Cloning and Characterization of Liver Progenitor Cells From the Scattered Cell Clusters in Primary Culture of Porcine Livers

Takayoshi Tokiwa,* Taisuke Yamazaki,* Masashi Ono,† Shin Enosawa,‡ and Takashi Tsukiyama†

*Department of Liver Cell Biology, Kohno Clinical Medicine Research Institute, Tokyo 140-0001, Japan
†Kita-Shinagawa Hospital, Kohno Clinical Medicine Research Institute, Tokyo 140-0001, Japan
‡Department of Innovative Surgery, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan

The scattered cell clusters that can differentiate into hepatocytes or biliary epithelial cells have been isolated from primary cultures of adult porcine livers. We have generated 11 clonal cell lines from this system and identified liver progenitor cells (LPCs) among the clonal lines. These clonal lines expressed c-kit, HNF-1, HNF-6, and/or CK19 mRNA. An immunocytochemical study of the clonal lines indicated that clonal line CL-11 expressed liver epithelial cell markers CK14, vimentin, CK18, and BD-1. The expression of albumin and α1-antitrypsin (α1-AT) mRNA was only upregulated in CL-11 among the clonal lines when they were grown as aggregates. Under these conditions, CL-11 also exhibited ammonia metabolic activity and several indicators that suggest hepatocytic differentiation, including the upregulation of liver-specific genes such as dipeptidyl peptidase IV, CYP1A1, and CYP3A4 mRNA, and the downregulation of biliary cell markers such as γ-glutamyltranspeptidase (GGT), CK19, and HNF6 mRNA. After culturing CL-11 in Matrigel, the expression of GGT and HNF6 mRNA was upregulated. These results indicate that CL-11 has dual potential: the ability to differentiate as hepatocytes or as bile duct cells. The isolation of scattered cells could provide a simple method to generate LPC lines from adult livers.

Key words: Progenitor cells; Adult pig liver; Cell cluster; Cloning; P450

INTRODUCTION

The presence of liver progenitor cells (LPCs) is supported by the fact that a population of oval cells, which have the potential to differentiate into either hepatocytes or biliary epithelial cells, arises in the rat liver when hepatocytes are unable to proliferate after a liver injury (20). If LPC lines could easily be isolated from the liver and hepatocytes could easily be generated from the LPC lines, new possibilities for studying drug metabolism, gene regulation, and cell transplantation would be opened. It has been widely noted that the isolation of LPC lines from adult livers remains elusive, although several LPC lines, including oval cell lines, have been isolated in various in vitro systems (12,29). Using the NAIR-1 culture medium, we demonstrated that primary cultures of the nonparenchymal cell fraction from adult porcine livers produce scattered cell clusters that can differentiate into either hepatocytic or biliary epithelial lineage cells (9,24). This suggested that we could isolate LPC lines from the scattered cell clusters. In the current report, we describe their simple isolation and identification from this system.

MATERIALS AND METHODS

Cell Isolation and Culture

The cell suspensions, consisting of a nonparenchymal cell fraction, were prepared from 6–6.7-month-old abattoir porcine livers, as described previously (9,24,30), and then they were plated (1.5 × 104 cells/dish) onto type I collagen-coated 60-mm polystyrene dishes for primary culture. On the third day, the scattered cells derived from the cell clusters that attached to the dishes were scraped with pipette tips, collected, and used as sources of cloned cells. Single cells were isolated using micropipettes and placed in 96-well dishes as described previously (23). The clonal lines thus obtained were used for the experiments at passage 5 to 10. The culture medium NAIR-1 [DMEM/F12 (1:1) medium supplemented with 5% FBS, 10 ng/ml glucagon, 10 µg/ml insulin, 60 ng/ml hydrocortisone sodium acetate, 25 ng/ml EGF, 10 µg/ml transferrin, 50 nmol/L triiodothyronin, 5 ng/ml sodium selenate, 10 mmol/L nicotinamide, 0.1 mmol/L ascorbic acid 2-phosphate, 1 µg/ml α-tocopherol acetate, and 50 ng/ml linoleic acid] was employed to induce scattering until day 3 in the primary culture.
NAIR-3 medium [DMEM/F12 (1:1) medium supplemented with 5% FBS, 10 µg/ml insulin, 60 ng/ml hydrocortisone sodium acetate, and 25 ng/ml EGF] was used for the clonal culture (24). The medium was changed every other day, and the cells were cultured at 37°C in a humidified incubator with 5% CO₂.

**In Vitro Differentiation**

For studies of differentiation, the cells were grown in the NAIR-1 culture medium supplemented with or without 10 ng/ml human hepatocyte growth factor (HGF, PeproTech EC LTD, London, UK), 10 ng/ml human oncostatin M (OSM, PeproTech), or combinations of HGF and OSM. In order to form cell aggregates, the cells were inoculated at a density of 5 × 10⁵ cells into a 60-mm plastic dish coated with 2.5% w/v poly-(2-hydroxyethylmethacrylate) (poly-HEMA, Sigma, St. Louis, MO) (11,25). Cell aggregates were collected for RNA extraction on day 2 after inoculation. For the culture in Matrigel, 0.05 ml of Matrigel (BD Bioscience, San Jose, CA) was placed onto 96-well plates, permitted to set for 1 h, and 5 × 10⁴ cells were plated in NAIR-1 culture medium supplemented with 10 ng/ml HGF and 10 ng/ml OSM. Cells were recovered after 2–4 days for RNA extraction.

**RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

Total RNAs were isolated as described elsewhere (1). The cDNAs were synthesized with a first-strand cDNA kit (ReverTraAce-α, Toyobo Co., Ltd, Osaka, Japan). PCR amplification was performed with a 5-µl aliquot of reverse transcriptase reaction and in a total volume of 50 µl containing 0.2 mM dNTP, 0.25 µM primers, and 1.25 U Taq DNA polymerase (MBI Fermentas, Hanover, MD). The PCR primers used for amplification were as follows: α1-antitrypsin (α1-AT) (forward 5'-tcctctccacacctcaacc-3', and reverse 5'-cggtgtctttgtcaagctca-3', 245 bp); α-fetoprotein (AFP) (forward 5'-tcgcagtcggaaaccctct-3', and reverse 5'-tcactgaggtgacagcaagc-3', 248 bp); albumin (forward 5'-tgtgttgctgtgatgacttg-3',

![Figure 1.](image-url) RT-PCR analysis of the clonal cell lines grown in monolayers. G3PDH, internal loading control.
Table 1. Summary of the Immunocytochemistry Findings of the Clonal Cell Lines

<table>
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<tr>
<th>Marker</th>
<th>Clone No.</th>
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<tr>
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ne: not examined; −: no expression; ±: slightly expressed; +: expressed; ++: strongly expressed.

and reverse 5'-tcagcagcaatgacagacag-3', 884 bp); c-kit (forward 5'-gctgaaactgagacagcag-3', and reverse 5'-ttcatttgc-3', 500 bp); CK19 (first) (forward 5'-aagctacacgacagacagc-3', and reverse 5'-tattgcca-3', 310 bp); CK19 (nested) (forward 5'-tccgctacgacagacagc-3', and reverse 5'-ggcgcgctacgacagacag-3', 745 bp); CYP1A1 (forward 5'-ttcctcttgaatggaagtt-3', and reverse 5'-atccgacagctgatatgg-3', 191 bp); CYP3A4 (forward 5'-tctcagccagagacagagc-3', and reverse 5'-ggctactgaaagtgtg-3', 196 bp); dipeptidyl peptidase IV (DPPIV) (forward 5'-ttctttctcttggccttcca-3', 215 bp); γ-glutamyl transpeptidase (GGT) (forward 5'-gcctcgctcgggtccgtgtt-3', and reverse 5'-cctctgctcgggtccggtt-3', 183 bp); glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (forward 5'-accacagtc-3', and reverse 5'-tcaccccatgtggtgta-3', 452 bp); HNF1 (forward 5'-ttcttcagctgagcagcagc-3', and reverse 5'-gtcagctgagtcgtgtgtcta-3', 275 bp); HNF4α (forward 5'-tctgctcgggcaaaaagagactag-3', and reverse 5'-acatcagctgagtcgtgtgta-3', 372 bp); HNF6 (forward 5'-aaacacaaagactcactgagcactgc-3', and reverse 5'-gctagagcagaaaaatcactactc-3', 387 bp). The PCR conditions of α1-AT, AFP, albumin, c-kit, CYP1A1, CYP3A4, DPPIV, GGT, G3PDH, HNF1, HNF4α, and HNF6 were 25–40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. For CK19, the condition was a first PCR of 20 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 50 s, and extension at 72°C for 2 min, followed by a nested PCR with 30 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 50 s, and extension at 72°C for 2 min. When porcine primers are unavailable, we used human primers. In this case, the identity of the PCR products was confirmed by DNA sequencing using the Big dye terminator. As positive controls, mRNAs extracted from porcine fetal livers, bone marrows, livers, and hepatocytes were used.

Immunocytochemistry

Monolayer cultured cells were fixed in acetone/methanol at −20°C. Cell aggregates were fixed with neutral buffered 10% formalin and then were processed to par-

Figure 2. RT-PCR analysis of the clonal cell lines grown as aggregates. C, confluent cells; A, aggregates; G3PDH, internal loading control.
affin wax for sectioning. Rabbit anti-human albumin (DAKO-Japan Co., Kyoto, Japan), α1-AT (Ylem, S.R.L., Rome, Italy), and AFP (NeoMarkers, Fremont, CA), mouse monoclonal anti-swine vimentin (DAKO-Japan), mouse monoclonal anti-human cytokeratin (CK)14 (Chemicon International, Temecula, CA), CK18 (Progen biotechnik, Heidelberg, Germany), and CK19 (Progen biotechnik), and mouse monoclonal anti-rat BD1 and BD2 (gifts from Dr. Hixson) (6) were used as the primary antibodies. Immunostaining was carried out using a Histofine stain kit (Nichirei Corporation, Tokyo, Japan).

**Western Blotting**

Cellular extracts were analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (27). The proteins were transferred to clear blot membrane-P (Atto Co., Ltd, Tokyo, Japan), incubated with an antibody against α1-AT (Ylem, S.R.L.). After incubation with anti-rabbit IgG coupled with peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, CA), the proteins were visualized by detecting the peroxidase activity.

**Ammonia Metabolic Activity**

In order to determine the ammonia metabolic activity, ammonium chloride was added to the culture to a final concentration of 0.5 mM and the residual ammonia was measured at 2, 4, and 6 h using a blood ammonia analyzer (Amiecheck meter, Daiichi Chemical Co., Ltd, Kyoto, Japan).

**Data Analysis**

The data were analyzed using Student’s paired test.

**RESULTS**

**Isolation and Characterization of 11 Clonal Cell Lines**

We generated 11 clonal lines from the cloning of the scattered cells from the porcine liver nonparenchymal cell fraction. Oval cells are characterized by the coexpression of stem cell markers, such as CK19 (18) and c-kit (3), and hepatocytic lineage markers, such as albumin, AFP, and CK19 (28). All of the clonal lines expressed mRNA for c-kit (Fig. 1), but did not express AFP (data not shown) mRNA. Among these clonal lines, five expressed CK19 mRNA, but six did not (Fig. 1). Because human primers for amplification of CK19 were used, DNA sequencing of the PCR product was carried out. The nucleotide sequence of the product showed a high degree of homology with the nucleotide sequence of the human CK19 gene [645 of 704 nucleotides (92%)] (data not shown). It was thus confirmed that the PCR product represents porcine CK19. As
shown in Figure 1, many of the clonal lines expressed HNF1 (8/9) and HNF6 (9/10) mRNA. However, no expression of HNF4α mRNA was observed (data not shown). Liver epithelial cell (LEC)/oval cell markers were examined by immunocytochemistry. The clonal lines expressed vimentin or BD-1, or both, while CL-11 expressed CK14, vimentin, CK18 (Fig. 6e) and BD-1 (Fig. 6f), as summarized in Table 1. In addition, neither of the clonal lines expressed AFP, nor BD-2 (data not shown). These phenotypes remained stable throughout the experiments. Some of the clonal lines expressed albumin or α1-AT mRNA to varying extents in cells grown as a confluent monolayer, although CL-11 expressed neither of the two (Fig. 2). The gene expression patterns shown in Figure 2 indicate that the expression of albumin and α1-AT mRNA is upregulated only in CL-11 among the clonal lines when they are grown as aggregates. Based on these results, the differentiation potential of CL-11 was analyzed further. CL-16, which rarely expressed LEC/oval cell markers, was used as a control.

Growth and Morphology of CL-11 and CL-16

Two clonal lines had polygonal-shaped morphologies (Fig. 3). There was a small difference in the morphology between CL-11 and CL-16. Both CL-11 and CL-16 continued to grow in culture for at least 20 population doublings (>100 culture days) (Fig. 3).

Differentiation Potential of CL-11

When grown as a monolayer, CL-11 did not express a hepatocyte marker albumin mRNA, despite being cultured for 14 days in the presence of HGF and/or OSM (Fig. 4-1a), which has been demonstrated to promote hepatic differentiation in vitro (8). However, when these cells were grown as aggregates (A1 & A2), they expressed albumin mRNA (Fig. 4-1b). The expression levels of albumin mRNA in the cell aggregates were higher following 14 days of monolayer treatment with HGF and OSM (A2) than following nontreatment (A1), suggesting that HGF and/or OSM are also associated with the differentiation of CL-11 (Fig. 4-1b).

CL-11 expressed DPPIV mRNA when grown as aggregates (Fig. 4-2). CL-11 also expressed CYP3A4 and CYP1A1 mRNA at higher levels in the aggregate culture than did the cells in monolayers (Fig. 4-2). The expression of albumin (data not shown) and α1-AT (Fig. 4-3) in CL-11 was analyzed by a Western blot analysis and the results showed that the cells were positive for these markers when grown as aggregates. On the other hand, HGF and OSM promoted the expression of these markers in CL-11 aggregates.

Figure 4. RT-PCR analysis and/or Western blotting of CL-11 and -16. Cell lines: 1a, 1b, 2, and 3, CL-11; 4, CL-16. Analyses: 1a, 1b, 2, and 4, RT-PCR; 3, Western blot. (1a) Cells cultured in NAIR-1 medium supplemented with HGF or OSM alone, or a combination of HGF and OSM (Mix). (1b) C, confluent cells; A1, aggregates; A2, aggregates following 14 days of monolayer culture. (2) C, confluent cells; A, aggregates. (3) C, confluent cells; A, aggregates. (4) Mix, cells cultured in NAIR-1 medium supplemented with a combination of HGF and OSM; A1, aggregates; A2, aggregates following 14 days of monolayer culture. Hep, hepatocytes as positive control; G3PDH, internal loading control.
hand, CL-16 did not express albumin mRNA in the aggregate culture, even though they were precultured in the presence of HGF and OSM for 14 days (Fig. 4-4). Under these conditions, the expression of α1-AT, CYP3A4, CYP1A1, and DPPIV mRNA was neither expressed nor upregulated in CL-16 (data not shown).

The expression of GGT, CK19, and HNF6 mRNA was downregulated when CL-11 was grown as cell aggregates on poly-HEMA substrates (Fig. 5-1). When CL-11 was cultured in Matrigel, previously shown to favor bile duct cell differentiation (14), the cells changed their morphology from flat to spherical and formed ag-

![Figure 5. RT-PCR analysis of CL-11 grown as aggregates or in Matrigel. (1) aggregate culture; (2) culture in Matrigel. C, confluent cells; A, aggregates; Mat, culture in Matrigel; G3PDH, internal loading control.](image)

![Figure 6. Immunocytochemistry of CL-11 grown in monolayers or as aggregates in NAIR-1 medium supplemented with HGF and OSM. (a, b) CK19; (c, d) CK14; (e) CK18; (f) BD-1. (a, c, e, f) Monolayer culture; (b, d) aggregate culture. No counterstaining. Magnification: (a, c, e, f) 200×; (b, d) 400×. Scale bar: 25 μm.](image)
LIVER PROGENITOR CELL LINES

Cells and LECs and have been used as their markers (4,25). The presence of CK14 and vimentin in CL-11 indicates that CL-11 is closely related to oval cells or LECs.

CK19 has been used as a marker for biliary epithelial cells (9,17,25). During human development, it has been found that LPCs express CK19 and CK14, but not vimentin, and that the commitment of these LPCs to either hepatocytes or bile duct epithelial cell lineages results in the increased expression of one marker and the loss of the other marker (5). CL-11 expressed CK14, CK19, and vimentin when grown in monolayers. The downregulation of CK19 and vimentin suggests that the cells within the aggregates have differentiated along the hepatocytic lineage (21,22,25,26). We thus speculate that CL-11 is derived from hepatic stem cells that persist in the mature liver from fetal life.

Cytochrome P450, the key component of the microsomal monooxygenase system, is responsible for the liver oxidative metabolism of many xenobiotics (15). It is generally thought that cytochrome p450 induction is one of the best indexes of hepatocytic differentiation. In this study, we demonstrated the upregulation of CYP1A1 and CYP3A4 when CL-11 was grown as aggregates. This suggests that CL-11 retains the ability to metabolize xenobiotics and could be used as a new model to analyze the metabolic pathways and the hepatotoxicity of new drugs.

HNF6 is expressed in the mature hepatocytes and stimulates the transcription of liver-specific genes such as albumin and α1-AT (10,16). On the other hand, HNF6 is essential for differentiation and morphogenesis of the biliary tract (2). The forced expression of HNF6 in zebrafish produces biliary phenotypes (13). We found that CL-11 expressed HNF6 in the monolayer culture and that its expression was downregulated by aggregation and then upregulated after culturing in Matrigel, thus inducing biliary cell differentiation (14,19). These facts suggest that HNF6 may be a crucial factor in the determination of hepatocytic or biliary phenotypes in CL-11.

In this study, we attempted to isolate LPC lines from the scattered cell clusters, and one bipotential LPC line, CL-11, was obtained among 11 lines. Five cell lines, including CL-11, expressed oval cell markers CK19 and c-kit, but not AFP. This suggests the possibility that in addition to CL-11, some other clonal lines also have the nature of LPC lines. HGF is known to be involved in cell scattering (7) and to promote hepatic oval cell proliferation. The precise reason why CL-11 was less responsive to HGF in the differentiation study remains to be determined.

In the current study, we showed that scattered cell clusters can be used as a source of LPCs. This simple method of isolating bipotential LPC lines from adult liv-
ers may provide a novel model for investigating drug metabolism, gene regulation, and cell transplantation.

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