a low Ca^{2+} concentration [25]. In addition, medium containing a high concentration of amino acids such as Leibovitz 15 was known to support not only the prolonged maintenance of hepatic differentiated functions but also the increase in proliferation of primary cultured rat hepatocytes following stimulation by EGF [26]. Furthermore, KSFM includes transferrin and trace elements such as heavy metals and they were also reported to be necessary for hepatocyte proliferation [27]. Thus, KSFM may be suitable for the prolonged survival of human hepatocytes and the maintenance of their differentiated functions.

Nicotinamide is known to stimulate the growth of cultured rodent hepatocytes as well as maintaining hepatic differentiated functions [28, 29]. Furthermore, when the hepatocytes were cultured in the medium supplemented with 10 mM nicotinamide and EGF, it was reported that small hepatocytes appeared and formed colonies [30]. These cells were morphologically similar to but were less than half the size of mature hepatocytes. A single small hepatocyte could proliferate so rapidly that the number of cells may increase 100-fold over 2 weeks [17, 31]. Recently, Hino *et al.* reported that human small hepatocytes were isolated from surgically resected livers [32]. In the present experiment we observed the existence of small hepatocyte-like cells in the later cultures of human hepatocytes. As shown in Fig. 3, they did not exhibit clear demarcation as a colony or rapid proliferation. One of the reasons small hepatocytes did not markedly grow may be that most experiments were performed using hepatocytes isolated from relatively aged patients since the population of small hepatocytes was reported to decrease with increasing rodent age [33]. Human small hepatocytes actively proliferate in culture derived from the livers of relatively young patients [32]. Another factor may be the composition of the medium and further experiments are needed in this area.

The culture system presented in this report is a useful tool to facilitate the study of human differentiated hepatocytes. The development of a stable hepatocyte culture system from human livers has multiple potential benefits in the fields of medicine, basic science research, and biotechnology. For example, these cells could be transplanted to provide temporary liver support for patients suffering from hepatic failure. Recently, bioartificial livers have been developed and are clinically applicable as a bridge to liver transplants. Porcine hepatocytes and a transformed human liver cell line are used to fill the device as a bioreactant. Further improvements of the methods to culture proliferative human normal hepatocytes may significantly contribute to the development of devices that are more efficient.

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Kupffer Cell Targeting by Intraportal Injection of the HVJ Cationic Liposome

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Key Words
Gene transfer · Targeting · Kupffer cell · Hemagglutinating virus of Japan · HVJ liposome · Wistar rats

Abstract
The aim of this study was to target Kupffer cells (KCs) selectively and efficiently by the intraportal injection of fusigenic cationic liposomes with hemagglutinating virus of Japan components (HVJ cationic liposomes). Phosphorothioate FITC-oligodeoxynucleotides (FITC-ODNs) encapsulated in either HVJ cationic liposomes, HVJ anionic liposomes or conventional cationic liposomes without HVJ were transferred to the rat. FITC-ODNs in HVJ cationic liposomes administered via portal vein were selectively transfected to KCs for up to 24 h with no apparent cytotoxicity at higher transfection efficiency than FITC-ODNs in conventional cationic liposomes without HVJ administered via portal vein or tail vein. On the other hand, FITC-ODNs in HVJ anionic liposomes were observed mainly in hepatocytes, not KCs. This new method will be useful for the modulation of KCs activity in both basic research and clinical applications.

Introduction

Increasing evidence indicates that Kupffer cells play a pivotal role in the inflammatory response of the liver under a variety of stress conditions such as hepatic resection, ischemia-reperfusion and sepsis [1–4]. Therefore, development of an effective technique to modify the activity of Kupffer cells is thought to be important for experimental and clinical purposes, especially for use in combination with recently developed gene transfer techniques.

To date several modalities of gene transfer have been developed both in vitro and in vivo. Adenovirus vectors have a high transfection efficiency, although this approach is limited by the host immune response, resulting in short-term gene expression and failure of readministration [5, 6]. Furthermore, complicated procedures are required to construct new viral genomes containing desired foreign genes. Retroviral gene transfer enables long-term transgene expression, but transfects only proliferative cells and might cause carcinogenesis due to random insertion into the genome [7]. Both types of viral vectors can only carry DNAs of large size and are not suitable vectors for smaller DNA such as the oligonucleotides used in the new antisense or decoy technologies.

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Recently, fusigenic liposomes with hemagglutinating virus of Japan components (HVJ liposomes) were reported to have a high transfection efficiency in several organs in vivo: the blood vessels [8], heart [9, 10], brain [11], and kidneys [12]. In this vector system, the HVJ promotes fusion of the liposome with the surface membrane of targeted cells resulting in delivery of DNA directly into the cell [13]. Uehara et al. [14] reported that the HVJ cationic liposome method was successful for selective gene transfer to bile duct epithelial cells in vivo. On the other hand, Kupffer cells have never been selectively targeted, although direct injection of HVJ liposomes into the liver was performed [15]. Some reports have been published on gene transfer intravenously to Kupffer cells by conventional cationic liposomes without HVJ [16–20]. In this study, we compared the efficiency of transfection to Kupffer cells of FITC-labeled oligonucleotides in HVJ cationic liposomes to that in HVJ anionic liposome or conventional cationic liposomes without HVJ.

Materials and Methods

FITC-Oligodeoxynucleotide and Preparation of HVJ Liposomes and Conventional Cationic Liposomes without HVJ

The FITC-oligodeoxynucleotide (FITC-ODNs) was prepared by labeling a phosphorothioate oligodeoxynucleotide (18 mer: 5′-TCCAGCTTCGTAGCTAGC-3′) with fluorescein isothiocyanate on the 3′ end. Cationic liposomes were prepared as follows: phosphatidylcholine, dioleoylphosphatidylethanolamine, sphingomyelin, cholesterol and 3[N-(N′,N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-cholesterol) were mixed in a molar ratio of 5:5:5:12:3 in chloroform. The use of the cationic lipid DC-cholesterol is crucial for producing particles bearing a positive charge. The lipid mixture in 500 μl of chloroform was deposited on the sides of a flask by removal of the chloroform in a rotary evaporator. The dried lipid was then hydrated in 200 μl of TE (pH 8.0) containing FITC-ODN (100 nmol). Liposomes containing ODN were prepared by agitating and shaking this mixture. The mixture of liposomes and ODN was passed through cellulose acetate membrane filters (pore size: 0.45 and 0.20 μm) to form unilamellar cationic liposomes. Purified hemagglutinating virus of Japan (HVJ) was inactivated by ultraviolet irradiation (110 erg/mm²/s) just before use. The liposome suspension mixed with the inactivated HVJ was incubated at 4°C for 10 min and then at 37°C for 30 min. The HVJ cationic liposome complex was collected after removal of the free HVJ by sucrose density gradient centrifugation at 62,800 *g* at 4°C for 90 min. HVJ cationic liposomes were used in the following experiments in vitro or in vivo within 24 h. To prepare the anionic liposomes with HVJ (HVJ anionic liposome), we used negatively charged lipids as liposome components: phosphatidylcholine, phosphatidylserine and cholesterol (molar ratio of 5:1:4). The use of phosphatidylserine instead of DC-cholesterol is crucial. The other steps were the same as for preparation of HVJ cationic liposomes. To compare the in vivo transfection efficiency of HVJ cationic liposomes with that of cationic liposomes without HVJ, we used DMRIE-C Reagent® (Life Technologies, Rockville, Md., USA) as conventional cationic liposomes without HVJ. Five hundred microliters of DMRIE-C Reagent was incubated with 100 nmol of FITC-labeled oligonucleotide in 500 μl of balanced saline solution (BSS: 137 m*M* NaCl, 5.4 m*M* KCl, 13 m*M* Tris-HCl, pH 7.6) for 15 min at room temperature and used for the experiments in vivo immediately.

In vitro Transfection

An alveolar macrophage cell line (NR8383: CRL-2192) was obtained from American Type Culture Collection and was maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Five hundred thousand cells were inoculated per well of a 4-well chamber plate on the day before transfection. The cells were then washed three times in phosphate buffered solution (PBS) and transfected with the HVJ cationic liposomes containing 5 μ*M* of FITC-ODN suspended in 2 ml of balanced saline solution (BSS: 137 m*M* NaCl, 5.4 m*M* KCl, 13 m*M* Tris-HCl, pH 7.6). The transfected cells were incubated at 37°C for 15 min and 24 h, washed three times with PBS, fixed with 99.5% ethanol at 4°C for 30 min and observed using a fluorescence microscope (Olympus BH2-RFK) to examine the transfection efficiency. The cytotoxicity of the liposomes was determined by comparison of the number of viable cells stained by trypan blue before and after the transfection. Four experiments were performed to evaluate transfection efficiency and cytotoxicity, respectively.

In vivo Transfection

All studies were conducted in accordance with the institutional guidelines of Kyoto University. Laparotomy of male Wistar rats weighing 250 g was performed under diethyl ether anesthesia, and the loop of the duodenum was exposed. Either HVJ cationic liposomes or HVJ anionic liposomes containing FITC-ODN (10 μ*M*) suspended in 1 ml of BSS were injected intraportally with a 30-gauge needle. BBS alone was also injected intraportally as a control. The injected HVJ liposomes were allowed to incubate in the liver intraportally by clamping the portal vein for 15 min or intravenously via the tail vein using a 30-gauge needle. One milliliter of the conventional cationic liposomes without HVJ containing FITC-ODN (10 μ*M*) was injected intravenously or intraportally in the same way as HVJ cationic liposomes were injected. Six rats were used for each liposome group. Furthermore, possible hepatic damage due to gene transfer by HVJ cationic liposome or HVJ anionic liposome was assessed by determining the plasma levels of asparate aminotransferase (AST) and alanine aminotransferase (ALT) 24 h after transfection, using three rats for each group. Morphometric changes were also evaluated in all samples after staining with hematoxylin and eosin.

In vivo Analysis of FITC-ODN

Rats were sacrificed 1 or 24 h after injection of liposomes. The liver was placed in embedding medium (Tissue Tek®, Sakura, Torrance, Calif., USA) and snap frozen in liquid nitrogen. Five-micrometer sections were obtained using a cryostat, fixed in cold acetone and observed by fluorescence microscopy. Immunohistochemical staining was carried out using the avidin-biotin-peroxidase complex method in order to identify the localization of transfected cells. Briefly, after fixation, sections were pretreated with 0.3% H_2O_2 in methanol and normal goat serum. Monoclonal antibody to tissue macrophage specific glycoprotein (Chemicon International, Temecula, Cal-

if., USA) was diluted 1:400 and incubated with the specimen for 90 min at room temperature. Subsequently, freshly prepared avidin-biotin-peroxidase complex (Vector Laboratories Inc., Burlingame, Calif., USA) was applied for 30 min. The histochemical reaction for peroxidase was visualized by using an H_2O_2–3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co, St. Louis, Mo., USA). The sections were then slightly counterstained with hematoxylin. To evaluate the transfection efficiency, at least 1,000 cells that were macrophage specific glycoprotein positive were quantified and FITC-positive cells were counted in five sections from each harvested liver, and the transfection efficiency of each liver was expressed as the mean value of these five sections (%). For statistical analysis we performed ANOVA (analysis of variance), followed by post hoc comparison with Bonferroni/Dunn test.

Preparation of HVJ Cationic Liposomes Containing pCAG-lacZ and in vivo β-Galactosidase Assay

The β-galactosidase expression vector (pCAG-lacZ) was constructed as previously described [14]. HVJ cationic liposomes containing pCAG-lacZ (200 μg), previously incubated with 64 μg of a nuclear protein (the high mobility group 1 (HMG-1)), were prepared as described above for the HVJ cationic liposomes containing FITC-ODN. The HVJ cationic liposomes were diluted in 1 ml of BSS and injected intraportally as described above. Rats were sacrificed 3 days after transfection of the lacZ gene. Sections (5 μm) of the liver frozen in Tissue Tek® were fixed with 1.25% glutaraldehyde in PBS for 10 min, rinsed three times for 5 min each time, and incubated with 0.1% Triton X-100 in PBS at 37°C for 10 min. Then they were rinsed with PBS and incubated with 5-bromo-4-chloro-3-indolyl β-*D*-galactoside, or X-Gal (2 mg/ml), 3.3 m*M* $K_3Fe(CN)_6$, 3.3 m*M* $K_4Fe(CN)_6$, and 1 m*M* $MgCl_2$ at 37°C overnight. The sections were rinsed twice for 5 min each time and counterstained with hematoxylin. To identify the location of the transfected cells, Kupffer cell immunohistochemistry was performed in serial sections as described above. Three rats were used for evaluation.

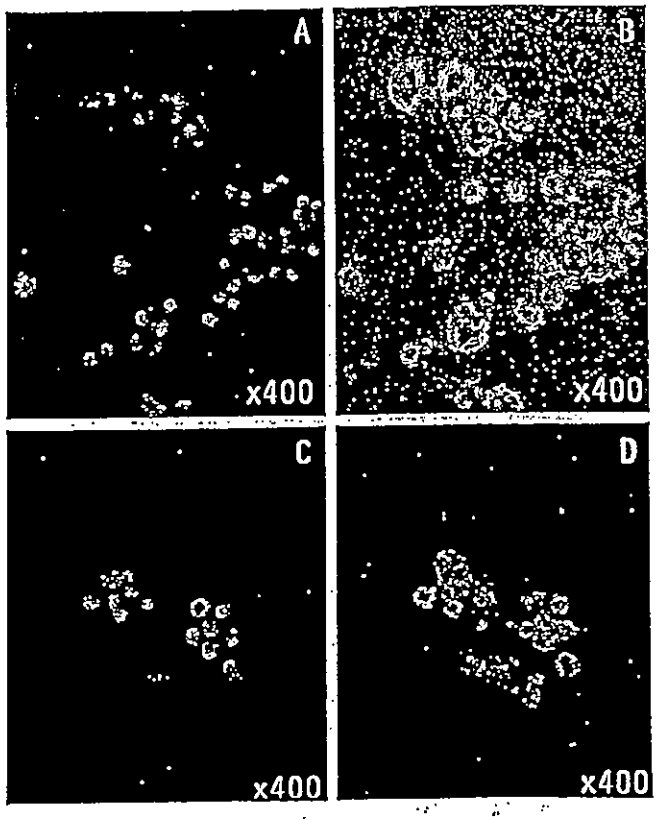

Fig. 1. Fluorescence (**A**) and phase-contrast (**B**) micrographs of NR8383 cells 15 min after transfection of HVJ cationic liposomes containing 5 m*M* of FITC-ODN. ×400. Fluorescent signals were detected in the cell nuclei. Similar results were obtained in the cells 24 h after the injection, as shown in (**C**) and (**D**).

Results

Transfection Efficiency in vitro

Fluorescent signals of FITC-ODN were detected in over 90% of NR8383 cells 15 min and 24 h after transfection (fig. 1A, C). Comparison of fluorescence and phase-contrast images revealed that the fluorescence was mainly localized in the cell nuclei (fig. 1A–D). The percentage of viable cells was above 90% until 3 days after the transfection.

Transfection Efficiency and Localization after HVJ Anionic Liposome Injection Intraportally and HVJ Cationic Liposome Injection Intravenously in vivo

Using HVJ anionic liposomes, fluorescent signals were detected mainly in hepatocytes 1 h after the injection of the liposomes via the portal vein (fig. 2A). Comparison of fluorescence with hematoxylin and eosin staining confirmed that cells bearing fluorescence were hepatocytes and that the main intracellular site of localization of the fluorescence was the nuclei (fig. 2B). Similar localization and the same fluorescence intensity were observed until 24 h after the injection (fig. 2C, D). In contrast, using HVJ cationic liposomes, fluorescent signals were detected mainly in sinusoidal lining cells 1 h after the injection of the liposomes via the portal vein (fig. 3A). These cells proved to be identical to Kupffer cells as shown by immunohistochemical staining using monoclonal antibody to tissue macrophage specific glycoprotein (fig. 3B). No fluorescence was detected in hepatocytes or other nonparenchymal cells. The same results were observed 24 h after the injection (fig. 3C, D). When the HVJ cationic liposomes were injected intravenously, the fluorescent signals were detected in Kupffer cells and the transfection efficiency 1 h after the injection is by far lower than that by intraportal injection (fig. 4A, B). The transfection effi-