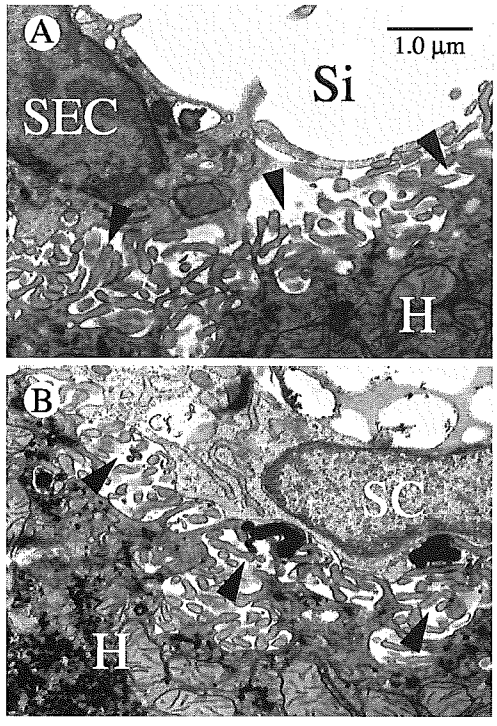

Fig. 4. Ultrastructural analysis of sinusoidal interstitium in regenerating liver. The liver 7 days after PHx was fixed and observed by EM. (A) Electron micrograph shows sinusoidal interstitium (space of Disse) between SEC and hepatocyte. (B) Stellate cell is localized in sinusoidal interstitium. Arrowheads indicate space of Disse. No BM as defined by electron microscopy was observed in sinusoidal interstitium. H, hepatocyte; SEC, sinusoidal endothelial cell; SC, stellate cell; Si, sinusoid. Scale bar, 1.0 μm.

disappeared by 12 weeks of age (data not shown). Therefore, although the investigators did not provide details, the mice used were likely less than 12 weeks old. Laminin α1 is a subunit of laminin-1 that has been well studied [36]. Interestingly, the expression of α1 is restricted in fetal and adult tissues [45,46]. Laminin α1-deficient mice die at early stages of embryogenesis [47]. The null embryos cannot form Reichert's membrane, an extraembryonic BL. The roles of α1 in late embryogenesis and adult tissues are still unknown.

Stellate cells are regarded as the principal matrix-producing cell of the liver [48–50]. We also found that the cellular source of laminin α1 was stellate cells but not SECs and Kupffer cells. Maher et al. showed that an antibody to laminin-1 stained stellate cells isolated from normal rat liver [51]. To further examine whether stellate cells express laminin α1, we also tried to isolate stellate cells from normal and regenerating livers. However, the expression of laminin α1 was not observed in cultured stellate cells (unpublished data). Since Loreal et al. also showed that cultured stellate cells lacked α chain [52], the expression of laminin α1 may be regulated under the restricted condition. The expression of laminin α1 in sinusoids reached maximum at 6 days and disappeared by 10 days after PHx. In rodents, hepatocytes replicate during the first 2–3 days of regeneration after PHx [27,28]. Following this replication, SECs seem to invade clusters of replicated hepatocytes and form new sinusoids [1]. Normal architecture of the liver lobule is restored by 7–10 days after PHx [2]. The present result showed that the appearance of the α1 chain in the sinusoid might be correlated with the period of sinusoidal reconstruction. Although Kim et al. showed that laminin increased at the early stages of hepatic regeneration [53], the investigators did not provide details about α chains. In vitro studies also revealed that α1-containing laminin promotes adhesion and spreading of SECs. The SECs isolated from regenerating liver elongated pseudopodia on α1-containing laminin more than those from normal liver did. This result suggests that α1-containing laminin is a key mediator to regulate the function of SECs in sinusoidal reconstruction. In cell adhesion, laminin α1 binds to cell-surface receptors, such as integrins and dystroglycan [5,6]. In hepatic regeneration, SECs seem to increase the expression of laminin receptors and activate them. In the future, we will examine the expression and distribution of laminin receptors in normal and regenerating livers. Open fenestrations are a conspicuous feature of SECs. McGuire et al. reported that laminin-1-containing α1 chain failed to maintain the fenestrations in vitro [54]. However, since the number of fenestrations decreases to less than half by 3 days and recovers to control level by 2 weeks after PHx [43], the accumulation of laminin α1 may be necessary for the formation of fenestration during sinusoidal reconstruction. A current model of BL postulates a polymeric network formed by laminins and collagen IV [55]. Laminin-1 containing the α1 chain can self-assemble into polygonal lattices in vitro through calcium-dependent interactions between short arms [55]. Although the deposition of laminin α1, β2, γ1, and nidogen-1 was observed in the sinusoids of regenerating liver, EM did not reveal the existence of BL. The discontinuous BLs in sinusoidal interstitium of cirrhotic liver [30] may be due to the other α chain containing laminins.

Laminin α5 was ubiquitously distributed in all hepatic BLs but not sinusoidal interstitium. Hepatic laminin α chains that had not been detected in previous studies could have been the α5 chain [39,40]. Since laminin α5-deficient mice die during late embryogenesis [56], we could not analyze its roles in adult mouse liver. The distribution of laminin α4 and the localization of SECs were identical in the regenerating liver, indicating that SECs were expressing the α4 chain. Thyboll et al. reported that targeted deletion of laminin α4 causes the impairment of microvessel maturation [57]. Although the adhesion activity of α4-containing laminin to hepatic cells was weaker than that of α1-containing laminin, α4 chain may be necessary to maintain hepatic architectures such as open fenestrations of SECs. Laminin α2 chain was mainly observed in the hepatic artery but was not a major component of hepatic BLs. Although it was reported that the expression of laminin α2 in hepatic vessels responds to regeneration and aging [40], this remarkable change of α2 chain was not

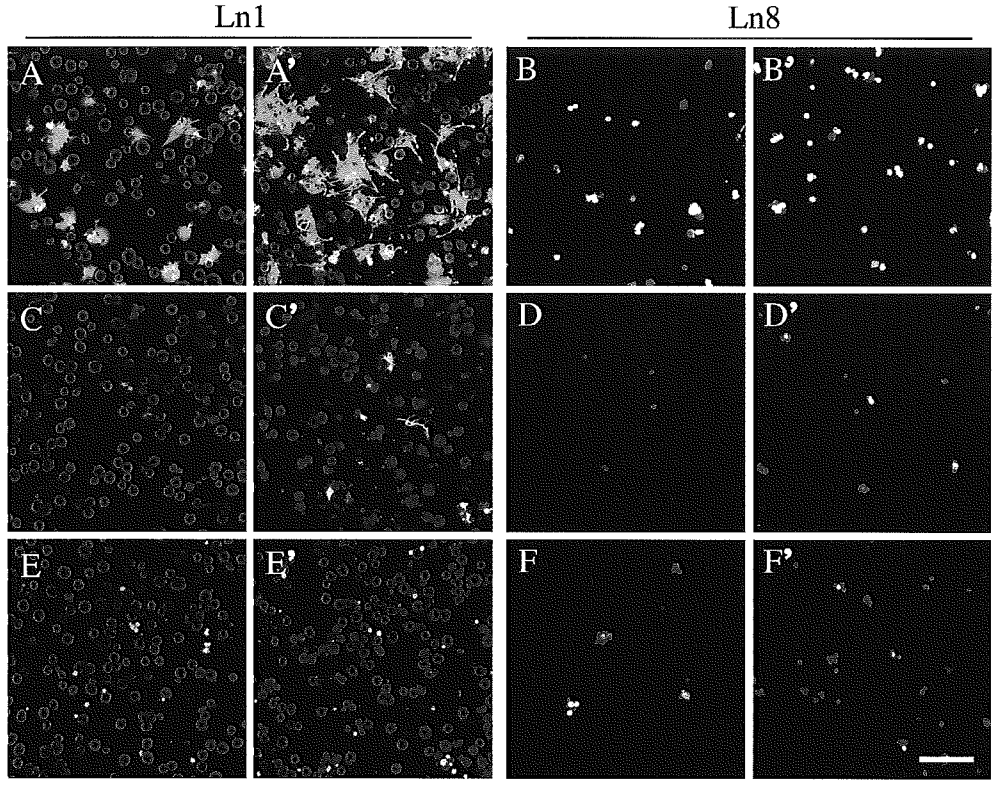

Fig. 5. Attachment and spreading of hepatic cells on surfaces coated with laminin isoforms. Hepatic cells were isolated from normal (A–F) and regenerating rat livers (A′–F′). Cover glasses were coated with 10 μg/ml of α1- or α4-containing laminin (Ln1 or Ln8) and incubated with isolated hepatic cells at 37°C for 3 h. Adherent cells were doubly stained with rabbit antibody to rat albumin to label hepatocytes (all panel, Red), and mouse monoclonal antibodies to SE-1 to label SECs (upper panel, Green), desmin to label stellate cells (middle panel, Green), and ED1 to label Kupffer cells (lower panel, Green). Hepatocytes attached to α1-containing laminin but did not spread. The SECs isolated from regenerating liver elongated pseudopodia on α1-containing laminin more than did those from normal liver. The stellate cells isolated from regenerating liver spread on α1-containing laminin, but Kupffer cells did not. The hepatic cells could weakly attach to α4-containing laminin but did not exhibit cell spreading. Scale bar, 100 μm.

observed in our study. Although laminin γ3 could assemble with α2, α4, α5, β1, and β2 [12,58], it was not observed at the mRNA level in mouse liver [59].

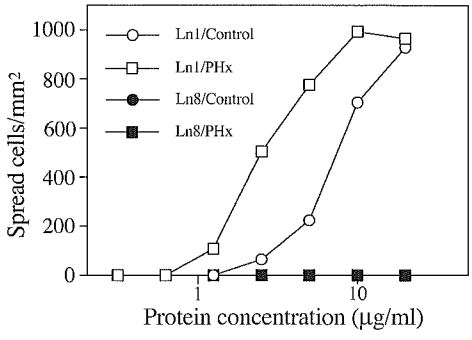

Fig. 6. Quantification of spread SECs on laminin isoforms. 96-well plates were coated with increasing concentrations of α1 (Ln1: open symbols)- or α4 (Ln8: closed symbols)-containing laminin and incubated with the SEC-enriched fraction at 37°C for 1 h. Cells adhering to the plates were stained and counted under the microscope. The spreading of SECs was quantified as described in Materials and methods. SECs isolated from regenerating liver (PHx: square symbols) spread on α1-containing laminin more readily than did cells from normal liver (Control: circle symbols). The hepatic cells did not spread on α4-containing laminin. Similar results were obtained in three independent experiments.

Laminin-5 (α3, β3, γ2) underlying BECs has unique biological activities such as promoting cell scattering, migration, and adhesion [60–62]. It has also been identified as a component of the hemidesmosomes, an anchoring device between epidermis and connective tissue [62–64]. Although it is unclear whether there are hemidesmosomes in bile ducts, laminin-5 seems to mediate tight adhesion of BECs to BL. Laminin β2 is known to be expressed in restricted regions, such as neuromuscular junction and kidney glomerulus [65–67]. Interestingly, our results showed that β2 chain but not β1 is ubiquitously distributed in all hepatic BLs and sinusoidal interstitium. In the present experiment, we could not find the partners of β2 that are present in sinusoids of normal liver. As the γ2 chain was reported to be secreted as a single chain in tumor cells [68], laminin β2 may also be present as monomer in sinusoids.

PHx is an artificial stimulus to study hepatic regeneration. Liver exposed to actual hepatic toxins undergoes either acute or chronic injury. Like PHx, acute injury causes hepatic regeneration associated with remodeling of ECM. In contrast, chronic injury causes hepatic fibrosis and cirrhosis associated with ectopic deposition of ECM. Definition of the roles of laminins in liver may not only elucidate

important aspects of hepatic regeneration, but may also provide a better understanding of the mechanisms of fibrosis.

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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.

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).

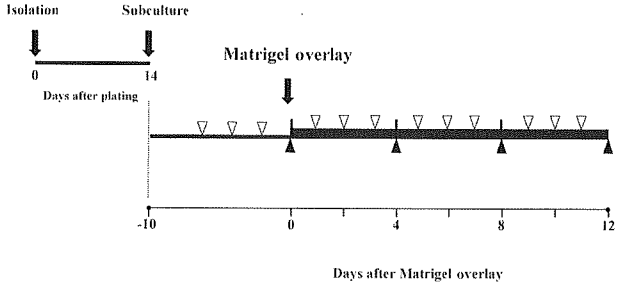

Figure 1 Illustration of experimental schedule. Hepatic cells were isolated from a rat and cultured in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 10 mmol/L nicotinamide, 1 mmol/L Asc2P, 10 ng/mL epidermal growth factor, hormones and antibiotics. Small hepatocyte 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 $500 \times g$ for 5 min at 4°C. The supernatants were collected and recentrifuged at $1500 \times g$. 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 $1800 \times g$ for 15 min at 4°C. The supernatants were collected and recentrifuged at $9000 \times g$ for 10 min. Then the supernatants were collected and centrifuged at $105\,000 \times g$ 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 $3–5 \times 10^3$ 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

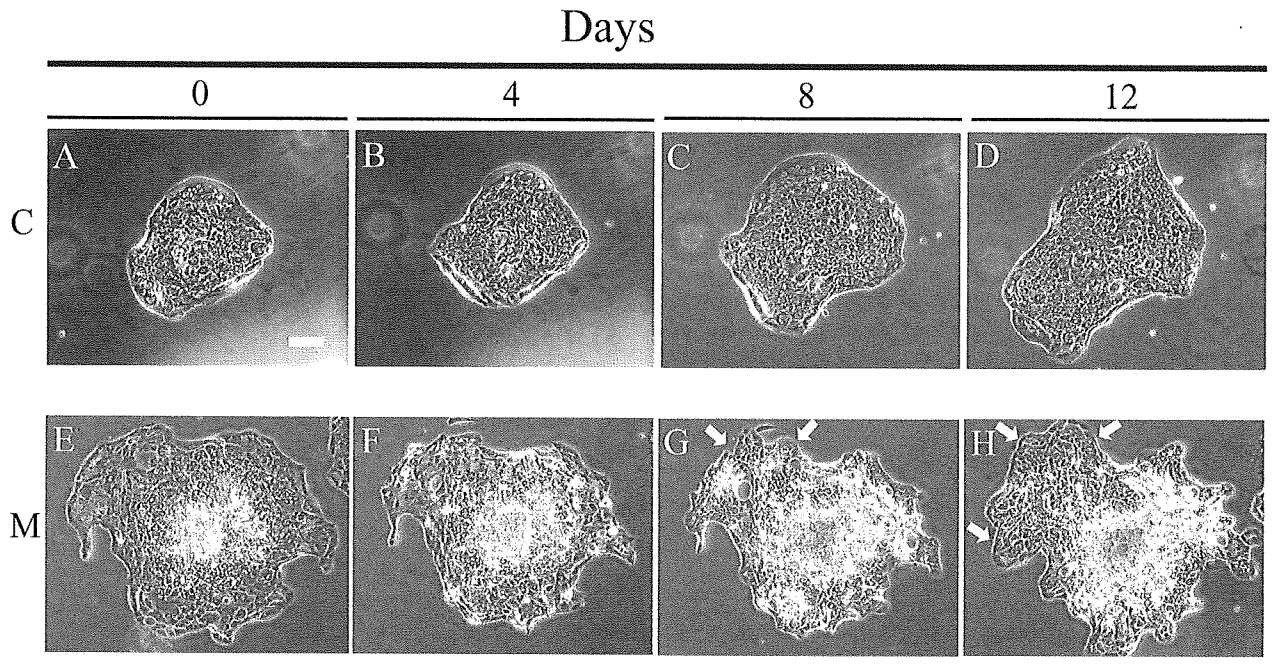

Figure 2 Phase-contrast micrographs of a replated small hepatocytes (SH) colony treated with (e–h) or without (a–d) Matrigel. Photos of the same colony were taken at days 0 (10 days after subculture; a, e), 4 (b, f), 8 (c, g), and 12 (d, h) after Matrigel treatment. White arrows (g, h) show the expansion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

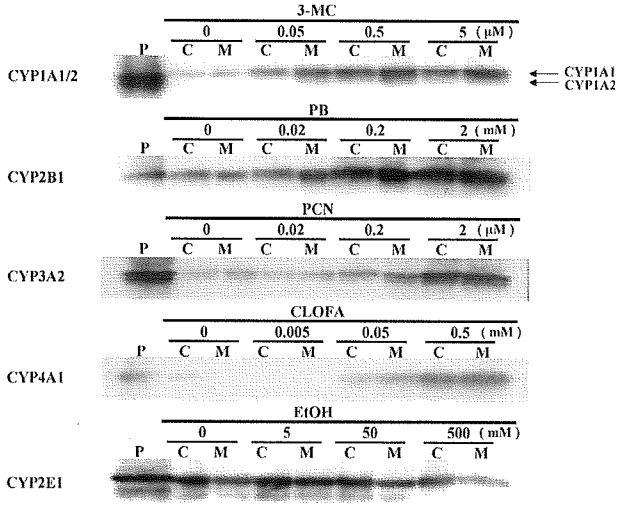

Figure 3 Immunoblots for cytochrome P450 (CYP) proteins induced by various chemicals in small hepatocytes treated with 'M' or without Matrigel 'C'. CYP1A1/2, CYP2B1, CYP3A2, CYP4A1, and CYP2E1 proteins were induced by 3-MC, PB, PCN, CLOFA, and EtOH, respectively. Cells were treated with the chemicals 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.

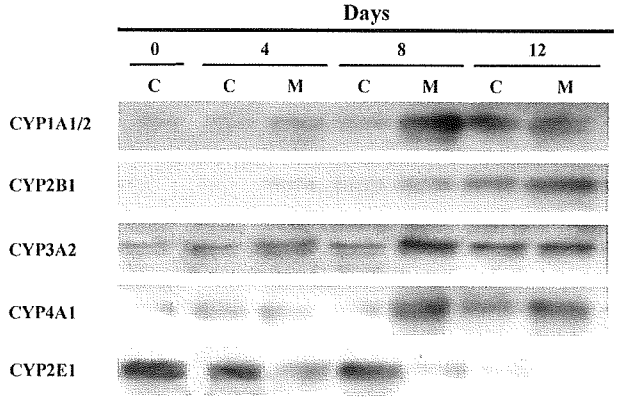

Figure 4 Time course of cytochrome P450 (CYP) expression induced by various chemicals in small hepatocytes treated with 'M' or without Matrigel 'C'. Expression of CYP1A1/2, CYP2B1, CYP3A2, CYP4A1, and CYP2E1 was induced by 5 μM 3-MC, 2 mmol/L PB, 2 μmol/L pregnenolone-16α-carbonitrile, 0.5 mmol/L clofibric 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.

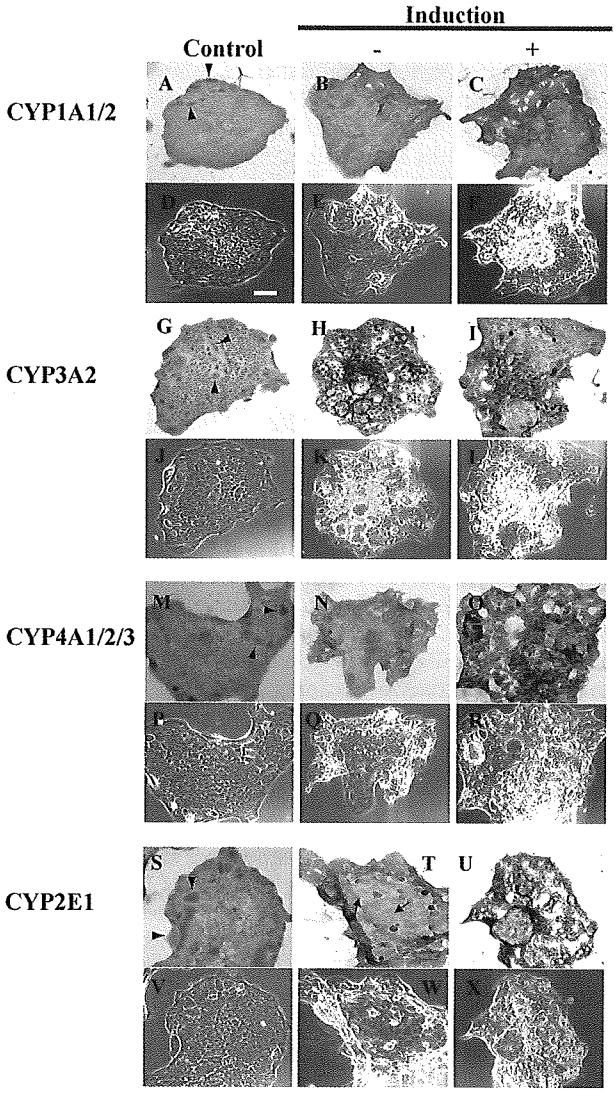

Figure 5 Immunocytochemistry for cytochrome P450 (CYP) in small hepatocytes (SH) colonies treated with various chemicals. The cells were cultured in serum-free medium for 14 days after replating and treated with 500 μg of Matrigel 4 days before fixation (b, c, h, i, n, o, t, u). Expression of CYP1A1/2, 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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Review article

Hepatocyte transplantation for total liver repopulation

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Abstract
Hepatocyte transplantation (HT) is an attractive therapeutic alternative to liver transplantation. A number of experiments have shown the feasibility of total liver parenchymal cell replacement by transplanted hepatocytes. In this review, we would like to highlight researches and clinical reports of HT for liver repopulation. Cellular source of clinical HT should be safety. Immortalized cells, hepatic stem cells, and other stem cells have been used for an experimental model for HT. The exact mechanism of the cell engraftment after HT has not been completely understood, although there were some markers to detect and investigate transplanted cells. In order to achieve liver repopulation following HT, a mild hepatic damage may need to facilitate cell engraftment and replace the host liver by transplanted cells. Hormonal factor may use for the same purpose. Despite the results of preclinical studies promising clinical benefits for cell therapy, the clinical experience of HT has been disappointing, except in a few cases. HT may become an alternative for liver transplantation in the future; however, many efforts should made before establishing an effective method for HT and liver replacement therapy.

Key words Hepatic stem cells · Hepatocyte transplantation · Liver repopulation

Introduction

Hepatocyte transplantation is an attractive therapeutic alternative to liver transplantation. A number of experiments have shown the feasibility of total liver parenchymal cell replacement by transplanted hepatocytes.[1–5] However, researchers are still looking for more efficient methods of hepatocyte transplantation to employ for clinical treatment.

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The most recent important finding is the discovery of which liver stem cells can be derived from bone marrow cells.[6,7] Part of the phenomenon can be explained by cell fusion;[8,9] however, genetic disorders can be corrected by either cell engraftment or cell fusion.

Despite preclinical studies that suggest the potential benefits of hepatocyte transplantation, a clinical protocol for such transplantation has not yet been established. A number of issues must be resolved for total liver replacement by hepatocyte transplantation to succeed. The source cells should be few in number and proliferate vigorously without transformation. A cell marker should be identified to detect and evaluate transplanted cells. Techniques for manipulation and transplant procedures need to be safer and more effective than they are now. We reviewed the literature to investigate experimental and clinical experiences of hepatocyte transplantation.

Potential cell source

Normal hepatocytes can be an ideal cell source for hepatocyte transplantation. However, very few healthy donor cells are available. Therefore, researchers have tried to increase cell numbers by culturing hepatocytes. The obstacle to this strategy is that mature normal hepatocytes hardly proliferate in classical culture conditions.[10] Therefore, various strategies have been employed to increase the cell numbers. These include attempting to develop immortalized cells by gene transduction, and searching for hepatic stem cells or an alternative stem cell source that will transdifferentiate into hepatocytes (Table 1).

Immortalized cells

A number of genes have been successfully transferred into hepatocytes to proliferate eternally in vitro. The

Table 1. Potential cell sources

	Reference
Immortalized hepatocytes	
SV 40 large T-antigen	Isom et al.[11] 1981
Thermolabile mutant SV40 large T, antigen	Fox et al.[12] 1995
Hepatitis C virus core protein	Ray et al.[13] 2000
hTERT	Wege et al.[14] 2003
$p19^{ARF}$	Mikula et al.[16] 2004
C/EBP alpha	Soriano et al.[17] 1998
Truncated Met	Amicone et al.[18] 1997
Hepatic stem cells	
Oval cells	Yasui et al.[22] 1997
Small hepatocytes	Gordon et al.[23] 2002
Other stem cells	
Hematopoietic cells	Petersen et al.[6] 1999
Pancreatic stem cells	Dabeva et al.[25] 1997
Salivary gland progenitor cells	Okumura et al.[26] 2003
Umbilical cord blood cells	Kakinuma et al.[27] 2003
Cell fusion	Wang et al.[8] 2003
	Vassilopoulos et al.[9] 2003
Myelomonocytic cells (cell fusion)	Willenbring et al.[28] 2004
Embryonic stem cells	Yamamoto et al.[29] 2003
Low asialoglycoprotein receptor cells	Ise et al.[30] 2004

SV, simian virus; hTERT, human telomerase reverse transcriptase; C/EBP, CCAAT/enhancer binding protein

initial experiment, using simian virus (SV) 40 large T-antigen transferred into hepatocytes, in a rat model resulted in immortality of the cells, but with tumor development.[11] This strategy appears to have been ignored for a decade because of its tumorigenesis; however, hepatocytes conditionally immortalized using thermolabile mutant SV40 large T-antigen were successfully transplanted into rats without tumor development.[12] Hepatitis C virus core protein promotes an immortalized phenotype in primary human hepatocytes, although possible tumorigenesis has not yet been investigated.[13] Transformation, using the human telomerase reverse transcriptase (*hTERT*) gene, has been shown to immortalize human fetal hepatocytes without disrupting their differentiation potential,[14] although the mature hepatocyte itself cannot be immortalized. Inactivation of $p16^{INK4a}$ expression in accord with *hTERT* transformation may immortalize mature hepatocytes, as it does other cells.[15] Other genes, such as $p19^{ARF}$, *C/EBP* alpha, and truncated *Met*, have been used to successfully immortalize murine hepatocytes,[16–18] but have not yet been tried for human hepatocytes.

Hepatic stem cells

The existence of hepatic stem cells has been debated for a long time, because the stem cell-like properties of mature hepatocytes are sufficient for them to regenerate without a stem cell population. However, once the mature hepatocyte is unable to proliferate, due to hepatic damage, as in the D-Galactosamine (D-GalN)-treated rat liver, Cu^{2+}-deficient rat pancreas, or retrorsine-treated rat, hepatic stem cells emerge to restore liver regeneration.[2,19,20] This peculiar condition is rarely seen in the clinical setting, except during the process of hepatocarcinogenesis.

Two types of liver progenitor cells have been discovered. Oval cells are considered to be progenitor cells of both hepatocytes and cholangiocytes.[2] On the other hand, small hepatocytes (SHs) are considered to be progenitor cells of hepatocytes.[21] Both cell types have been used as cell sources for transplantation and engraftment to the host liver, with various functions being shown.[22,23] The difference between these cell types is that the basic phenotype of the oval cell is for cholangiocytes and that of SH is for hepatocytes. In the clinical setting, SHs are preferable to oval cells for various reasons, as outlined below.

Hepatocytes have a wide variety of functions and are regulated in various ways to achieve each function. Hepatocyte function is tightly regulated at the transcription level, and liver-enriched transcription factors regulate the expression of many genes associated with protein production and detoxification in a hierarchical manner.[24] Basically, SHs express all the liver-enriched transcription factors that need to be expressed in the mature hepatocyte. On the other hand, oval cells do not express all of these factors. Therefore, it is a reasonable hypothesis that SHs would achieve full hepatocyte func-

Table 2. Markers to detect exogenous transplanted hepatocytes in the recipient liver

	Reference
Markers	
DPPIV	Thompson et al.[31] 1991
HBsAg	Gupta et al.[32] 1994
Y-chromosome	Krishna Vanaja et al.[33] 1998
111-indium	Gupta et al.[35] 1994
	Bohnen et al.[36] 2000
99m-technetium	Gupta et al.[37] 1981

HBsAg, hepatitis B virus antigen; DPPIV, dipeptidyl peptidase IV

tion more rapidly than oval cells. Another reason to avoid the clinical use of oval cells for cell transplantation is their malignant potential.

Other stem cells

The natural plasticity of stem cells means that they could generate hepatocyes in vivo. Hematopoietic cells, pancreatic stem cells, salivary gland stem cells, and umbilical blood cells generate functional hepatocytes in vivo after cell transplantation.[6,25–27] Cell fusion could be the reason why stem cells with different origins can transdifferentiate into other cell types, and cell fusion is sufficient for treating some gene disorders.[28] Therefore, most stem cells in any organ could be feasible cell-source candidates for cell transplantation to manage liver disease in the clinical setting. However, an efficient method for the transdifferentiation of stem cells with other origins to hepatocytes should be developed before such cells can be used clinically. Another potential cell source could be embryonic stem cells.[29] However, the efficacy and safety of this approach should be carefully determined before clinical use can begin. Recently, a selective hepatocyte population with low asialoglycoprotein receptor-expressing cells was suggested to have the ability to repopulate the host liver after cell transplantation.[30] This approach could be used for clinical treatment by selecting a small cell population that could effectively repopulate the host liver.

Markers to detect exogenous transplanted hepatocytes in the recipient liver

A marker for donor cells is important to evaluate the cells' fate after transplantation (Table 2). In animal models, various genetic markers are available when using such genetically deficient animals as the dipeptidyl peptidase IV (DPPIV)-deficient rat[31] and the hepatitis B virus surface antigen (HBsAg) transgenic mouse model.[32] This approach cannot be used for clinical treatment in humans. The Y chromosome can be used in the case of sex-mismatch cell transplantation if a specimen is available for molecular evaluation.[33,34] However, as this approach needs tissue specimens, an invasive approach to obtain them is unavoidable. Cell labeling, with either 111-indium[35,36] or 99m-technetium,[37] may be an ideal strategy to detect donor cells after cell transplantation, without the need for an invasive approach. Indeed, 111-indium has been used for a clinical study to detect the transplanted cells in a 5-year-old child.[36] However, it can be used only in the early peroid after cell transplantation, because the label may be transferred to other cells after exposure for longer periods. Without a method to detect the transplanted cells, the exact mechanism by which hepatocyte transplantation ameliorates liver function will be difficult to determine.

Method for enhancement of liver repopulation

The purpose of hepatocyte transplantation is to support hepatocyte function that has been disrupted by either a congenital gene abnormality or acquired cell damage. The liver is an ideal place for the transplanted hepatocyte to work properly. However, very limited space is available under normal conditions without any manipulation. Therefore, manipulation is necessary to make a space for hepatocyte engraftment (Table 3).

Animal models created by gene manipulation

The engraftment of hepatocytes after transplantation has been considered to be limited, because the proliferation of mature hepatocytes is believed to be limited and does not last forever. Hepatocyte transplantation of the urokinase plasminogen activator (*uPA*) gene into the liver, under control of the albumin promoter gene, in a transgenic mouse (uPA-alb) model showed massive engraftment of transplanted hepatocytes, which replaced nearly all parenchymal cells.[38] Subsequently, similar extensive liver repopulation was seen in the fumaryl acetoacetate hydrolase (FAH)-knockout mouse model, which is a model of human hereditary tyrosinemia type 1.[39] These models showed that hepatocyte transplantation could be feasible for the replacement of liver tissue, as an alternative to liver transplantation. In addition, the results indicated the existence of highly proliferative mature hepatocytes.

The strategy for efficient hepatocyte transplantation is to make a space for the engraftment and increase the proliferative activity of the donor cells. In the initial genetically manipulated models, such as the uPA-alb and FAH models, the manipulation resulted in critical damage to the recipient liver and produced a space for

Table 3. Methods for enhancement of liver repopulation

	Reference
Animal models	
Alb-uPA transgene	Sangren et al.[38] 1991
FAH mutant	Overturf et al.[39] 1996
Bcl-2 transgene	Mignon et al.[40] 1998
Bcl-x(L) transgenic donor cell	Mitchell et al.[41] 2002
p27kipl-deficient donor cell	Karnezis et al.[42] 2001
Hepatotoxic agents	
Retrorsine and hepatectomy	Laconi et al.[43] 1998
CCl_4 and hepatectomy	Gupta et al.[44] 1999
DDC	Camargo et al.[45] 2004
Radiation	
With hepatectomy	Guha et al.[46] 1999
With ischemic reperfusion	Malhi et al.[47] 2002
With FasL	Takahashi et al.[48] 2003
Others	
HGF	Kato et al.[49] 1996
Thyroid hormone	Oren et al.[52] 1999

uPA, urokinase-type plasminogen activator; FAH, fumarylacetate hydrolase; CCl_4, carbon tetrachloride; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; FasL, Fas ligand; HGF, hepatocyte growth factor

donor cells to proliferate in the liver. The resistance of hepatocytes to apoptosis was generated by the gene transfer of either *Bcl-2*[40] or *Bcl-xL*,[41] and the donor cells successfully repopulated the host liver after transplantation. In this model, Fas-mediated apoptosis killed mature hepatocytes in the recipient liver and the transgenic hepatocytes resisted Fas ligand-induced apoptosis and proliferated to repopulate the host liver.

Genetically manipulated donor cells with knockout of the $p27^{Kipl}$ gene enhanced liver repopulation in the FAH model.[42] The deficiency of the $p27^{Kipl}$ gene enhanced cell proliferation, because loss of $p27^{Kipl}$ activates cyclin-dependent kinase (Cdk2)-kinase activity, which promotes cell-cycle progression. These studies using engineered cells show that the basic strategy for liver repopulation is to damage the recipient liver and enhance the proliferation of donor cells.

Hepatotoxic agents

Pyrrolidine alkaloids, such as retrorsine, are hepatotoxic and block the hepatocyte cell cycle in the late S and/or G2 phase. Although the halflife of pyrrolidine alkaloids is very short, their inhibitory effect on hepatocyte proliferation lasts for several weeks. Therefore, a retrorsine model has been successfully used with transplanted hepatocytes to repopulate the liver after cell transplantation combined with partial hepatectomy.[43] In this model, donor cells are the only cells that can proliferate after stimulation for liver regeneration, as proliferation of the host liver is inhibited by the retrorsine treatment. Hepatic toxins have also been used instead of genetically manipulated models to ablate liver cells and make a space for the engraftment of hepatocytes after cell transplantation. Carbon tetrachloride (CCL_4), which depletes perivenous hepatocytes (zone 3 hepatocytes), has been used to enhance liver repopulation.[44] The concept of requiring space for liver repopulation has been proven. Another hepatic toxin, 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC),[45] has been employed in a model of hematopoietic stem cell transplantation.[28] In this model, myelomonocytic cells were sufficient to produce functional hepatic repopulation.

Radiation

In the clinical strategy of liver repopulation, it is preferable to induce only mild hepatic damage. After radiation-induced liver damage, it has been shown that massive liver repopulation is accomplished by hepatocyte transplantation combined with partial hepatectomy.[46] Radiation can inhibit host hepatocyte proliferation in the recipient of hepatocyte transplantation. However, the surgical procedure is necessary to stimulate liver regeneration, and this is an obstacle to clinical application. Non-surgical alternatives have been investigated, and successful liver repopulation by cell transplantation has been demonstrated in an ischemia-reperfusion model after irradiation.[47] *FasL* gene transfer to the host liver also resulted in significant liver repopulation when combined with irradiation after cell transplantation.[48] These results indicate that irradiation of the liver prior to hepatocyte transplantation is feasible for liver repopulation therapy.

Enhancement of donor-cell proliferation

Hepatocyte growth factor (HGF) and a thyroid hormone have been used for the enhancement of donor-cell proliferation after hepatocyte transplantation. HGF administration after hepatocyte transplantation improves donor-cell proliferation.[49] However, it does not enhance liver repopulation in the CCl_4-treated hepatocyte transplantation model.[44] Serum HGF levels are markedly high after partial hepatectomy.[50,51] Therefore, it is a matter of debate whether additional HGF can enhance cell proliferation in liver regeneration when the HGF level is already high. On the other hand, a thyroid hormone (triiodothyronone) has been shown to stimulate liver repopulation in a retrorsine model without partial hepatectomy.[52] Other hepatic mitogens should be tested to see whether they stimulate liver repopulation in models for clinical treatment.

Table 4. Clinical reports of hepatocyte transplantation

Applications	Number of patients	Outcome	Reference
Chronic liver failure			
Chronic liver disease	10	Transplanted cells detectable for 1 to 6 months	Mito et al.[53] 1992
Chronic end-stage liver disease	5	Three recovered	Strom et al.[54] 1997
Acute liver failure			
Fulminant hepatic failure	7	Three recovered	Habibullah et al.[55] 1994
Acutse liver failure	19	Two recovered; six liver transplantations; 11 died	Strom et al.[1] 1999
TPN sepsis and HCV	2	Both died	
Acute liver failure	5	All died within 52 days	Bilir et al.[56] 2000
Hereditary metabolic liver disease			
Familial hypercholesterolemia	1	Stable for 18 months	Grossman et al.[57] 1994
Familial hypercholesterolemia	5	Three improved	Grossman et al.[58] 1995
A1-antitrypsin deficiency	2	Liver transplantation	Strom et al.[59] 1997
Crigler-Najjar syndrome type I	1	Liver transplantation after 4 years	Fox et al.[60] 1998
OTC deficiency	1	One died after 42 days	Strom et al.[59] 1997
	1	One had liver transplantation after 6 months	Horslen et al.[61] 2003
	1	One had liver transplantation after 7 months	Mitry et al.[62] 2004
Glycogen storage disease type Ia	1	9 Months, improved	Muraca et al.[63] 2002
Infantile Refsum's disease	1	18 Months, improved	Sokal et al.[64] 2003
Factor VII deficiency	1	Not described	Horslen et al.[65] 2004
Bile salt export protein deficiency	1	Not described	Horslen et al.[65] 2004

OTC, ornithine transcarbamoylase; TPN, total parenteral nutrition

Clinical reports of hepatocyte transplantation

Despite the results of preclinical studies promising clinical benefits for cell therapy, the clinical experience of hepatocyte transplantation has been disappointing, except in a few cases (Table 4). The first clinical hepatocyte transplantation was reported by Mito et al.,[53] in 1992, who transplanted autologogous hepatocytes in ten patients with chronic liver disease. Although the transplanted hepatocytes survived in the host body, no clinical benefit of the hepatocyte transplantation was seen. Strom et al.[54] reported hepatocyte transplantation in five patients with chronic liver disease. Three of the five patients survived; however, orthotopic liver transplantation eventually had to be conducted for all three patients.

Hepatocyte transplantation has been tested for acute liver failure. Habibullah et al.[55] transplanted human fetal hepatocytes in 7 patients with fulminant hepatic failure and reported that 3 patients recovered. Strom et al.[1] reviewed 30 cases of hepatocyte transplantation in the United States. Although 11 of 19 patients died, 2 of the patients recovered as a result of hepatocyte transplantation alone. An other clinical experience was reported by Bilir et al.[56] in 2000. Three of their patients survived for more than 48 h, although they would not have been expected to survive without treatment including liver transplantation. However, all their patients died within 52 days of the hepatocyte transplantation.

The greatest clinical benefit of hepatocyte transplantation could be for patients with hereditary metabolic liver diseases.[57–65] Most of such patients reported to have received hepatocyte transplantation received liver transplantation eventually; however, some of them showed improved clinical status for more than 6 months with the transplantation. Therefore, at present, hepatocyte transplantation is not an alternative for liver transplantation, but it could be a bridge to liver transplantation. Hepatocyte transplantation may become an alternative for liver transplantation in the future; however, this will not be easy. This dream may come true as a result of the many efforts currently being made to develop an effective method for hepatocyte transplantation and liver replacement therapy.

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