Side Population Cells From an Immortalized Human Liver Epithelial Cell Line Exhibit Hepatic Stem-Like Cell Properties

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The existence of hepatic stem cells in human livers is controversial. We investigated whether the side population (SP) cells derived from an immortalized human liver epithelial cell line THLE-5b possess the properties of hepatic stem-like cells. SP cells derived from THLE-5b were isolated using flow cytometry and were assayed for the expression of phenotypic markers by reverse transcription polymerase chain reaction and immunostaining. THLE-5b SP cells retained the capacity to generate both SP and non-SP cells, showed a capacity for self-renewal, and were more efficient in colony formation than non-SP cells. Neither the SP nor the non-SP cells formed tumors when transplanted into athymic nude mice or severe combined immunodeficient mice. The expression level of stem cell-associated markers such as an ATP-binding cassette membrane transporter, epithelial cell adhesion molecule, c-kit, Thy-1, and octamer binding transcription factor 4 was higher in SP cells than in non-SP cells. When cultivated as rotation-mediated aggregates, the expression of liver-specific genes including tryptophan oxygenase and CYP3A4 was up-regulated in SP cells, suggesting that THLE-5b SP cells have the ability to differentiate into a hepatocyte phenotype. One of the clonal cell lines derived from the SP cells expressed stem cell-associated markers. These results indicate that SP cells derived from THLE-5b possess hepatic stem-like cell properties and suggest that THLE-5b can be used as a model of normal human liver progenitor or stem cell line.

Key words: Side population, Immortalized cell line, Hepatic stem/progenitor cell

INTRODUCTION

There is increasing evidence for the presence of stem/progenitor-like cells in adult human liver (2,4,8,11,20,21); however, a population with stem cell properties has not yet been isolated (20). In most of these studies, primary cells were isolated from human liver tissues, cultured, and characterized. Major difficulties encountered in stem cell research based on the use of primary cells include limited access to fresh tissues and the short life span of the cells. An in vitro model is therefore essential to overcome these problems. Although some immortalized epithelial cell lines have been used as models of normal epithelial progenitor or stem cell lines (7,17), few adult human liver cell lines are available (18). Human hepatic stem/progenitor-like cell lines may provide the basis for valid reproducible in vitro human models for studies on the presence of stem/progenitor-like cells and their roles in human livers.

Normal organs are maintained by a hierarchical organization that includes stem cells, progenitor cells in various stage of differentiation, and differentiated cells (13,19). Stem cells are the only cells capable of self-renewal. In addition, stem cells or stem-like cells have more differentiation potentials than that of progenitor cells. Side population (SP) cells are identified by flow cytometry because of their unique ability to efflux Hoechst 33342 (5,6,27). This approach has been used to identify and isolate enriched stem cell populations from a variety of tissues. THLE-5b is a nontumorigenic adult human liver epithelial cell line (14). Previously, we reported that THLE-5b cells behave as liver progenitor-like cells (24,25). Moreover, in a preliminary work, we have shown the presence of SP cells in a cultured THLE-5b cell population (28).

In this study, we investigated hepatic stem-like cell properties of THLE-5b using SP cells that were sorted from these cells and assaying the expression of stem cell-associated markers. The aim was to determine whether

THLE-5b could be used as a model of normal human liver progenitor or a stem cell line.

MATERIALS AND METHODS

Human Studies

Approval for the use of human cell lines was obtained from the Human Studies Committee of Kohns Clinical Medicine Research Institute, Tokyo, Japan.

Cell Culture

THLE-5b, an SV40 large T antigen-immortalized human liver epithelial cell line (14,24,25), was unable to grow in soft agar (unpublished results), and no tumors were found in nude mice 6 months after subcutaneous injection of 5 million cells as described elsewhere (14). These data suggest that THLE-5b is only weakly transformed. Certified THLE-5b cells, free of mycoplasma contamination, were initially subcultured at a 1:5 ratio, and cells at passage (p)40 were used for this study. For cell cloning, single cells were isolated using micropipettes, inoculated into a 96-well culture plate, and then grown in larger dishes as clonal cell lines. THLE-5b cells and their clonal cell lines were routinely grown in Dulbecco's modified Eagle Medium (DMEM)/F12 (1:1) (Invitrogen, CA, USA) supplemented with 5% fetal bovine serum (FBS) and antibiotics. The human hepatocellular carcinoma cell line HuH-7 was used as a reference line and grown in Roswell Park Memorial Institute (RPMI 1640) medium (Invitrogen) supplemented with 10% FBS. Cells were grown in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C.

Colony-Forming Assay

Three thousand cells were inoculated into 60-mm dishes, and colonies formed after 6 days were fixed in 100% methanol and stained with Giemsa. Colony-forming efficiency was defined as the percentage of colonies formed per number of cells inoculated. The clonal growth rate (population doubling/day, PD/D) was defined as the mean log₂ of the number of cells of 18 randomly selected colonies divided by the number of days of incubation.

SP Analysis

Prior to reaching confluence, the cells were detached with trypsin and resuspended at 1×10⁶/ml in prewarmed RPMI 1640 or DMEM/F12 (1:1) medium supplemented with 2% FBS and 25 mM N'-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES). The cells were filtered through a 40-μm cell strainer and then incubated with 10 μg/ml Hoechst 33342 dye (Sigma Chemical, MO, USA) for 2 h at 37°C in a water bath with gentle agitation every 15 min. To identify SP cells, the cells were preincubated with 50–200 μM reserpine (Sigma), an inhibitor of

the ATP-binding cassette (ABC) transporter, for 20 min at 37°C before addition of the Hoechst 33342 dye. After incubation, the cells were collected by centrifugation and suspended in ice-cold Hanks' balanced saline solution (HBSS) supplemented with 2% FBS and 10 mM HEPES containing 2 μg/ml propidium iodide to identify dead cells. The cells were kept on ice until analysis by flow cytometry. The number of viable cells was measured by Hoechst 33342 dye exclusion using an EPICS ARTLA flow cytometer (Beckman Coulter, CA, USA). Hoechst 33342 was excited with a UV laser at 350 nm, and fluorescence emission of blue and red lights by the dye was measured using 450 DF10 and 675LP optical filters, respectively, as described previously (28).

Aggregate Culture

Single cells were plated at 5×10⁵ cells in a 25-ml Meyer's flask containing 3 ml of DMEM/F12 (1:1) medium coated with 2.5% poly-(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma) in 95% ethanol to prevent cells from attaching to the flask. Two different conditions were used to induce aggregate formation: (i) static culture on a poly-HEMA substrate (rotation-nonmediated aggregate culture) and (ii) rotation culture, in which the Meyer's flasks were rotated at 70 rpm on an orbital shaker in a chamber, on a poly-HEMA substrate (rotation-mediated aggregate culture), for 24 h. The cultures were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Western Blot Analysis

Western blot assays were done as described previously (23,29). Blots were probed with the following antibodies: rabbit anti-human ATP-binding cassette membrane transporter (ABCG2) (Gene TEX, CA, USA), mouse monoclonal anti-human epithelial cell adhesion molecule (EpCAM) (Thermo Fisher Scientific, Tokyo, Japan), and rabbit anti-chicken actin (Sigma, St. Louis, MO, USA).

Goat anti-mouse (Santa Cruz Biotechnology, CA, USA) or anti-rabbit IgG-horseradish peroxidase antibody (Cell Signaling Technology, MA, USA) served as the secondary antibody.

Immunocytochemistry

Cell monolayers were fixed in acetone/methanol (1:1), and cell aggregates were fixed with 2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.2) as smears on a microscope slide. Rabbit anti-human albumin (Dako-Japan), rabbit anti-human α1-antitrypsin (AAT) (Ylem, Rome, Italy), mouse anti-human cytokeratin 14 (CK14), CK18, CK19 (Thermo scientific), and tryptophane 2,3-dioxygenase (TO) (Abnova, CA, USA) were used as primary antibodies. Immunostaining was carried out using a Histofine stain kit (Nichirei Co., Tokyo, Japan) (12).

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

Total RNA was isolated from cells as described previously (23,25,29) and used as a template for synthesis of cDNA using a first-strand cDNA kit (ReverTraAce- α , Toyobo Co., Ltd., Osaka, Japan). A 5- μ l aliquot of cDNA was then amplified by PCR. The PCR reaction was carried out in a total volume of 50 μ l in the presence of 0.2 mM dNTP, 0.25 μ M primers, and 1.25U Taq DNA polymerase (MBI Fermentas, MD, USA). The PCR primers used for amplification are shown in Table 1. A 25- to 40-cycle program was used for all PCR reactions. PCR consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was assayed as an internal loading control.

Tumorigenicity Assay

THLE-5b SP, non-SP or CL-7 cells, were injected subcutaneously into four male nude mice (BALB/c, nu/nu) at a density of 5×10^6 cells/mouse. The mice were examined for the presence of tumors over a 6-month period. As a positive control, HuH-7 cells (10^6 cells/mouse) were injected subcutaneously into three nude mice. These mice were observed for 3 months. The tumorigenicity assay was also carried out in severe combined immunodeficient (SCID) mice. Briefly, cells were injected subcutaneously into three adult SCID mice at a density of 5×10^6 cells/mouse. The SCID mice were observed for 6 months for the appearance of tumor development.

Data Analysis

Student's *t* test was used to evaluate the statistical significance between experimental groups.

RESULTS

SP (THLE-5b SP) Cells Present in Cultured THLE-5b and Their Self-Renewing Ability

Flow cytometric analysis of the cultured cell line THLE-5b revealed the presence of SP cells, which represented 0.2% of the total cell population (Fig. 1A). SP and non-SP cells (Fig. 1B) were sorted from the SP and non-SP regions, respectively, subcultured six times over 35 days, and then reanalyzed by flow cytometry. As shown in Fig. 1C, cells cultured from the SP cells contained both SP (2.1%) and non-SP cells. In contrast, cells cultured from non-SP cells mostly contained non-SP cells, although they also contained a few SP cells (0.2%).

Colony-Forming Efficiency, Clonal Growth Rate, and Tumorigenicity of THLE-5b SP Cells

The colony-forming efficiency and clonal growth rate (PD/D) of THLE-5b SP cells were significantly higher than those of non-SP cells as shown in Table 2. No tumors were observed following injection of THLE-5b SP or non-SP cells into athymic nude mice or SCID mice (data not shown) over a period of 6 months even though injection of the positive control HuH-7 cells induced tumors within 3 months.

Table 1. Oligonucleotides Used as PCR Primers

Target cDNA	Sense	Antisense
AAT	5'-ctgggacagtgaatcgacaatgc-3'	5'-tctgtttcttgccctcttcggtg-3'
ABCG2	5'-ggcctcaggaagacttatgt-3'	5'-aaggaggtggtgtagctgat-3'
AFP	5'-accaaagttaattttactgaaat-3'	5'-gtttgtcctcactgagttggca-3'
Albumin	5'-agcggcacagcactctctaga-3'	5'-tccacacggaatgctgcatgg-3'
CD133	5'-gcgtaagatacttcaacgcacagg-3'	5'-cagctatcaatgttgatggcctgtc-3'
c-kit	5'-gtgaatctacttgagcctg-3'	5'-tcattcttgatgtctctggc-3'
CK19	5'-aagctaaccatgcagaacctcaacaccga-3'	5'-ttattggcaggtcaggagaagagcc-3'
CYP3A4	5'-ccaagctatgctcttcaccg-3'	5'-tcaggctccacttacggtgc-3'
EpCAM	5'-ctggccgtaaaactgctt-3'	5'-agcccatcattgttctggag-3'
G3PDH	5'-accacagtccatgccatcac-3'	5'-tccaccaccctgtgtgta-3'
Oct-4	5'-gacaacaatgaaatcttcaggaga-3'	5'-ttctggcgccggttacagaacca-3'
Thy-1	5'-ctagtggaccagagccttcg-3'	5'-tggagtgcacacgttaggt-3'
TO	5'-gggaactacgtgcatccttga-3'	5'-gtgcatccgagaacaacct-3'

AAT, α 1-antitrypsin; ABCG2, ATP-binding cassette membrane transporter; AFP, α -fetoprotein; CK19, cytokeratin 19; CYP3A4, Cytochrome P450 3A4; EpCAM, epithelial cell adhesion molecule; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; Oct-4, octamer binding transcription factor 4; TO, tryptophan 2,3-dioxygenase.

Figure 1. Flow cytometric analysis of the immortalized human liver epithelial cell line THLE-5b side population (SP) and non-SP cells. SP and non-SP cells (B) were sorted from the SP and non-SP regions (A), respectively, subcultured six times over 35 days, and then reanalyzed by flow cytometry. Cells cultured from the SP cells contained both SP (2.1%) and non-SP cells (C). In contrast, cells cultured from non-SP cells mostly contained non-SP cells, although they also contained a few SP cells (C). These SP cells were disappeared by treatment with reserpine [Reserpine (+)]. The results are representative of two independent experiments.

Phenotypic Properties of THLE-5b SP Cells

To determine the phenotypic properties of THLE-5b SP cells, we compared phenotypic expression of the indicated markers in THLE-5b SP cells with non-SP cells by RT-PCR analysis, Western blot, and immunostaining (Fig. 2). As shown in Fig. 2A, THLE-5b SP cells expressed higher levels of mRNA for the ABCG2 (stem cell marker), octamer binding transcription factor 4 (Oct-4, stem cell marker), Thy-1 (oval cell/hematopoietic stem cell marker), c-kit (oval cell/hematopoietic stem cell marker), and EpCAM (hepatic stem cell marker) than non-SP cells, but lower levels of CK19 mRNA (oval

cell/biliary epithelial cell marker) than non-SP cells. The expression level of CD133 (oval cell/hematopoietic stem cell marker) was almost the same in THLE-5b SP and non-SP cells. Neither the SP nor the non-SP cells expressed α -fetoprotein (AFP, hepatocellular carcinoma marker). Expression of ABCG2 and EpCAM in THLE-5b SP and non-SP cells was analyzed by Western blot (Fig. 2B), and the results showed that only THLE-5b SP cells are positive for these markers. Immunocytochemical study revealed that THLE-5b SP cells weakly expressed albumin and AAT and strongly expressed CK-14 (liver epithelial cell marker), CK-18 (hepatocyte marker), and

Table 2. Colony-Forming Rate, Clonal Growth Rate, Tumorigenicity of THLE-5b SP and Non-SP Cells

Cells	Colony-Forming Rate (%)	Clonal Growth Rate (PD/D)	Tumorigenicity in Nude Mice
SP	35.3 $\pm$ 2.4*	0.72 $\pm$ 0.1†	0/4
Non-SP	6.7 $\pm$ 0.9	0.40 $\pm$ 0.07	0/4
HuH-7	nd	nd	3/3

Colony-forming rate and clonal growth read: values are means $\pm$ SD of four experiments. Tumorigenicity in nude mice: SP and non-SP cells (5×10^6 cells for each) and HuH-7 cells (positive control; 1×10^6 cells) were injected subcutaneously into nude mice. Tumor formation was observed for 3–6 months after injection. PD/D, population doubling/day; nd, not done.

* $p < 0.001$ versus non-SP.

† $p < 0.01$ versus non-SP.

Figure 2. Expression of stem cell-associated and other markers in THLE-5b SP and non-SP cells. (A) Reverse transcription and polymerase chain reaction (RT-PCR) analysis of THLE-5b SP and non-SP cells. ABCG2, ATP-binding cassette membrane transporter; Oct-4, octomer binding transcription factor 4; pCAM, epithelial cell adhesion molecule; AFP, α -fetoprotein. G3PDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control. (B) Western blot analysis of THLE-5b SP and non-SP cells. Actin was used as a loading control. (C) Immunocytochemistry of THLE-5b SP cells. AAT, α 1-antitrypsin; control, primary antibody-minus control. The cells were not counterstained. Scale bars: 50 μ m. The results are representative of two independent experiments.

CK-19 (Fig. 2C). Taken together, these results suggest that THLE-5b SP cells are less differentiated than non-SP cells and have characteristics of stem/progenitor-like cells. Examination of the morphology of these cells by phase-contrast microscopy indicated that THLE-5b SP cells are composed of small cells that are mostly oval in shape (Fig. 3A), whereas the non-SP cells are composed of cells with differing morphologies that are either palmate-like or polygonal in shape (Fig. 3B).

Induction of Cell Differentiation

The ability of THLE-5b SP cells to undergo cell differentiation was assayed in cells grown as rotation-mediated and rotation-nonmediated aggregate cultures. Cells formed aggregates in both rotation-mediated and rotation-nonmediated cultures. The aggregation pattern in a rotation-nonmediated aggregate culture was somewhat different from that in a rotation-mediated aggregate culture. For up to 12 h, the cells in the former were loosely attached to each other and could be easily separated by

gentle pipetting. In contrast, the aggregates formed in rotation-mediated culture appeared as more compact (data not shown). The influence of the three culture conditions (rotation-mediated, rotation-nonmediated aggregate cultures and monolayer culture) on the gene expression of THLE-5b SP cells were examined by RT-PCR. The mRNA expression of albumin, AAT, Cytochrome P450 3A4 (CYP3A4), and TO was up-regulated when the cells were grown as rotation-mediated aggregates (Fig. 4). When THLE-5b SP cells were cultured as rotation-nonmediated aggregates, albumin and AAT were up-regulated (Fig. 4). These genes were only weakly expressed in THLE-5b SP cells under monolayer culture condition (Fig. 4).

Clonal Culture

We next cloned THLE-5b SP cells at a single-cell level in order to characterize these cells in further detail. Of 13 wells with only a single cell in 96-well plates, seven wells were with a clonal growth. Ultimately, four stable, clonal lines (CL-2, 5, 7, and 9) were chosen for analysis

Figure 3. Phase-contrast microscopy of THLE-5b SP and non-SP cells. The morphology of THLE-5b SP (A) and non-SP (B) cells was analyzed by phase contrast microscopy. Scale bars: 200 μ m.

Figure 4. Expression of hepatocyte-specific markers in THLE-5b SP cells. THLE-5b SP cells grown under two different aggregation culture conditions was assayed by reverse transcription and polymerase chain reaction (RT-PCR) and compared with that in cells grown as monolayer cultured cells (Mono), rotation-nonmediated aggregates (Aggregates I), or rotation-mediated aggregates (Aggregates II). AAT, α 1-antitrypsin; TO, tryptophan 2,3-dioxygenase. G3PDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control. The results are representative of two independent experiments.

and examined for the expression of phenotypic markers by RT-PCR. All of these clonal lines expressed ABCG2. Two of the clones, CL-7 and CL-9, expressed EpCAM whose expression was higher in CL-7 compared with CL-9 (Fig. 5A). The expression of phenotypic and differentiation markers was then further analyzed in clonal lines. The CL-7 cells expressed CD133, c-kit, Oct-4, Thy-1, CK19, and albumin, and the CL-9 cells weakly expressed c-kit and albumin and strongly expressed CK19. In contrast, the other two clonal lines (CL-2 and CL-5) were both negative for these markers. The level of expression of CK19 in CL-9 cells was higher than that in CL-7 cells (Fig. 5B). Based on these data, the CL-7 cells were chosen as a representative clonal line and their properties were investigated in more detail. The mRNA expression of the differentiation markers albumin, CYP3A4, and TO in CL-7 cells was up-regulated or induced when the cells were grown as rotation-mediated aggregates (Fig. 5C). When the CL-7 cells were grown as a monolayer, they only weakly expressed albumin and TO proteins. However, when they were grown as the aggregates, these proteins were strongly expressed (Fig. 5D). The CL-7 cells were relatively uniform in shape, and their morphology was more similar to that of THLE-5b SP than to the non-SP cells (Fig. 5E). In addition, no tumor formation was observed 6 months after injection of CL-7 cells (5×10^6 cells/mouse) into athymic nude mice or SCID mice (data not shown).

DISCUSSION

It has been reported that SP cells identify primitive progenitor cells in several tissues (5,6,27) and cell lines (11). There is currently controversy over the nature of SP cells (15). On the one hand, it has been suggested that SP cells in human and mouse epidermis lack stem cell properties (26). In this

Figure 5. Expression of stem cell and undifferentiated liver markers of clonal lines and their differentiation. (A and B) Reverse transcription and polymerase chain reaction (RT-PCR) analysis of clonal lines. (C) RT-PCR analysis of CL-7 cells grown as monolayer cultured cells (Mono) or rotation-mediated aggregates (Aggregates). EpCAM, epithelial cell adhesion molecule; ABCG2, ATP-binding cassette membrane transporter; Oct-4, octomer binding transcription factor 4; TO, tryptophane 2,3-dioxygenase. G3PDH (glyceraldehydes-3-phosphate dehydrogenase) was used as an internal control. The results are representative of two independent experiments. (D) Immunocytochemistry of CL-7 cells grown as monolayer cultured cells (Mono) or rotation-mediated aggregates (Aggregates). TO, tryptophane 2,3-dioxygenase. The cells were not counterstained. Scale bars: 50 μ m. (E) Phase-contrast microscopy of CL-7 cells. Scale bar: 200 μ m.

study, we investigated the stem-like properties of THLE-5b SP cells derived from the THLE-5b cell line. Self-renewal capability, an increase in colony-forming efficiency, and the expression of stem cell-associated markers are widely accepted criteria for the definition of adult stem cells (3). The SP cells in C6 glioma cell line can self-renew and produce both SP and non-SP cells, whereas the non-SP cells under the same culture conditions can produce non-SP cells only (13). In this study, we found that cells cultured from the SP cells contained both SP (2.1%) and non-SP cells. In contrast, cells cultured from the non-SP cells mostly contained non-SP cells, although they also contained a few SP cells, suggesting that THLE-5b SP cells can self-renew in culture. The exact reason why cells cultured from the non-SP cells contained a few SP cells as well as non-SP cells remains to be determined. Oligodendrocyte precursor

cells can be induced by extracellular signals to convert to a multipotent neural stem-like cells (13). It is likely that liver progenitor or precursor cells, which may be contained in the non-SP fraction, can be induced by extracellular signals to convert to hepatic stem-like cells. Sphere formation assay has been developed to study self-renewal capacity of certain stem cell types. Neural stem cells can proliferate and form floating spherical structures termed neurospheres when grown in serum-free medium supplemented with peptide growth factors (19). We are currently investigating whether the THLE-5b SP cells can grow as spheres in serum-free medium.

THLE-5b SP cells showed greater colony-forming efficiency and a higher clonal growth rate and expressed a higher level of stem cell-associated markers such as EpCAM, c-kit, Thy-1, and Oct-4 as well as albumin (oval

cell/hepatocyte marker) than non-SP cells. In addition, the expression of these markers was also observed in cells at the tip of the SP cell fraction obtained using flow cytometry (data not shown). The expression of stem cell-associated markers in this fraction of the SP cells suggests that this fraction of the SP cells contains the highest proportion of undifferentiated cells (10). The combined data strongly suggest that THLE-5b SP cells possess hepatic stem-like cell properties.

The existence of adult human liver progenitor cells has recently been demonstrated, and their phenotype has been defined. The expression of Oct-4, albumin, γ -glutamyl transpeptidase, AAT, CYP1B1, CK18, and CK19 has been reported in epithelial cell cultures of adult human liver (21). In our study, THLE-5b SP and CL-7 cells were induced to express albumin and AAT when cultured under aggregation culture condition but not when grown as monolayer cultures. It was concluded from a study of the gene expression profile of hepatic stem cells that the stem cell phenotype is defined by expression of EpCAM, neural cell adhesion molecule, CK19, c-kit, and claudin3, by low levels of albumin, and by lack of expression of AFP (20). The THLE-5b SP and CL-7 cells characterized in this study exhibited a similar gene expression profile to that of hepatic stem cells. Thus, these cells expressed EpCAM, CK19, c-kit, and low levels of albumin and did not express AFP. In contrast, THLE-5b SP and CL-7 cells differed from the hepatic stem-like cells described in other studies (4,9) by the presence of c-kit and Oct-4. Therefore, the properties of the THLE-5b SP and CL-7 cells, as defined in this study, appear to closely resemble those previously reported for hepatic stem cells (20). In addition, our findings strongly support the assumption that hepatic stem cells exist in adult human liver and suggest that EpCAM may be a useful marker for the isolation and identification of hepatic stem/progenitor-like cells.

It is often pointed out that cellular functions may be affected during the immortalization process. However, there is evidence to suggest that an immortalized human breast epithelial cell line with stem cell properties (7) and a mesenchymal stem cell line (17) retain much of their original phenotype. Furthermore, the stem-like properties of immortalized hepatic stem cells have been reported to be well maintained in a primate bipotential epithelial liver stem cell line (1) and in a fetal human progenitor liver epithelial cell line (16). It has been proposed that immortalized stem cell lines result either from transformation of a normal stem/progenitor-like cell or from acquisition of stem cell characteristics of a differentiated cell. We believe that liver stem/progenitor-like cells were inadvertently immortalized by the SV40 large T protein resulting in THLE-5b SP and CL-7 cells. Our results suggest that the stem-like properties of these cells are affected very little by immortalization.

To assess whether THLE-5b SP cells could differentiate to express hepatocytic functions, they were cultured as rotation-mediated or rotation-nonmediated aggregates. RT-PCR analysis revealed that more differentiated hepatocyte phenotypes were obtained after growth as rotation-mediated aggregates. Much attentions have been paid to spheroidal or multilayer aggregate culture systems reorganize hepatocytes themselves into three-dimensional structure normally seen in vivo (22–25). The present results suggest the potential of THLE-5b SP cells to engage in differentiation along into the hepatocyte phenotype. The aggregates formed in the rotation-mediated culture appear as more compact structures than those formed in the rotation-nonmediated culture. The spheroidal microenvironment established by cell contact may bring forth the cells along a pathway toward hepatocyte differentiation.

Unlike primary cultured cells, immortalized cells grow indefinitely in vitro, are phenotypically stable, and, importantly, allow repetition of experiments using these cell lines. To date, no nontumorigenic human liver cell lines possessing hepatic stem-like cell properties have been available. The THLE-5b SP and CL-7 cells described in our study could be used for the study of liver cell differentiation and/or carcinogenesis as well as provide a model of normal liver progenitor or stem cell lines. Current studies are underway to explore the engraftment capacity of these cells in athymic or SCID mice.

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