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Hepatic Organoid Formation in Collagen Sponge of Cells Isolated from Human Liver Tissues

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ABSTRACT

We examined whether small hepatocytes (SHs), which are hepatic progenitor cells, could be isolated from a normal human liver and whether human hepatic cells could form hepatic organoids in a collagen sponge. Normal liver tissues were obtained from resected specimens from nine patients who underwent hepatic resection. Isolated hepatic cells were plated on dishes and a collagen sponge. More than 1 month later, SH-like cells appeared and proliferated on the dishes, whereas cell aggregates were formed in the sponge and showed characteristic tissue architecture: columnar and/or cuboidal epithelial cells lined the surface of the sponge. Clusters of epithelial cells with a large cytoplasm and ductular structures were observed under the lining cells. The lining and ductular cells were positive for cytokeratins 7 and 19, which indicated they were biliary epithelial cells (BECs), and the epithelial cells forming clusters were positive for the anti-human hepatocyte antibody, identifying them as hepatocytes. Some lining cells were positive for both the hepatic marker and the BEC markers. The cells in the collagen sponge actively proliferated and the hepatocytes excreted albumin into the medium. Thus, hepatic organoids could be reconstructed in a collagen sponge by normal human liver cells.

INTRODUCTION

Bioartificial liver (BAL) devices supporting a large mass of metabolically active hepatocytes are thought to be necessary for the successful treatment of patients with severe acute liver failure. Many researchers have attempted to improve the differentiated hepatic functions of such devices.[1–5] In those experiments, primary hepatocytes, immortalized hepatic cells, or hepatoma-derived cells were used as bioreactors of BAL and, to maintain the hepatic functions, various materials and devices such as microcarrier beads, multiporous microcarriers, polyurethane foam, and porous resin have been produced and used as carriers of cells. Furthermore, devices for packing carriers have been developed such as hollow fibers, a multicompartment capillary fiber, a synthetic biodegradable polymer scaffold, and a three-dimensional perfused microarray bioreactor. Although the materials and devices have been improved, hepatic functions of the cells are not good enough to reach the level of clinical application. Therefore, the development of methods for maintaining highly differentiated functions of cells has

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TABLE 1. PATIENT PROFILES

Patient no.	Primary disease	Age (years)	Sex	Weight (g)	Yield ($\times 10^6$ cells/g)
1	Liver metastasis (colon)	54	M	9.0	2.46
2	Hepatocellular carcinoma	73	M	5.8	3.03
3	Gallbladder carcinoma	23	F	12.0	3.20
4	Hepatocellular carcinoma	68	M	8.5	2.50
5	Liver metastasis (colon)	59	M	5.4	2.07
6	Liver metastasis (colon)	51	M	15.4	1.86
7	Hepatocellular carcinoma	57	M	11.2	1.89
8	Hepatocellular carcinoma	72	M	11.4	1.30
9	Liver metastasis (lung)	64	M	5.5	2.65

been required. Three-dimensional culture is well known to be a method by which hepatocytes can keep their differentiated functions well via spheroid formation[6,7] and coculture with fibroblasts.[8,9] The formation of hepatic organoids, which are reconstructed by the cells isolated from a normal adult rat liver, has been reported.[10,11] The tissues reported consisted of epithelial cells, which are both mature hepatocytes (MHs) and biliary epithelial cells (BECs), and connective tissues. Although Lázaro *et al.*[12] reported that a hepatic organoid could be formed by human fetal hepatocytes after long-term culture, there is no report that cells from a normal human adult liver reconstruct hepatic tissues *in vitro*.

We have reported that rat small hepatocytes (SHs), which are hepatic progenitor cells, appear and proliferate in a medium supplemented with nicotinamide and epidermal growth factor (EGF).[13,14] SHs have high growth ability compared with MHs and can reconstruct hepatic organoids interacting with hepatic nonparenchymal cells (NPCs) such as stellate (Ito) cells and liver epithelial cells (LECs).[15] The morphological changes of SHs are correlated with hepatic maturation and can be induced by extracellular matrix (ECM).[15,16] We reported that rat SHs could proliferate in a collagen sponge after isolated SH colonies were replated.[17] The cells could become large ones that were morphologically and functionally differentiated hepatocytes. In addition, some ductlike structures consisting of cytokeratin 19 (CK19)-positive cells were formed. In the present experiment we examined whether proliferating SHs could be observed in the cells isolated from a normal human liver and whether human hepatic cells could form hepatic organoids in a collagen sponge.

MATERIALS AND METHODS

Liver specimens from patients

Normal liver tissues were obtained from resected specimens from nine patients who underwent hepatic resections at Kyoto University Hospital (Kyoto, Japan) and Sapporo Medical University (Sapporo, Japan), under informed consent and with the approval of the Kyoto University and Sapporo Medical University Ethics Committees, respectively. As shown in Table 1, four patients had liver metastasis of colon or lung cancer, four had hepatocellular carcinoma, and one had gallbladder carcinoma. All tissues were obtained from the patients without any viral infections. The mean age of the patients was 57.9 ± 15.2 years old, and the mean wet weight of the liver tissues was 9.4 ± 3.5 g.

Isolation of human hepatocytes

Hepatocytes were isolated from liver tissues by the injection method, using a 10-mL disposal injector with a 27-gauge needle. The isolation procedure was previously described.[18] Briefly, the liver tissue was examined for small vessels and Hanks' balanced salt solution (HBSS; GIBCO-BRL Invitrogen, Grand Island, NY) supplemented with EGTA (Dojindo Chemical Laboratories, Kumamoto, Japan) was poured into the vessels. After the washout of blood, HBSS containing 0.05% collagenase (Wako Pure Chemical, Osaka, Japan) and 0.5% dispase (Godo Shyusei, Tokyo, Japan) was poured into the vessels. This procedure was repeated many times and the swollen tissue was soaked in the enzyme solution during the procedure. The isolated cells were collected into centrifugal tubes. The cell suspension was centrifuged 50 $\times$ *g* for 1 min and the pellet (hepatocyte fraction) was suspended in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with insulin (0.5 mg/mL), 10^{-7} M dexamethasone, and antibiotics. The cell density was adjusted to 1×10^5 viable cells/mL. Nondigested liver tissues in the supernatant, which was collected after the first 50 $\times$ *g* centrifugation of the cell suspension, were soaked and stirred for 30 min at 37°C. Digested cell suspension was subjected to 50 $\times$ *g* centrifugation for 5 min. After this procedure was repeated several times, cells were resuspended in the

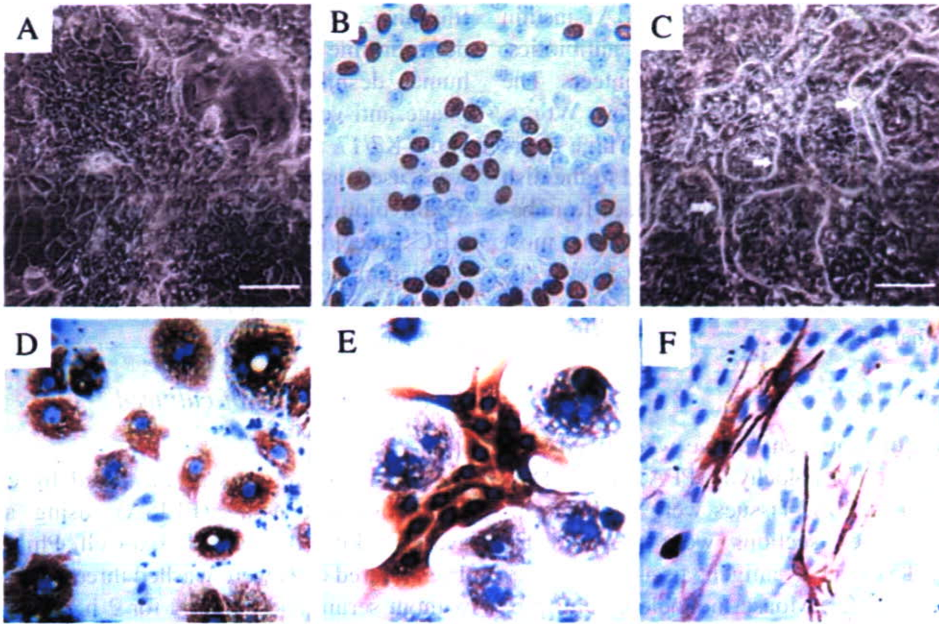

FIG. 1. Photographs of cells isolated from human liver tissues. Phase-contrast photographs of cells on dishes were taken on day 63 (**A**) and day 60 (**C**). (**B**) Cells were fixed with cold ethanol on day 60. Before fixation, 10 mM BrdU was added to the medium for 24 h and immunocytochemistry for BrdU was performed. Counterstaining was done with hematoxylin. Darkened nuclei were positive for BrdU. (**D–F**) A mixture of hepatocytes and hepatic NPCs was cultured on collagen-coated dishes and fixed on day 1 (**D** and **E**) and day 5 (**F**). Immunocytochemistry for anti-Hep (**D**), anti-CK19 (**E**), and anti-desmin antibodies (**F**) was carried out. Details are described in Materials and Methods. Scale bars: 50 μm.

medium. The cell density was adjusted to 1×10^5 viable cells/mL (NPC fraction).

An equal volume of each fraction (hepatocyte fraction:NPC fraction, 1:1) was suspended in a small volume of the medium and 1 mL of the cell suspension was slowly poured onto collagen sponge to avoid overflowing. Helistat (1 × 1.5 cm; COLLA-TEC, Plainsboro, NJ) was used as collagen sponge and directly placed on a 35-mm dish without any pretreatment. The cells were cultured in DMEM supplemented with 20 mM HEPES, 25 mM $NaHCO_3$, L-proline (30 mg/L), 10% human serum (HS), 5% fetal bovine serum (FBS; HyClone, Logan, UT), 10 mM nicotinamide (Katyama Chemical, Osaka, Japan), 1 mM ascorbic acid 2-phosphate (Asc2P; Wako Pure Chemical), hepatocyte growth factor (HGF, 20 ng/mL; Genzyme/Techne, Minneapolis, MN), EGF (10

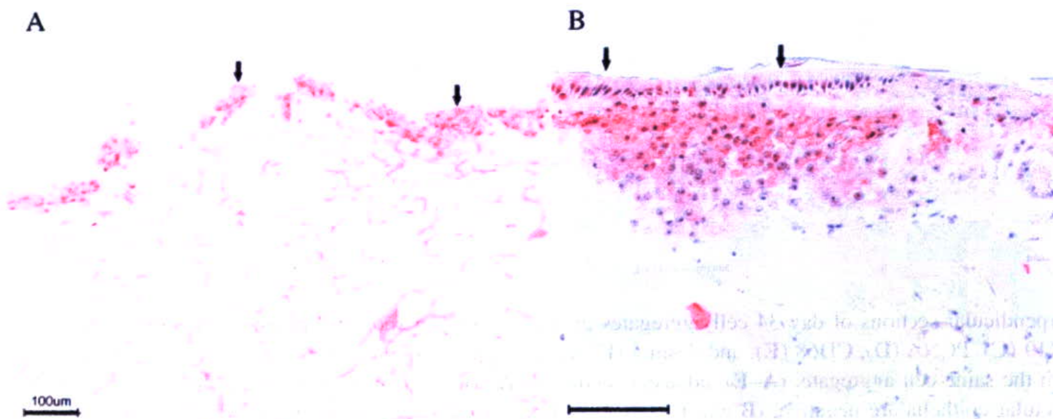

FIG. 2. Perpendicular sections of a collagen sponge on day 7 (**A**) and day 40 (**B**). Hematoxylin–eosin staining was carried out. Arrows in (**A**) show mature hepatocytes. Arrows in (**B**) show lining columnar epithelia. Scale bars: 100 μm.

ng/mL; Collaborative Research, Lexington, MA), insulin (0.5 mg/mL), 10^{-7} M dexamethasone, and antibiotics. HS was obtained from several healthy volunteers. The remnant was plated on dishes (Corning Glass Works, Corning, NY) coated with rat tail collagen. Three hours after plating, 2 mL of the medium was added to the dish for the purpose of preventing the sponge surface from becoming dry. Several days later, we confirmed that most cells attached to the collagen sponge and that a few cells were observed around the sponge. The medium was changed every other day.

Immunocytochemistry

Cells grown on the collagen sponge were fixed with cold ethanol or 4% paraformaldehyde (PFA; Merck, Darmastadt, Germany) in PBS. Tissues were paraffin embedded and 5-μm-thick sections were prepared for immunostaining. To retrieve antigen, tissues were microwaved in citrate buffer. Mouse monoclonal anti-proliferating cell nuclear antigen (PCNA), anti-bromodeoxyuridine (BrdU), anti-human hepatocyte (Hep), anti-human desmin, anti-human CD68, anti-human macrophage, anti-von Willebrand factor (vWF), anti-CK19, and anti-CK7/17 antibodies (Dako, Copenhagen, Denmark) were used as the primary antibodies, followed by the avidin–biotin peroxidase complex method (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA). 3′-Diaminobenzidine (Tokyo Kasei Industries, Tokyo, Japan) was used as a substrate. The cells were then counterstained with hematoxylin.

Albumin synthesis of cultured human hepatocytes

Albumin secretion was measured by enzyme-linked immunosorbent assay (ELISA), using a commercial available kit (Albuwell II; ExoCell, Philadelphia, PA). The cultured cells were washed three times with medium without serum and cultured for 2 h, after which the tis-

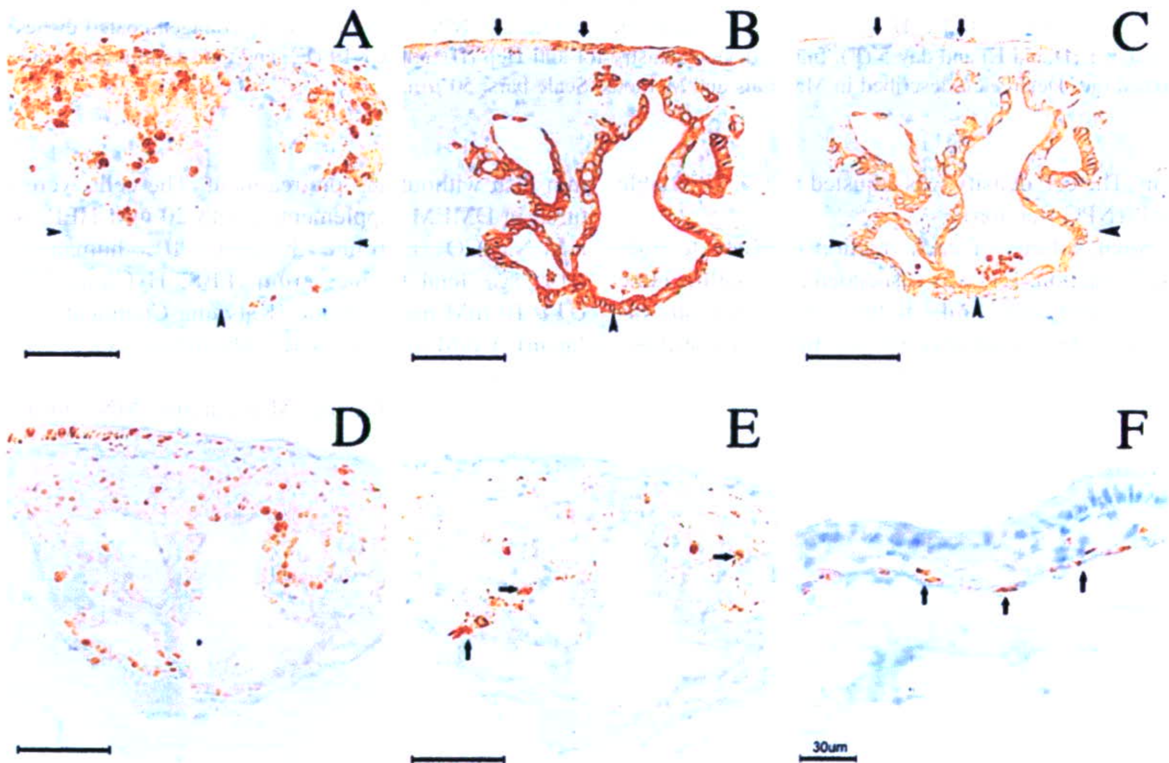

FIG. 3. Perpendicular sections of day 34 cell aggregates grown in collagen sponge. Immunocytochemistry for anti-Hep (**A**), CK7 (**B**), CK19 (**C**), PCNA (**D**), CD68 (**E**), and desmin (**F**) was carried out. Light brown staining is positive. All sections were prepared from the same cell aggregate, (**A–E**), adjacent sections.) (**A**) Hep was used as a marker for hepatocytes. Arrowheads show that ductular epithelia are negative. (**B** and **C**) CK7 and CK19 were used as markers for BECs. Arrows show the lining columnar epithelia and arrowheads show the ductular epithelia. All cells of both structures are positive. (**D**) PCNA was used as a marker for cell proliferation. (**E**) CD68 was used as a marker for Kupffer cells. Arrows show positive cells. (**F**) Desmin was used as a marker for stellate/myofibroblasts. Arrows show positive cells. Scale bars: (**A–E**) 100 μm; (**F**) 30 μm.

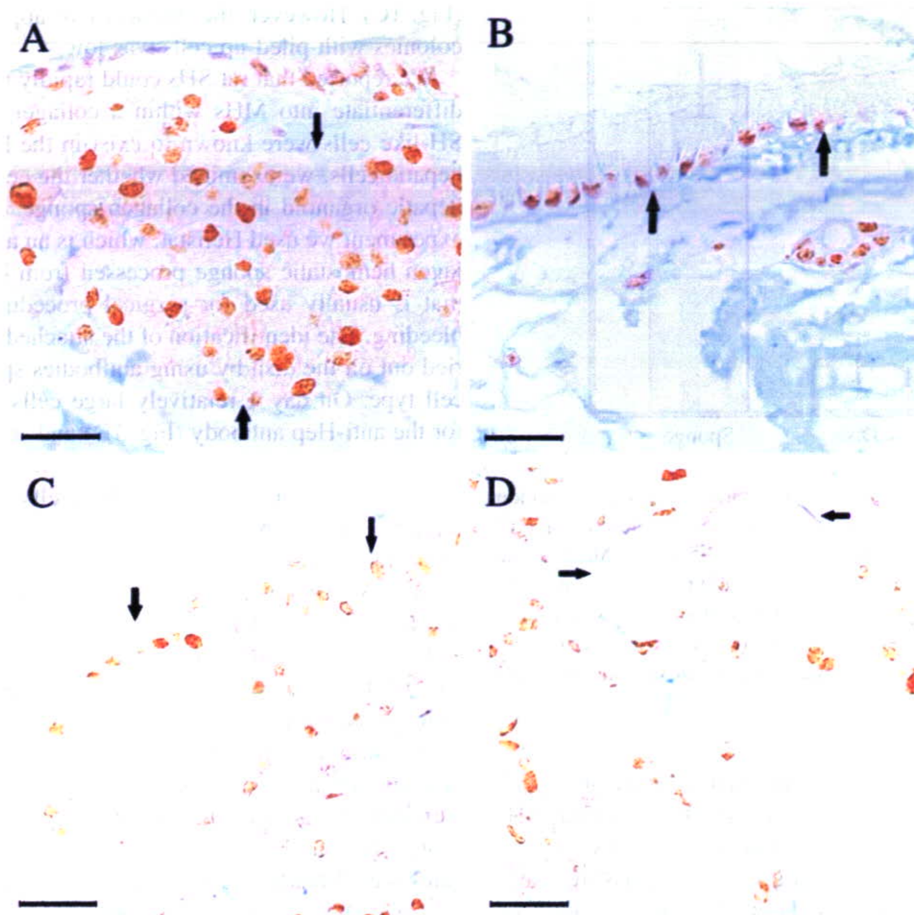

FIG. 4. Immunocytochemistry for PCNA of cells in collagen sponge. The cells were fixed with 4% PFA on day 34 (**A** and **B**) and day 40 (**C** and **D**). The nuclei of many hepatocytes (**A**; surrounded by arrows), columnar epithelia (**B**; arrows), cells forming ductlike structures (**C**; arrows), and cells forming vessels/capillaries (**D**; arrows) are positive for PCNA. Scale bars: 30 μm.

sue culture supernatant was collected and centrifuged at $1 \times 10^4 \times g$ for 10 min. The supernatant was kept at $-30°C$ until use. Three dishes were used per experiment and three independent experiments were carried out.

RESULTS AND DISCUSSION

Normal hepatic cells were isolated from liver specimens of nine patients (Table 1) and cultured in DMEM supplemented with 10% HS, 5% FBS, 10 mM nicotinamide, 1 mM Asc2P, HGF (10 ng/mL), EGF (10 ng/mL), and hormones. The average yield of hepatocytes per 1 g wet weight of the tissue was 2.33×10^6 cells and the viability of the cells was more than 85%. When cells of the hepatocyte and NPC fractions were plated on dishes and cultured in medium supplemented with all additives, SH-like cells, the morphology of which was quite similar to that of rat SHs, appeared in many dishes al-

TABLE 2. PERCENTAGE OF PCNA-POSITIVE CELLS BY CELLS FORMING A SPECIFIC STRUCTURE

Cells	*PCNA-positive cells (%)*[a]
Hepatocytes[b]	67.3 ± 11.2
Biliary epithelial cells (duct formation)[b]	80.3 ± 15.3
Biliary epithelial cells (sheet formation)[b]	83.3 ± 9.3
Vessel or capillary-like structure[c]	71.3 ± 17.7

[a]Numbers indicate averages ± standard deviation.
[b]More than 100 cells were examined.
[c]About 50 cells were counted, as the number of structures was low.

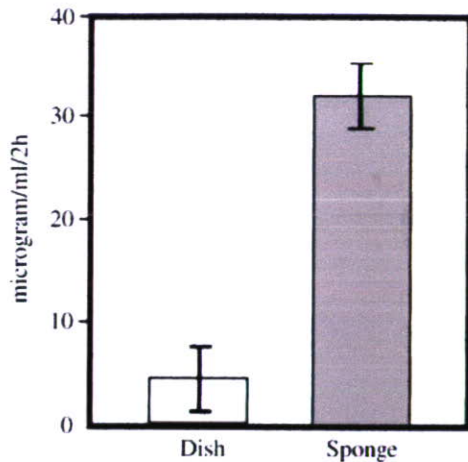

FIG. 5. Albumin secretion into culture medium of human cells cultured in collagen-coated dishes (open column) and collagen sponge (shaded column). On day 35 the medium of the cells was exchanged for serum-free medium and, 2 h later, the medium was collected. The amount of albumin was measured by ELISA. Four independent experiments were performed. Each column shows the average of three dishes and the error bars show standard deviations.

though the number of cells fluctuated between experiments (Fig. 1A). Even 2 months after plating, human SH-like cells could slowly proliferate as shown by the fact that many cells took bromodeoxyuridine (BrdU) into their nuclei (Fig. 1B). Without HS, nicotinamide, or HGF, SH-like cells did not appear. The mixture of 5% FBS and 10% HS was also reported to be necessary for the growth of human SH-like cells.[19] Although the authors of that report pointed out that SH-like cells required the conditioned medium of 3T3 cells for their proliferation, we found that SH-like cells expanded without the conditioned medium. When the cells were cultured for more than 2 months, piled-up cells could be observed (Fig. 1C). However, the frequency of appearance of the colonies with piled-up cells was low.

We reported that rat SHs could rapidly proliferate and differentiate into MHs within a collagen sponge.[17] As SH-like cells were known to exist in the human normal hepatic cells, we examined whether the cells could form hepatic organoid in the collagen sponge. In the present experiment we used Helistat, which is an absorbable collagen hemostatic sponge processed from bovine tendon that is usually used for surgical procedures to control bleeding. The identification of the attached cells was carried out on the dish by using antibodies specific to each cell type. On day 1 relatively large cells were positive for the anti-Hep antibody (Fig. 1D) and some clusters of small epithelial cells were positive for the anti-CK19 antibody. These were BECs (Fig. 1E). Cells with a little cytoplasm and long processes, which might have been stellate cells, were positive for anti-desmin (Fig. 1F). In the present experiment, hepatocytes (Hep^+), BECs ($CK19^+$), and Kupffer cells ($CD68^+$) accounted for about 70, 3.7, and 2% of the cells, respectively. Stellate and endothelial (vWF^+) cells were rarely observed. When the cells were plated on the collagen sponge, many MHs settled on the surface of the sponge on day 7 (Fig. 2A), but they gradually disappeared from the sponge (data not shown). Although it was difficult to identify each cell type on the sponge by phase-contrast microscopy, many cell aggregates were clearly found on the sponge about 1 month after plating. As shown in Fig. 2B, perpendicular sections of the portion of the aggregates showed that layers of cells were formed in the upper part of the sponge. On the surface facing the medium there was a continual monolayer of columnar/cuboidal epithelial cells, the nuclei of which were along the basal membrane (Fig. 2B). These cells morphologically resembled the epithelial cells of bile ducts and gallbladder and were positive for CK7 and CK19 (Fig. 3B and C), which may be BECs. Below the

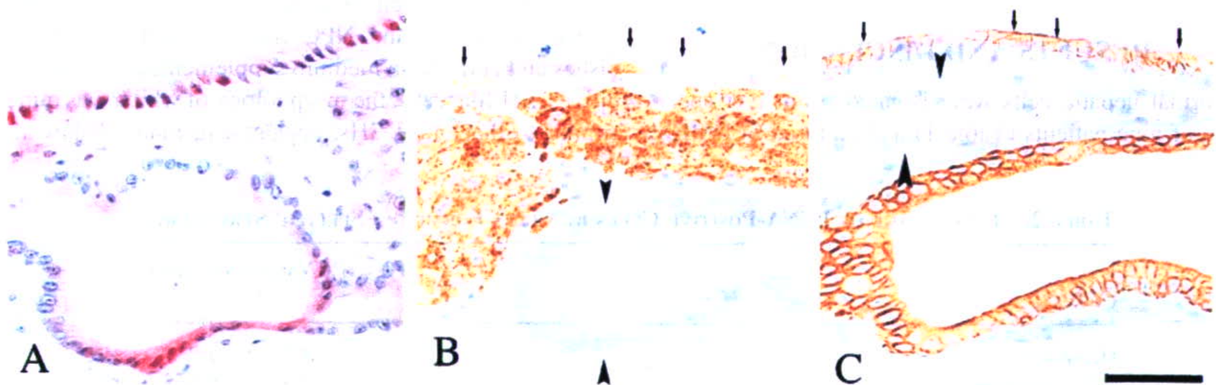

FIG. 6. Coexpression of hepatic and biliary markers in the cells. Shown are day 34 perpendicular sections of cell aggregates grown in collagen sponge. Adjacent sections were prepared and used for staining. Hematoxylin–eosin staining (**A**) and immunocytochemistry for Hep (**B**) and CK19 (**C**) were performed. Some lining BECs [arrows in (**B**) and (**C**) coexpress hepatic (**B**) and biliary (**C**) marker proteins, whereas BECs forming a ductlike structure do not express the hepatic marker (arrowheads, **B**) and hepatocytes are not stained with anti-CK19 antibody (**C**). Scale bar: 50 μm.

lining cells, there was a cluster of relatively large cells that were positive for anti-Hep antibody (Fig. 3A). Although most of the cells were mononucleate, some were binucleate cells. A thin layer of connective tissue usually separated the cluster of hepatocytes from lining BECs. Ductlike structures of various sizes consisting of cuboidal and/or flattened cells were formed in the sponge and they consisted of both CK7- and CK19-positive cells. Some vessel- or capillary-like structures that consisted of flattened thin cells were formed within the sponge (Fig. 4D). Although the morphology of the structure was similar to that of vessels or capillaries, CD31, CD34, vVW, CK7, and CK19 were not clearly stained (data not shown). Some Kupffer cells were scattered in and near hepatocyte clusters (Fig. 3E) and a few stellate cells (desmin$^+$) were observed under the clusters of hepatocytes (Fig. 3F).

The growth of cells was examined by PCNA staining (Figs. 3D and 4). Even more than 40 days after plating, nuclei of the epithelial cells were positive for PCNA. We measured the number of PCNA-positive cells by the type of cells; hepatocytes forming clusters (Fig. 4A), lining BECs (Fig. 4B), BECs forming ductlike structures (Fig. 4C), and cells forming capillary-like structures (Fig. 4D) accounted about 67, 80, 83, and 71% of the cells, respectively (Table 2). When MHs were plated on the sponge without NPC fractions, it was difficult to find the proliferating MHs, although many MHs survived in the sponge even after 1 month (data not shown). We also examined albumin secretion of the cells. Albumin secretion of the cells on the sponge decreased with time in culture and then, 2 to 3 weeks later, reversed to increase (data not shown). As shown in Fig. 5, on day 35, the amounts of albumin produced by the cells on the collagen-coated dish and the collagen sponge were 4.7 ± 3.2 and 32.8 ± 3.2 μg/mL per 2 h, respectively. This result may be coincident with the fact that MHs gradually disappeared from the sponge in the early culture period and that the clusters of hepatocytes appeared in the later culture period. We terminated most experiments within 2 months because the sponge gradually melted and could not support the three-dimensional structure.

It is of interest that coexpression of CK19 and Hep was found in some lining columnar/cuboidal cells on the surface of the sponge (Fig. 6). However, cells forming ductlike structures were negative for the anti-Hep antibody. Furthermore, hepatocytes forming clusters showed positivity for neither CK7 nor CK19. In the present experiment, although about 4% of the plating cells were BECs, ductlike structures were not observed on the collagen-coated dish. On the other hand, when rat SHs were plated on the sponge, some large CK19-positive cells were positive for albumin.[17] Michalopoulos *et al.*[10,11] reported that transdifferentiation of MHs into BECs might occur when primary rat hepatocytes were cultured in a pleated roller bottle with rotation. Under their culture conditions, CK19-positive cells with hepatic marker proteins appeared in the hepatic organoid. Thus, some CK19-positive cells that appeared in the sponge might have been derived from hepatocytes. In addition, although we do not have any direct proof, there is a possibility that both proliferating hepatocytes and BECs came from SHs, which were included in the isolated hepatic cells. Auth *et al.*[20] reported that isolated human BECs could develop three-dimensional structures in between collagen gels. When autologous human hepatocytes were cocultured with a small number of BECs in the collagen sandwich, the ductular formation of BECs was enhanced. In our culture system a small number of BECs was initially included in the isolated cells. Therefore, there is a possibility that the ductular formation of the BECs might have been enhanced by the coculture of MHs.

For the purpose of the reconstruction of functional hepatic organoids, the structure of the reformed organoids should be similar to liver lobules *in vivo*. In the present experiment, although cells derived from normal adult livers could proliferate and form hepatic organoids in the collagen sponge, the tissues were located mainly on the upper parts of the sponge and the sponge was never filled with cells. Therefore, to use collagen sponges as a scaffold, the size of the spaces between fibers and the thickness of the collagen should be considered and newly designed materials will be necessary to create a BAL. On the other hand, although vessel-like structures and bile ductlike structures were formed, tubelike structures were not found and the connection between bile canaliculi of hepatocytes and bile ductlike structures was not observed. For the enlargement of the organoids, the formation of vessels may be necessary to supply oxygen and nutrients inside the organoids. A method for growth factor-induced vascularization has been commonly used in the field of tissue engineering.[21,22] A scaffold and/or carrier combined with angiogenic factors such as vascular endothelial growth factor and/or fibroblast growth factor may improve *in vitro* organoid formation.

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HEPATOLOGY

Expression of cytochrome P450 enzymes in hepatic organoid reconstructed by rat small hepatocytes

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Abstract

Background and Aims: Small hepatocytes (SH), which are hepatic progenitor cells, were isolated from an adult rat liver. SH in a colony sometimes change their shape from small to large and from flat to rising/piled-up. The morphological changes of SH may be correlated with hepatic maturation. Cytochrome P450s (CYP) are drug-metabolizing enzymes and the expression is one of hepatic differentiated functions. However, it is well known that the re-expression and maintenance of CYP activity are very difficult in cultured hepatocytes. We investigated the expression of CYP and the enzymatic activities in long-term cultured SH.

Methods: SH were isolated from adult rat livers and SH colonies were collected, replated on new dishes, and then cultured. CYP1A1/2, CYP2B1, CYP3A2, CYP4A1, and CYP2E1 were induced by the addition of 3-methylcholanthrene, phenobarbital, pregnenolone-16α-carbonitrile, clofibric acid, and ethanol, respectively. Immunocytochemistry, immunoblots, and enzyme activities were examined.

Results: SH could differentiate into mature hepatocytes by the addition of Matrigel and re-express constitutive CYPs. The expression of CYP1A1/2, CYP2B1, CYP3A2, and CYP4A1 dose-dependently increased and the amounts gradually increased with time in culture, especially in the cells treated with Matrigel. Activities of CYP1A, CYP2B, CYP3A and CYP2E in SH treated with Matrigel induced by each of the inducers were approximately 120-fold, 2.8-fold, 6.4-fold and 0.8-fold higher than in the control.

Conclusion: The matured SH could re-express the constitutive CYP and recover inducibility, not only of protein expression but also of enzyme activities.

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Key words: enzyme activity, extracellular matrix, maturation, progenitor cells, proliferation.

INTRODUCTION

Small hepatocytes (SH), which are known to be hepatic progenitor cells, have been identified as proliferating cells with hepatic characteristics.[1–3] Recently, we showed that a single SH could clonally proliferate and form a large colony.[4,5] Some SH colonies changed their shapes from flat to rising/piled-up cells with time in culture. The rising/piled-up cells were large and tall, possessed many mitochondria, peroxisomes with a crystalline nucleoid, and glycogen granules.[4] In such colonies non-parenchymal cells (NPC) invaded under the colony and an accumulation of extracellular matrix (ECM) between hepatocytyes and NPC was observed. Therefore, we suspected that SH could differentiate into mature hepatocytes (MH) that interacted with hepatic NPC and ECM.[4] In a recent study[5] we demonstrated that replated SH colonies could attach to a new dish and continue proliferating. Moreover, the addition of Matrigel, which is derived from Engelbreth-Holm-Swarm sarcoma, could induce the alteration of the cell shape, from flat to rising/piling-up and from small to large. The most important change was the dramatic improvement of hepatic differentiated functions such as

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serum protein secretions and the expression of amino acid metabolizing and urea cycle enzymes.[4,6] In addition, the changes were correlated to the increased expression of liver-enriched transcriptional factors (LETF) such as hepatocyte nuclear factor (HNF) 4α, HNF6, CCAAT/enhancer binding protein (C/EBP)α, and C/EBPβ.[5]

Cytochrome P450 (CYP) constitutes a superfamily of mono-oxygenases that participate in the metabolism of endogenous substrates and play a key role in the detoxification as well as in the metabolic activation of xenobiotics.[7-9] The CYP involved in xenobiotic metabolism are most highly expressed in the liver. *In vivo*, many of the constitutive CYP are actually inducible by xenobiotics such as aromatic hydrocarbons, phenobarbital, ethanol, and peroxisome proliferators. However, when primary rodent hepatocytes are placed into the traditional culture system, the constitutive CYP promptly disappear and it is very difficult to maintain their activities for a long time.[10] In addition, the induction of not only CYP proteins but also their enzymatic activities has never been shown in primary rat hepatocytes cultured for about a month.

In the present study we investigated whether the matured SH treated with Matrigel expressed constitutive CYP, whether the CYP expression could be induced or enhanced by the administration of appropriate agents, and then whether the induced CYP possessed activity. The results showed that SH cultured for a long time possessed CYP1A1 in their cytoplasm and that the CYP expression was much enhanced in the matured SH and could be induced by an appropriate agent.

METHODS

Isolation and culture of small hepatocytes

Male Sprague–Dawley rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan), weighing 250–400 g, were used. All animals received humane care and the experimental protocol was approved by the Committee of Laboratory Animals according to university guidelines. Details of the isolation and culture procedure of the cells were previously described.[4] Finally, 1.8×10^6 viable cells were seeded on a 100-mm dish (Corning Glass Works, Corning, NY, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 20 mmol/L HEPES, 25 mmol/L $NaHCO_3$, 30 mg/L L-proline, 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA), 10 mmol/L nicotinamide (Katayama Chemical, Osaka, Japan), 1 mmol/L ascorbic acid 2-phosphate (Asc2P; Wako Pure Chemical, Tokyo, Japan), 10 ng/mL epidermal growth factor (EGF; Collaborative Research, Lexington, MA, USA), 0.5 mg/L insulin, 10^{-7} mol/L dexamethasone, and antiboiotics. After 4 days of culture, 1% dimethyl sulfoxide (DMSO; Aldrich Chemical, Milwaukee, WI, USA) was added to the medium.

Subculture of small hepatocyte colonies

To collect the colonies, they were detached from dishes 14 days after plating. The method used for the subculture of SH colonies was previously described.[5] The colonies ($3–5 \times 10^3$ colonies/60-mm dish) were plated on dishes coated with rat tail collagen. One day after replating, the medium was replaced with serum-free DMEM supplemented with 1% DMSO. Ten days after the subculture, the cells were overlaid with growth factor-reduced Matrigel (500 μg/dish; Becton Dickinson, Bedford, MA, USA).

Photographs of cells

Morphological changes of SH colonies were observed and recorded using a phase-contrast microscope equipped with a CCD camera (Olympus Optical, Tokyo, Japan).

Induction of CYP proteins

3-Methylcholanthrene (3-MC; Wako Pure Chemical, Tokyo, Japan), phenobarbital (PB; Wako Pure Chemical), pregnenolone-16α-carbonitrile (PCN; Sigma Chemical), clofibric acid (CLOFA; Ayerst Laboratories, New York, NY, USA), and ethanol (EtOH; Katayama Chemical) were used as CYP inducers. To enhance CYP expression, the medium containing the agent was renewed every day for three consecutive days before harvest (Fig. 1).

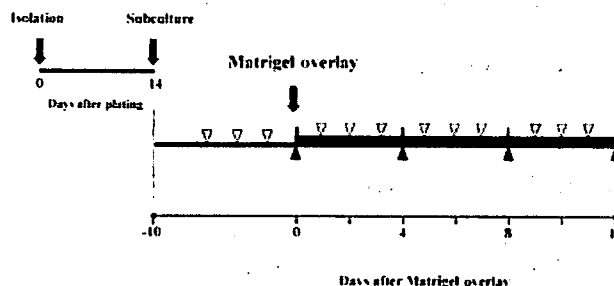

Figure 1. Illustration of experimental schedule. Hepatic cells were isolated from a rat and cultured in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 10 mmol/L nicotinamide, 1 mmol/L Asc2P, 10 ng/mL epidermal growth factor, hormones and antibiotics. Small hepatocyte colonies were isolated from the culture dishes at 14 days after plating and replated on rat tail collagen-coated dishes. One day after replating, the medium was replaced by serum-free DMEM. Ten days later, the cells were overlaid with Matrigel (500 μg/dish). White arrowheads indicate the timing of the chemical treatment. Black arrowheads indicate the timing of the cell harvest.

Immunoblots for CYP proteins

The dishes were washed with PBS twice and then treated with 700 μL of MatriSperse Cell Release Solution (Becton Dickinson) for 15 min at 37°C. Thereafter, 300 μL of buffer solution (10 mmol/L HEPES [pH 7.2], 0.25 mol/L sucrose, 0.5 mmol/L $MgCl_2$) was added to the dish. The cells were scraped and collected into microcentrifuge tubes. After pipetting several times with a microsyringe (Hamilton Com, Reno, NV, USA), homogenates were centrifuged at 500 × *g* for 5 min at 4°C. The supernatants were collected and recentrifuged at 1500 × *g*. The supernatant were kept at –80°C until use. Protein concentrations were measured using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). Samples (15 μg/lane) were separated by 10% SDS-PAGE. Rabbit anti-CYP1A2, anti-CYP3A2, goat anti-CYP2B1, anti-CYP4A1, and anti-CYP2E1 (Daiichi Pure Chemical, Tokyo, Japan) antibodies were used for immunoblots. The details were previously described.[5]

CYP enzyme activities

Cells treated with chemicals were harvested at day 32 after isolation (8 days after Matrigel treatment). The cells were washed with PBS twice and then treated with MatriSperse for 15 min at 37°C. After an addition of 1.5 mL of buffer solution (10 mmol/L HEPES [pH 7.2], 0.25 mol/L sucrose, 0.5 mmol/L $MgCl_2$) to the dish, the cells were scraped off and collected into 2.0 mL tubes. Cells were pipetted with a microsyringe and then centrifuged at 1800 × *g* for 15 min at 4°C. The supernatants were collected and recentrifuged at 9000 × *g* for 10 min. Then the supernatants were collected and centrifuged at 105 000 × *g* for 1 h at 4°C. The pellets were suspended with 200 μL of the buffer solution. Concentrations of the proteins were measured and the samples were kept at –80°C until use. A reaction mixture (NADPH Regenerating System, Gentest, Woburn, MA: 0.5 mg/mL of the protein, 1.3 mmol/L $NADP^+$, 3.3 mmol/L glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mmol/L magnesium chloride) and drug substrates in 0.1 mol/L potassium phosphate buffer (pH 7.4) were incubated at 37°C. 7-Ethoxyresorufin-*O*-deethylase activity (5 μmol/L 7-ethoxyresorufin as a substrate) catalyzed by CYP1A was fluorometrically measured according to the method of Burke and Mayer with some modifications.[11] Briefly, high-performance liquid chromatography (HPLC) was performed with a CAPCELL PAK C_{18} UG120 column (Shiseido, Tokyo, Japan) and the metabolites were detected with a fluorescence detector (Ex. 575 nm, Em. 595 nm). Activities of testosterone-6β-hydroxylation and -16β-hydroxylation (substrate concentrations; 120 μmol/L) catalyzed by CYP3A and CYP2B were determined using HPLC with a Cosmosil percentage C18-AR column (Nacalai Tesque, Kyoto, Japan) and a UV detector (240 nm). Activity of chlorzoxazone 6-hydroxylation (100 μmol/L) catalyzed by CYP2E was determined using HPLC with an Inertsil ODS-3 column (GL Sciences, Tokyo, Japan) and a UV detector (280 nm).[12,13]

Immunocytochemistry for CYP proteins in small hepatocyte colonies

Cells were fixed with cold absolute ethanol at 4 days after the Matrigel treatment. Sheep anti-CYP1A2, rabbit anti-CYP3A2, anti-CYP2E1, and anti-CYP4A1/2/3 antibodies (Chemicon, Temecula, CA, USA) were used as the primary antibodies. The details were previously described.[5]

Statistics

Statistical analysis was performed using Student's *t*-test. A *P*-value of 0.05 was considered significant.

RESULTS

Matrigel effects on morphology of small hepatocyte colonies

Small hepatocytes began dividing from day 3 and rapidly proliferated to form colonies.[4,5] When many colonies grew to consist of 30–50 cells (14 days after plating), we harvested SH colonies from dishes and 3–5 × 10^3 colonies were replated on the dishes. The replated SH colonies attached to the dishes and most cells in the colonies could continue proliferating (Fig. 2). Although the colonies were accompanied by some NPC at the time of replating, their growth was gradually suppressed. When SH could actively proliferate maintaining a monolayer (Fig. 2a–d), the colonies were treated with Matrigel. SH attached to Matrigel and rapidly changed their morphology from flat to rising/piling-up. Although most cells covered by Matrigel in the colony changed shape, some SH restarted to proliferate from the edge of the colony (Fig. 2g,h, arrowheads).

Expression of CYP proteins induced by various chemicals

Fetal serum is known to include unknown factors.[14,15] Thus, to exclude the influence of the factors on CYP expression, replated cells were cultured in serum-free medium. To determine effective concentrations of the chemicals, we examined the dose-dependent expression of CYP (Fig. 3). At day 8 after Matrigel treatment, the cells were exposed to 3-MC, PB, PCN, CLOFA, and EtOH at concentrations of 0–5 μmol/L, 0–2 mmol/L, 0–2 μmol/L, 0–0.5 mmol/L, and 0–500 mmol/L, respectively. Although a single administration of 3-MC was enough for the induction of CYP1A1/2, for the other chemicals 3 days of consecutive administration was necessary to obtain maximal induction. CYP 1A1 and 1A2, 2B1, 3A2, and 4A1 were dose-dependently induced in the cells with or without Matrigel. The expression of CYP was greater in the cells with Matrigel than in the cells without it. However, dose-dependent induction of CYP2E1 expression by EtOH was not

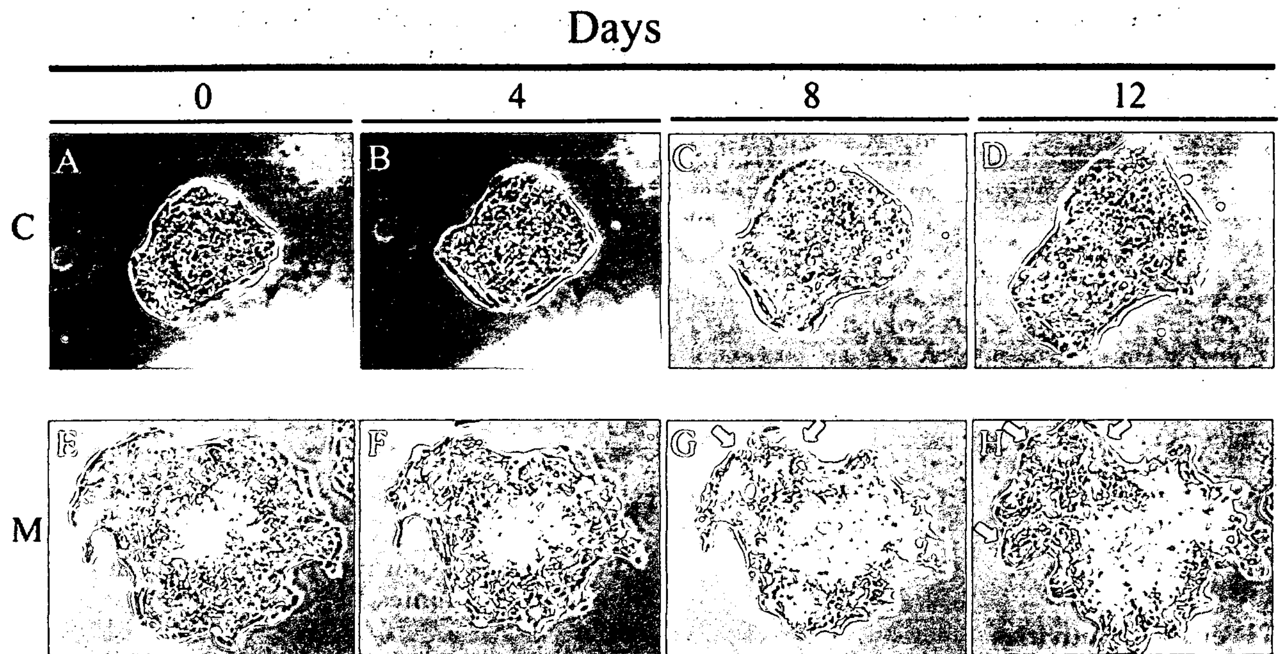

Figure 2 Phase-contrast micrographs of a replated small hepatocytes (SH) colony treated with (e–h) or without (a–d) Matrigel. Photos of the same colony were taken at days 0 (10 days after subculture; a, e), 4 (b, f), 8 (c, g), and 12 (d, h) after Matrigel treatment. White arrows (g, h) show the expansion of SH from the edge of the colony. All photos show the same magnification. Scale bar, 100 μm.

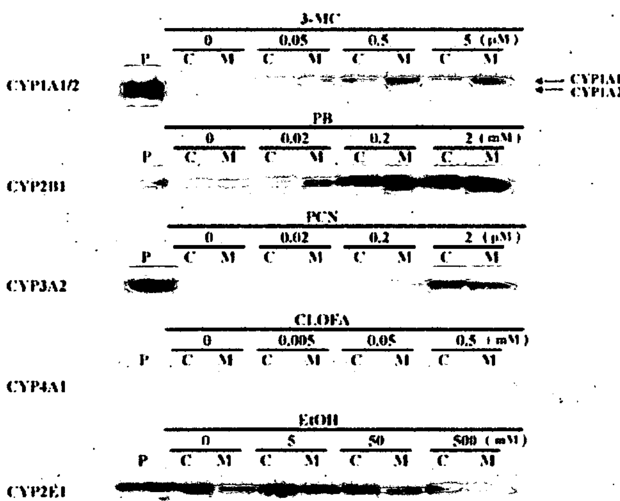

Figure 3 Immunoblots for cytochrome P450 (CYP) proteins induced by various chemicals in small hepatocytes treated with 'M' or without Matrigel 'C'. CYP1A1/2, CYP2B1, CYP3A2, CYP4A1, and CYP2E1 proteins were induced by 3-MC, PB, PCN, CLOFA, and EtOH, respectively. Cells were treated with the chemicals or a vehicle for 3 consecutive days. At 8 days after the Matrigel treatment, cells were harvested. Samples (15 μg/lane) were separated by 10% SDS-PAGE. 'P' shows the commercially available positive control specific for CYP1A2, CYP2B1, CYP3A2, and CYP2E1. Positive control for CYP4A1 was prepared from isolated rat hepatocytes.

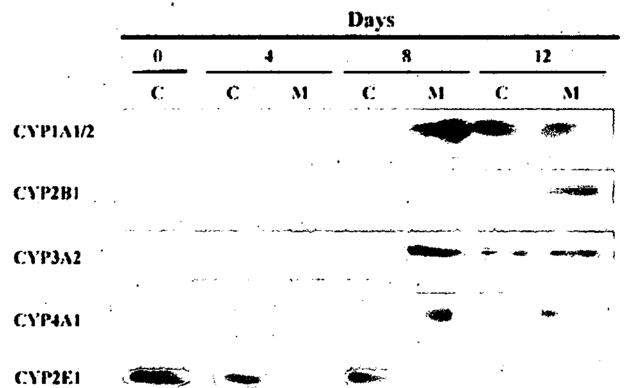

Figure 4 Time course of cytochrome P450 (CYP) expression induced by various chemicals in small hepatocytes treated with 'M' or without Matrigel 'C'. Expression of CYP1A1/2, CYP2B1, CYP3A2, CYP4A1, and CYP2E1 was induced by 5 μM 3-MC, 2 mmol/L PB, 2 μmol/L pregnenolone-16α-carbonitrile, 0.5 mmol/L clofibric acid, and 50 mmol/L ethanol (EtOH), respectively. The agents were treated for 3 consecutive days and then cells were harvested. Samples (15 μg/lane) were separated by 10% SDS-PAGE.

observed. Although the concentrations of 10 μmol/L 3-MC, 5 mmol/L PB, 5 μmol/L PCN, 1 mmol/L CLOFA, and 1 mol/L EtOH, were also examined, dose-dependent expression was not observed in the present experiment.

As shown in Fig. 4, CYP 1A1, 3A2, and 2E1 could be expressed in the cells even at day 0. Although, with time in culture, the induced expression of CYP 1A1,

2B1, 3A2, and 4A1 increased in the cells treated with or without Matrigel, the expression in the cells with Matrigel was much larger than in the cells without Matrigel. In addition, CYP 1A2 expression was detected in the cells with Matrigel. However, at day 12 the amount of CYP protein was not increased compared to that of each protein at day 8. In contrast, much more CYP 2E1 was induced in the cells without Matrigel than in the cells with Matrigel. Furthermore, the strength of the induction decreased with time in culture in both cells with and without Matrigel.

CYP activities of small hepatocytes

We investigated whether the expressed CYP in the SH really had enzymatic activities. As shown in Table 1, 7-ethoxyresorufin-*O*-deethylase activity induced by 3-MC in the cells treated with Matrigel was 121.2-fold higher than in the control. Amounts of testosterone-16β-hydroxylation induced by 2 mmol/L PB and testosterone-6β-hydroxylation induced by 2 μmol/L PCN in the cells treated with Matrigel were 2.8-fold and 6.4-fold larger than in the control, respectively. In contrast, chlorzoxazone 6-hydroxylase activity in the cells with Matrigel was less than in the control. As a large number of cells were necessary, we could not measure CYP4A activity in the present experiment.

Immunocytochemistry for CYP isozymes in small hepatocyte colonies

We carried out the immunostaining for CYP to examine whether CYP expression was correlated with the morphological changes of SH. As shown in Fig. 5, rising/piling-up cells in the colonies treated with Matrigel were strongly stained with CYP. The expression of CYP was much stronger in the treated cells than in the control (Fig. 5c,i,o,u). Some large cells, which seemed to be MH accompanying the replated colonies, were faintly positive (Fig. 5a,g,m,s).

DISCUSSION

Primary hepatocytes obtained from laboratory animals and humans have been used as an *in vitro* assay system to examine whether xenobiotics are inducers of CYP enzymes. With the use of the cultured cells, some problems still remain to be resolved; the most important one is that CYP enzyme activities rapidly disappear from the cells. Primary rodent hepatocytes were reported to lose the expression of CYP mRNA and proteins within 48 h after plating when they were traditionally cultured.[16,17] Therefore, many researchers have tried to improve culture conditions of hepatocytes in order to maintain constitutive and inducible expression of CYP. As the expression of the liver-specific genes is regulated by various factors, soluble agents, cell–cell interaction, and ECM have been used in the culture. When the cells were cultured on ECM such as collagen gel,[18–22] laminin[23] and Matrigel,[24–27] the liver-specific functions could be maintained for about a week. Kocarek *et al.*[16] showed that hepatocytes cultured on Matrigel could re-express mRNA of several constitutive CYP for 5 days. Gómez-Lechón *et al.*[22] showed that hepatocytes entrapped in collagen gels could survive for 3 weeks and maintain biotransformation activities of CYP1A, CYP2A, CYP2B, and CYP3A. However, there is no report that proliferated hepatocytes can recover the constitutive expression of CYP and that the expression can be induced by the administration of particular chemicals even after hepatic cells are cultured for more than a month. In the present study, we showed that SH could acquire the inducibility of CYP of not only proteins but also enzyme activities. Until now, to supply hepatocytes used for pharmacological and pharmaceutical investigations it has been necessary to isolate mature hepatocytes for every experiment because there are few cell lines possessing hepatic differentiated functions, especially CYP enzyme activities. Furthermore, the number of obtainable cells depends on the number of hepatocytes in individuals because a method of proliferating hepatocytes with differentiated functions has not been established. However, by using SH, these

Table 1 Cytochrome P450 activities induced by the agents in small hepatocytes

P450 Isozymes	Inducer	Liver microsomes† (pmol/min/mg protein)	Control‡ (pmol/min/mg protein)	Matrigel − (pmol/min/mg protein)	Matrigel + (pmol/min/mg protein)
CYP1A	3-MC(5 μM)	159.3	8.0	137.2 [17.1]§	972.0 [121.2]
CYP2B	PB(2 mM)	118.7	23.3	36.5 [1.7]	66.2 [2.8]
CYP3A	PCN(2 μM)	1808.0	45.1	82.5 [1.8]	289.9 [6.4]
CYP2E	EtOH(50 mM)	279.2	235.0	625.9 [2.7]	182.5 [0.8]

Five days after the Matrigel treatment, each inducer was added to the medium. The medium containing the inducer was renewed each day for 3 consecutive days. The activities of CYP1A, CYP2B, CYP3A, and CYP2E were determined as 7-ethoxyresorufin-*O*-deethylation, testosterone-16β-hydroxylation, testosterone-6β-hydroxylation, and chlorzoxazone 6-hydroxylation, respectively, as described in the Methods section.

†Liver microsomes from untreated male rats were purchased from Xeno Tech, LLC. ‡Control shows the activities in small hepatocytes cultured without inducers at day 18 after subculture. §Values in square blackets show the enhancement compared to each control.

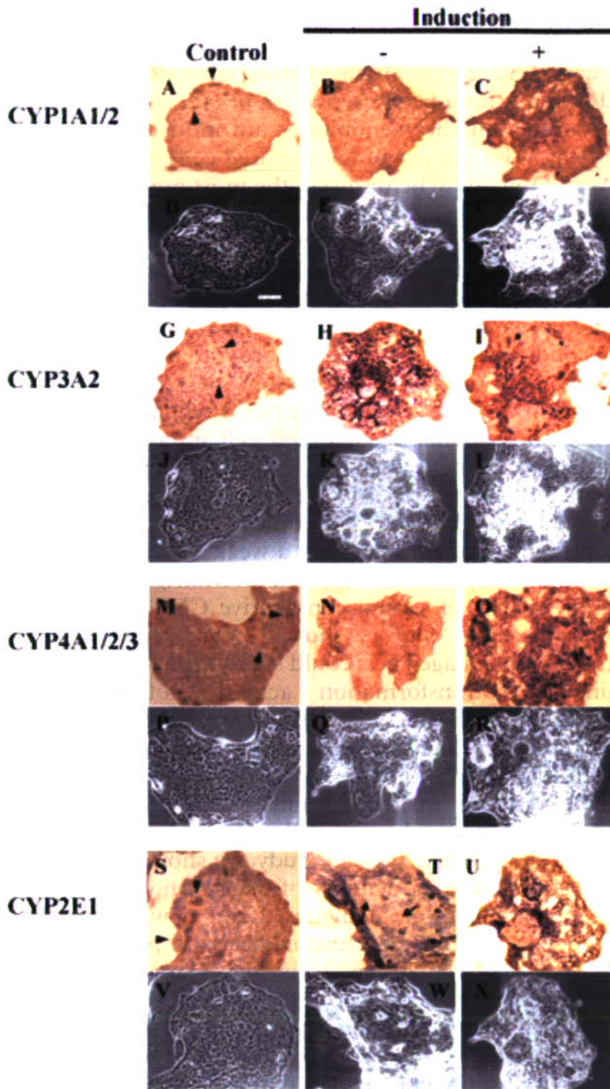

Figure 5 Immunocytochemistry for cytochrome P450 (CYP) in small hepatocytes (SH) colonies treated with various chemicals. The cells were cultured in serum-free medium for 14 days after replating and treated with 500 μg of Matrigel 4 days before fixation (b, c, h, i, n, o, t, u). Expression of CYP1A1/2, CYP3A2, CYP4A1/2/3, and CYP2E1 was induced by 5 μmol/L 3-MC (c), 2 μMOL/L pregnenolone-16α-carbonitrile (i), 0.5 mmol/L clofibric acid (o), and 50 mmol/L ethanol (EtOH) (u), respectively. The agents were treated for 3 consecutive days before fixation. Each phase-contrast photograph corresponds to the one above it. The cells were fixed with cold absolute ethanol and immunocytochemistry was carried out. The cells were counterstained with hematoxylin. Arrowheads in controls show cells that are large and/or binucleate and are strongly stained compared to SH. Although the cells shown by arrows in (t) are large and/or binucleate, the degree of brown staining of the cells is similar to that of SH. All photos show the same magnification. Scale bar, 100 μm.

problems may be resolved because SH can be isolated from adult rodents and they can continue proliferating for a long time. When we collect SH colonies and replate them on the new dishes, SH rapidly proliferate. After SH proliferate and reach a number, Matrigel treatment can induce their maturation. Thus, use of this culture system may reduce the number of animals required for experiments.

There are three advantages of this chemically defined culture system: first, this culture system can exclude serum from the medium at the time of chemical treatments. It is known that serum contains factors that may modulate CYP expression.[28,29] Once SH form a colony of a certain size, they can continue proliferating without serum. Therefore, after subculture of SH colonies, we could avoid the influence of serum. Second, we can minimize the effects of solvents on the CYP activity. As prototypical inducers are often lipophilic in nature, solvents are required for their solubilization in the medium. However, the potential influences of solvents on CYP activity have been pointed out.[30–32] In our culture system, as DMSO is already added to the culture medium, for many agents that have a lipophilic nature DMSO may function as a solvent. In addition, as the 1% concentration of DMSO is relatively high, we can use higher concentrations of the agents in the experiments compared to the concentrations that have hitherto been used. Third, whenever we need well-differentiated hepatocytes, we can prepare any number of cells that is required because SH colonies can be cryopreserved for more than 1 year.[33] We confirmed that the thawed cells could attach to the dishes and differentiate into mature ones when the cells were treated with Matrigel following the same protocol as shown in this experiment (data not shown).

LETF are the key transacting elements in control of hepatic gene expression.[34] Recent studies on gene promoter and enhancer sequences have revealed that several different LETF, including HNF1α, HNF1β, HNF3, HNF4, C/EBPα, C/EBPβ, and DBP, might regulate constitutive and inducible CYP expression. Furthermore, in most cases, two or more factors are known to be involved in liver-specific gene expression.[35] Regulation of the CYP2 family has been most extensively studied. Of the CYP2 family, 2E1 was the only isozyme whose regulation of gene expression might be dependent on HNF1α. No effects were observed with HNF4, C/EBPα, and C/EBPβ.[36] As shown in our previous study,[5] the differentiated hepatocytes induced by Matrigel could strongly express LETF such as HNF4, HNF6, C/EBPα, and C/EBPβ, whereas the expression of HNF1α was suppressed. In the present experiment, SH constitutively expressed CYP1A1/2, 2B1, 3A2, and 2E1, and in the differentiated cells treated with Matrigel their expression increased. In addition, dose-dependent expression of CYP isozymes by each agent was observed in the cells. The patterns of CYP induction by appropriate agents were similar to those in freshly isolated hepatocytes.[37] However, CYP2E1 expression decreased with time in culture and, the cells treated with Matrigel dramatically lost their expression. *In vitro* transcription and transactivation studies have reported that CYP2E1 is controlled in part by HNF1α.[36,38] Therefore, this

decreased expression of CYP2E1 might be related to the suppression of HNF1α expression.

The appearance of SH-like cells has been reported in experimentally injured animals. In rodents treated with hepatotoxins such as D-galactosamine,[39] allyl alcohol,[40] retrorsine,[41] and dipin,[42] clusters of SH-like cells were observed in the livers and the cells could differentiate into MH. Gene expression, especially of CYP isozymes, of the SH-like cells that appeared in the livers was reported to be different from those of MH.[41] Therefore, SH-like cells and/or their progeny may escape from the toxicity of the agents. In the present experiment we showed that the expression pattern of CYP in SH was different from that in MH and that some CYP activities were low in SH. Although the morphological and cytological appearances of the 'SH-like cells' observed *in vitro and in vivo* are very similar, their equivalence has not been proved. As specific markers of SH have not been clarified, the precise origin or location within the liver cannot be defined either. When the marker of SH is found and the equivalence of SH *in vitro* and SH-like cells *in vivo* can be proved, the mechanisms of the appearance of SH *in vivo* may be easily investigated and the origin of hepatic stem/progenitor cells will be revealed. However, further experiments will be necessary to clarify the characteristics of SH.

In conclusion, this culture system may be useful for CYP induction studies as an *in vitro* system. When SH are isolated from human livers, the cells will be useful in not only pharmacological and toxicological studies, and for the development of new drugs, but also in regenerative medicine such as cell transplantation and gene therapy, and a bioartificial liver assist system.

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Coordinated Movement of Bile Canalicular Networks Reconstructed by Rat Small Hepatocytes

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Abstract—Hepatocytes *in vivo* have a potential for liver regeneration, but it has been very difficult to reconstruct hepatic organoids *in vitro*. Recent studies have shown that small hepatocytes (SHs) can reconstruct hepatic organoids including functional bile canaliculi (BC). In the present study we analyzed the movement of BC formed in the hepatic organoids, focusing on the coordination of contraction and dilation among cells and the mechanism producing the coordination. Hepatic cells, including SHs, were isolated from an adult rat liver and cultured. Time-lapse images of BC movements were taken and analyzed in cells treated with or without cytochalasin B (CB). Time-lapse images revealed that all BC, regardless of region contracted in a coordinated manner. Actin filaments were observed along the BC even after the BC networks treated with CB dilated markedly. Microinjection of dye was also carried out to investigate the flow thorough BC. Secreted fluorescein from the injected cell flowed along BC, and gap junctional protein connexin 32 was expressed along BC networks, suggesting cell-to-cell communication. Thus, groups of hepatocytes in the hepatic organoids act in a coordinated manner through intercellular communication.

Keywords—Gap junctional communication, Actin, Cytochalasin B.

INTRODUCTION

Liver regeneration is a unique capability of the liver *in vivo*.[5,16] It is well known that hepatocytes can proliferate to recover the lost tissue after two-thirds partial hepatectomy. Although hepatocytes *in vivo* have the potential for liver regeneration, it has been very difficult to reconstruct hepatic tissues *in vitro*. However, reconstruction of hepatic organoids is needed for the transplantation of tissue-engineered organs.[37]

Recently, two approaches for the culture of hepatic organoids have been demonstrated.[15,20] Hepatic progenitor cells, identified as proliferating cells with hepatic characteristics, were found and named small hepatocytes (SHs).[17,18,21] Although primary hepatocytes rapidly lose their function with time in culture, SHs can reconstruct hepatic organoids interacting with hepatic nonparenchymal cells (NPCs) such as liver epithelial cells and stellate cells.[20] For hepatic organoid formation, extracellular matrices, especially components of basement membrane such as laminin and type IV collagen, were shown to be important and treatment with EHS gel (Matrigel®) could induce the maturation of SHs.[36] On the other hand, Michalopoulos *et al.*[15] reported that they developed hepatic organoids consisting of hepatocytes and fenestrated endothelium when hepatic cells on polystyrene beads were cultured in roller bottles. Furthermore, when cells isolated from a rat liver were cultured in the roller bottles with rotation, the cells formed a characteristic and reproducible tissue architecture composed of a superficial layer of biliary epithelial cells, an intermediate layer of connective tissue and hepatocytes, and a basal layer of endothelial cells.[14] These experiments demonstrated the formation of hepatic organoids, and revealed the structural features of the organoids. Although the formation of hepatic organoids has been morphologically investigated, tissue-level functions of the cells are not well analyzed.

Bile canalicular contraction is one of the tissue-level functions of the liver, because it is achieved by the coordination of adjacent hepatocytes. Bile canaliculi (BC) are tubular structures that form the most proximal channels of the biliary tree and carry bile secreted by hepatocytes. To expel bile through BC, a series of hepatocytes along BC must contract in a coordinated fashion. Therefore, the hepatic tissue needs to have communications and integration among neighboring hepatocytes.

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Abbreviations: SH, small hepatocyte; BC, bile canaliculi; CB, cytochalasin B; NPC, nonparenchymal cell; Cx, connexin; ROI, region of interest; FD, fluorescein diacetate; DAPI, 4′, 6-diamidino-2-phenylindole; CMFDA, CellTracker™ green 5-chloromethyl-fluorescein diacetate; MRP2, multidrug-resistance associated protein 2.

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It has been difficult to reconstruct hepatic organoids possessing BC networks with motile activity. However, we recently demonstrated that SHs could form BC with motility and secretory function.[35] BC were formed and three-dimensionally extended BC networks were developed in the hepatic organoids. In the reconstructed tissues, BC could continuously contract and dilate. Although the motility of BC was demonstrated, coordination of the movements was not analyzed. To understand the mechanism of bile transportation, the coordination of BC movements should be clarified. Thus, in the present study we analyzed movements of BC formed in the hepatic organoids and verified that BC movements were coordinated. We found that BC contractions occurring in different regions could act synchronously, and that intercellular communication through gap junctions might be important for the coordination of BC movements.

MATERIALS AND METHODS

Isolation and Culture of SHs and NPCs of the Rat

The cells were isolated from male Sprague-Dawley rats (250–450 g; Nippon Bio-Supp. Center, Tokyo, Japan) using Seglen's[32] two-step liver perfusion method with some modifications.[20] All animals used in the experiments received humane care and the experimental protocol was approved by the Committee of Laboratory Animals following Keio University guidelines. Details of isolation and culture of liver cells were previously described.[20] The cells were suspended in DMEM (Sigma-Aldrich Co., St. Louis, MO) with 20 mM HEPES, 25 mM $NaHCO_3$, 30 mg/l L-proline, 0.5 mg/l insulin, 10^{-7} M dexamethasone, 10% FBS, 10 mM nicotinamide (Sigma-Aldrich), 1 mM ascorbic acid 2-phosphate (Wako Pure Chemical, Tokyo, Japan), 10 ng/ml EGF (BD biosciences, Bedford, MA) and antibiotics, and the number of viable cells was counted. The cells were inoculated on culture dishes (4.5×10^5 viable cells/35-mm dish; Corning Glass Works, Corning, NY) coated with rat-tail collagen (50 μg of dried tendon/0.1% acetic acid) and placed in a humidified, 5% CO_2/95% air incubator at 37°C. Medium was changed to remove dead cells about 3 h after inoculation, and the medium was subsequently changed every other day. After day 4 (96 h after plating), 1% DMSO (Sigma-Aldrich) was added to the culture medium.

Time-Lapse Microscopy for the BC Contractions

Cells were placed in a humidified 5% CO_2/95% air chamber (Sankei Co., Tokyo, Japan) at 37°C, and photographed using a phase-contrast microscope (TE300; Nikon, Tokyo, Japan) equipped with a CCD camera (Roper Scientific, Trenton, NJ). Images of the BC networks formed in a colony with piled-up cells were recorded at 5-s intervals for 3 h. The sequential images (2,160 frames/colony and 17 colonies) were analyzed using the MetaMorph® imaging system (Universal Imaging Corporation™, Downingtown, PA).

Analysis of BC Movements

In this experiment, 17 colonies including well-developed BC networks were photographed using a phase-contrast microscope equipped with a time-lapse device. Measurement of BC movements was based on variations of translucent areas coinciding with BC networks in the sequence of phase-contrast micrographs. To quantify the BC areas, a specific threshold value that distinguished BC from cytoplasm was chosen with reference to translucent belts seen under phase-contrast microscopy. The threshold value was set using the first image of the time-lapse series because pixel intensity of the translucent belts varied in each time-lapse series. The threshold value was adjusted to fit thresholding areas to the translucent belts. For the quantitative analysis of the BC movement in the time-lapse series, thresholding was applied to the first time-lapse image and the same threshold value of the light intensity was applied to the other sequential images so that consistent thresholding applied. Next, four regions of interest (ROIs) were set for each image in the time-lapse series. To investigate the movements of various parts of the BC network, ROIs were randomly placed throughout the colony in the first image, avoiding neighboring cells. The same ROIs were set in the other sequential images of the same time-lapse series. We then measured the thresholding areas within each ROI to investigate the width of each BC. The thresholding areas were normalized by dividing the measurement of each time by that of the initial time. Variations of the normalized thresholding areas were compared to analyze the BC movements. Therefore, the results accurately represent BC movements without any biased data. The variations in the thresholding areas reflect the width of the BC. In this experiment, BC in 68 ROIs (4 ROIs/colony and 17 colonies) were analyzed.

To quantify the coordination of BC movements, a correlation coefficient was calculated for each colony. The correlation coefficients between the values of each ROI during the observation period (10 min for fast movements, 30 min for slow movements) were calculated. The correlation coefficient R_{xy} between pairs of the thresholding area in each ROI is given as

$$R_{xy} \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2 \sum (y_i - \bar{y})^2}} \quad (1)$$

where $\bar{x}$ and $\bar{y}$ are mean thresholding areas in each ROI. R_{xy} ranges from -1 to $+1$, and -1 and $+1$ show strong associations in a negative and positive manner, respectively.

FD Treatment

In hepatocytes, fluorescein diacetate (FD) is metabolized by intracellular esterase, to become fluorescein. The

cytoplasmic fluorescein is actively transported into the BC via multidrug-resistance associated protein 2 (MRP2). To visualize the lumen of the BC networks, FD was added to the medium at a final concentration of 2.5 μg/ml. After 15-min incubation, the cells were rinsed three times with a medium containing no FD and then photographed using a phase-contrast microscope equipped with a fluorescence device.

CB Treatment and Immunocytochemistry for Actin

To investigate the effects of cytochalasin B (CB; Sigma-Aldrich) on the behavior of BC networks, SH colonies were treated with CB. Sequential photographs were taken soon after the addition of CB to the medium. The CB solution was prepared from stock solution (25 mg/ml in DMSO). The cells were treated with a final concentration of 0.1, 1.0, 5.0, and 10.0 μg/ml CB in modified DMEM for 3 h. After images were obtained, the cells were fixed in cold absolute ethanol and kept at −20°C until use. The actin filaments were visualized by incubation with Alexa Fluor 594 phalloidin (Molecular Probes, Eugene, OR) for 1 h.

Immunofluorescent Staining for Gap Junctional Protein Cx 32

The cells cultured on coverslips coated with rat-tail collagen were fixed in cold acetone for 5 min and double-immunofluorescent staining for connexin 32 (Cx 32) and actin was carried out. We used a mouse anti-Cx 32 antibody (Zymed Laboratories Inc., South San Francisco, CA) as the primary antibody and Alexa Fluor 594 phalloidin (Molecular Probes). An Alexa Fluor 488 anti-mouse IgG antibody (Molecular Probes) was used as the secondary antibody. The dishes were mounted with 90% glycerol containing 1 g/l *p*-phenylenediamine and 1 mg/l 4′, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

Microinjection of Dye into the Piled-Up Cells and Transportation of Fluorescence

Before dye microinjection, the medium was replaced with L-15 (Invitrogen Co., Grand Island NY) medium. To observe flow through BC, 10 μM CellTracker™ green 5-chloromethyl-fluorescein diacetate (CMFDA; Molecular Probes) was microinjected (Eppendorf Micromanipulator system; Eppendorf, Hamburg, Germany) into one of the piled-up cells in each colony. When CMFDA is microinjected into cells, esterase in their cytoplasm hydrolyzes nonfluorescent CMFDA to fluorescent 5-chloromethylfluorescein. In hepatocytes, ATP-dependent multidrug transporters excrete the glutathione-conjugated fluorescent dye into BC.[30] Fluorescent images of the cells were recorded at 5-s intervals for 5 min using a fluorescence microscope equipped with a time-lapse system (Roper Scientific). More than 10 microinjections were performed.

RESULTS

BC Formation and Quantification of the BC Area

SHs started to proliferate 2–3 days after inoculation and then formed colonies. Although SH colonies appeared as flat clusters in the early period of the culture, they expanded to form large colonies, some of which were composed of piled-up cells. The piled-up cells had the appearance of mature hepatocytes. In the areas between the piled-up cells BC formed and developed into anastomosing networks as we previously reported.[35] One-fourth of the SH colonies displayed the piled-up cells that formed BC by day 30.[19]

BC networks were detected as translucent belts under phase-contrast microscopy [Fig. 1(A)]. We ascertained whether the translucent belts represented the width of the BC using FD as a probe to visualize BC networks. The BC in the piled-up SHs are functional as an apical domain including the secretory functions for bilirubin and fluorescein as we previously reported.[35] In addition, secreted fluorescein was not diffused after rinsing with the medium because the BC were sealed by tight junctions as previously reported by immunocytochemistry for tight junction-associated protein ZO-1.[35] Therefore, secreted fluorescein can indicate the

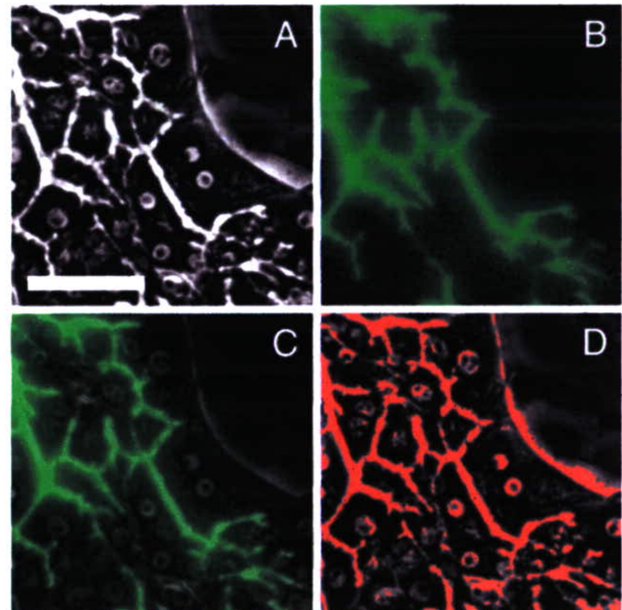

FIGURE 1. Corresponding images of an SH colony in which piled-up cells formed BC networks. A: phase-contrast micrograph of the cells at day 30 shows translucent networks between the cells representing BC. B: fluorescent image of the cells after FD treatment shows metabolized fluorescein secreted into BC. C: the merged image of the phase-contrast and fluorescent image indicates that the translucent networks under phase-contrast microscopy coincide with the fluorescent networks of fluorescein. D: the merged image of the phase-contrast and thresholding images. Thresholding to the phase-contrast image was applied to clarify the region of the BC, and is shown in red. Scale bar, 50 μm.