Isolation and Characterization of a Stem Cell Population from Adult Human Liver

Maria Beatriz Herrera, Stefania Bruno, Stefano Buttiglieri, Ciro Tetta, Stefano Gatti, Maria Chiara Deregibus, Benedetta Bussolati, Giovanni Camussi, M.D.

STEM CELLS®
TISSUE-SPECIFIC STEM CELLS

Isolation and Characterization of a Stem Cell Population from Adult Human Liver

MARIA BEATRIZ HERRERA,[a,b] STEFANIA BRUNO,[a,b] STEFANO BUTTIGLIERI,[a,b] CIRO TETTA,[c] STEFANO GATTI,[d] MARIA CHIARA DEREGIBUS,[a,b] BENEDETTA BUSSOLATI,[a,b] GIOVANNI CAMUSSI[a,b]

[a]Department of Internal Medicine, [b]Research Centre for Experimental Medicine (CeRMS), University of Torino, Torino, Italy;[c] Fresenius Medical Care Deutschland GmbH, Bad Homburg, Germany; [d]University of Milano, Division of Liver Transplantation, Ospedale Maggiore Istituto di Ricovero e Cura a Carattere Scientifico, Milano, Italy

Key Words. Progenitor cells • Hepatocytes • Liver injury • Mesenchymal stem cells • Pluripotent differentiation

ABSTRACT

Several studies suggested the presence of stem cells in the adult normal human liver; however, a population with stem cell properties has not yet been isolated. The purpose of the present study was to identify and characterize progenitor cells in normal adult human liver. By stringent conditions of liver cell cultures, we isolated and characterized a population of human liver stem cells (HLSCs). HLSCs expressed the mesenchymal stem cell markers CD29, CD73, CD44, and CD90 but not the hematopoietic stem cell markers CD34, CD45, CD117, and CD133. HLSCs were also positive for vimentin and nestin, a stem cell marker. The absence of staining for cytokeratin-19, CD117, and CD34 indicated that HLSCs were not oval stem cells. In addition, HLSCs expressed albumin, α-fetoprotein, and in a small percentage of cells, cytokeratin-8 and cytokeratin-18, indicating a partial commitment to hepatic cells. HLSCs differentiated in mature hepatocytes when cultured in the presence of hepatocyte growth factor and fibroblast growth factor 4, as indicated by the expression of functional cytochrome P450, albumin, and urea production. Under this condition, HLSCs downregulated α-fetoprotein and expressed cytokeratin-8 and cytokeratin-18. HLSCs were also able to undergo osteogenic and endothelial differentiation when cultured in the appropriated differentiation media, but they did not undergo lipogenic differentiation. Moreover, HLSCs differentiated in insulin-producing islet-like structures. In vivo, HLSCs contributed to regeneration of the liver parenchyma in severe-combined immunodeficient mice. In conclusion, we here identified a pluripotent progenitor population in adult human liver that could provide a basis for cell therapy strategies. STEM CELLS *2006;24:2840–2850*

INTRODUCTION

The regenerative capacity of the liver after partial hepatectomy or chemical injury is well known [1]. However, the cell types involved in hepatic regeneration are still undefined, and a contribution of both mature hepatocytes and resident stem cells has been suggested. Evidence from several studies indicates the presence of resident stem cells in the adult liver [2, 3]. Results based primarily on models of liver injury and carcinogenesis in experimental animals led to the concept that a potential stem cell compartment is located within the smallest branches of the intrahepatic biliary tree [2, 4]. Proliferation of cells located in this area and defined as "oval cell response" has been shown to contribute to regeneration of biliary cells and hepatocytes [2, 5–7]. Oval cells express markers of both fetal hepatocytes and biliary cells and are bipotent progenitors capable of generating hepatocytes and bile duct cells [2, 5–8]. The most likely candidate for the oval cell niche is the canal of Hering [9–11]. Moreover, oval cells express some hematopoietic markers such as c-kit, CD34, Sca-1, and CD90 [8, 12–15]. However, recent studies suggest that oval cells are a resident population rather than bone marrow-derived cells [16, 17].

Besides oval cells, a cell population referred to as "small hepatocytes" (small hepatocyte-like progenitor cell) has been described in rat liver [18]. These cells are apparently responsible for the regeneration of rat liver after partial hepatectomy in animals exposed to retrorsine [18, 19]. Small hepatocytes may represent a less differentiated liver population that is phenotypically distinct from hepatocytes and is more resistant to some forms of toxic injury [20]. The location of these cells in the liver is presently unknown.

Correspondence: Giovanni Camussi, M.D., Cattedra di Nefrologia, Dipartimento di Medicina Interna Ospedale Maggiore S. Giovanni Battista, Corso Dogliotti 14, 10126, Torino, Italy. Telephone: +39-011-6336708; Fax: +39-011-6631184; e-mail: giovanni.camussi@unito.it Received February 27, 2006; accepted for publication August 22, 2006; first published online in STEM CELLS *EXPRESS* August 31, 2006. 1066-5099/2006/$20.00/0 doi: 10.1634/stemcells.2006-0114

In humans, the counterpart to oval cells has been identified in the so-called "ductular reaction" [21, 22]. Epithelial cells with the morphological appearance and immunohistological markers consistent with oval cells have been identified in the regenerative areas in patients with chronic liver injury or submassive hepatic necrosis [14, 23–29]. Cells with a similar phenotype have also been identified in human fetal liver. Several studies have isolated and characterized these human fetal liver cells as bipotent progenitors [26–31]. These studies suggest that the human counterpart to rodent fetal liver progenitors exists, and may represent a source of cells for liver regeneration.

The aim of the present study was to isolate and characterize resident progenitor cells from adult human liver. For this purpose, liver cells were cultured under stringent culture conditions, and a population of human liver stem cells (HLSCs) was identified and characterized. HLSCs were phenotypically different from oval cells and showed self-renewing capability and multilineage differentiation potential.

Materials and Methods

Isolation and Culture of Human Liver Stem Cells and Human Mesenchymal Stem Cells

Human hepatocytes were isolated from fresh surgical specimens of patients undergoing hepatectomies. Healthy liver tissue (5–20 g) was used to isolate hepatocytes by collagenase digestion as described. Briefly, liver tissues were isolated and perfused with 350 ml of a warm (37°C) calcium-free buffer (Gibco Liver Perfusion Medium; Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Then, liver tissues were digested in Gibco Liver Digest Medium at 37°C. This resulted in blanching, softening, and dissociation of hepatic tissue and provided complete digestion of the liver in 10–12 minutes. The hepatocytes were released by mincing and pipetting with a large-bore pipette. The cell suspension was filtered through a sterile 100-μm nylon mesh into a beaker placed on ice, sedimented by centrifugation at 50*g* for 5 minutes, resuspended, and washed two to three times in cold wash medium (Gibco Hepatocyte Wash Medium; Invitrogen).

The initial plating consisted of Williams Medium E (Invitrogen) supplemented with glutamine and with 5% fetal calf serum (FCS; Euroclone, Wetherby, U.K., http://www.euroclone.net). Unattached cells were poured off 2–3 hours later and replaced with hepatocyte serum-free medium (Gibco Hepatozyme-SFM; Invitrogen), a highly modified Chees' medium supplemented with 1.25 μg/cm^2 collagen to provide a sandwich matrix. Cultures were refed with Hepatozyme-SFM (without collagen) at 24 hours and every 48 hours thereafter. Hepatocytes were seeded at a density of 1.0–1.5 $\times$ 10^5 viable cells (80% viable cells determined by the trypan blue [Invitrogen]) per cm^2 onto collagen-coated culture plates in Hepatozyme-SFM maintained at 37°C, 5% CO_2 for 2 weeks. Human cryopreserved normal hepatocytes obtained from Cambrex Bio Science Verviers S.p.r.l. (Verviers, Belgium; http://www.cambrex.com) were also used.

After 2 weeks of culture, hepatocytes died, and then medium was substituted by α-minimum essential medium/endothelial cell basal medium-1 (α-MEM/EBM) (3:1) (Gibco/Cambrex) supplemented with L-glutamine (5 mM), Hepes (12 mM, pH 7.4), penicillin (50 IU/ml), streptomycin (50 μg/ml) (all from Sigma, St. Louis, http://www.sigmaaldrich.com), FCS (10%), and horse serum (HS, 10%) from Invitrogen. Individual attached cells were identified on the culture dish after another 3 weeks. When colonies were evident, cloning rings were placed around them, and they were subcultured to an individual well of a 24-well culture plate. The expanded cells were transferred to a T-75 flask and analyzed when they approached confluence. Human mesenchymal stem cells (hMSCs) were isolated from bone marrow and cultured as previously described [32].

Colony-Forming Unit-Fibroblast Assay

The assay was performed using a modification of a previously described protocol used to evaluate the frequency of MSCs in fresh bone marrow [33]. Briefly, human cryopreserved or fresh normal hepatocytes were seeded at a concentration of 1.0–1.5 $\times$ 10^5 viable cells/cm^2 under the culture conditions described above. Medium was changed at day 15. On day 20, the number of colonies displaying five or more cells was scored under an inverted microscope.

Growth Kinetics

Growth curves describing culture kinetics were generated as previously described [33]. The growth area occupied by a primary HLSC culture, corresponding to 25 cm^2, was assumed as 1, as a matter of simplification. When the second passage took place, the split ratio at passage 1 (1:3) was multiplied by that value, meaning that at the end of passage 1, the cumulative growth area was 3 (i.e., three times the growth area occupied by a primary culture). At the end of the second passage, the split ratio at passage 2 (1:3) was multiplied by the cumulative growth area at passage 1 ($3 \times 3 = 9$). This procedure was repeated for each passage, providing a theoretical growth curve that is directly proportional to the cell number. Growth was evaluated in six different HLSC clones.

Immunofluorescence

Cytofluorometric analysis was performed as described [34], and the following antibodies, all phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated, were used: anti-CD105, -CD29, -CD31,-CD34, -CD146, -CD44, and -CD117 (Dako Denmark A/S, Copenhagen, Denmark, http://www.dako.com); -CD73, -CD45, -CD90, -CD14, and -CD144 (BD Biosciences Pharmingen, San Jose, CA, http://www.bdbiosciences.com/pharmingen); -CD133 (Miltenyi Biotec, Auburn, CA, http://www.miltenyibiotec.com); and monoclonal antibodies and PE-conjugated goat antibodies against mouse IgG (Dako Denmark A/S) secondary antibody (Ab) when needed. All incubations were performed in 100 μl of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and 0.1% sodium azide, at 4°C, and cells were washed twice between incubations. For each sample, 10,000 cells were analyzed on a FACScan cytometer (BD Biosciences Pharmingen). Gating was constructed based on negative controls, and compensation controls were included in all analyses performed. Population percentages and numbers were generated for gated populations from each experiment using Cell Quest software (BD Biosciences Pharmingen).

Indirect immunofluorescence was performed on HLSCs cultured on chamber slides (Nalge Nunc International, Rochester, NY, http://www.nalgenunc.com), fixed in 4% paraformaldehyde containing 2% sucrose, and, when needed, permeabilized

with Hepes-Triton X-100 buffer [35]. The following monoclonal antibodies were used: anti-α-fetoprotein (AFP), anti-glucose transporter 2 (Glut2), anti-albumin, anti-STRO-1 (R&D Systems, Abington, U.K., http://www.rndsystems.com), anti-cytokeratin-19 (CK19), anti-α-smooth muscle actin (α-SMA) (Dako Denmark A/S), anti-CK18 (Chemicon International, Temecula, CA, http://www.chemicon.com), anti-cytokeratin-8, and anti-vimentin (Sigma). Anti-insulin (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), anti-von Willebrand factor, anti-nestin, anti-osteocalcin, and anti-osteopontin rabbit polyclonal antibodies (Sigma), and anti-neural cell adhesion molecule (NCAM) (BD Biosciences Pharmingen) rat polyclonal antibodies were used. Omission of the primary antibodies or substitution with nonimmune rabbit, rat, or mouse IgG was used as control where appropriate. FITC-conjugated anti-mouse and anti-rabbit IgG (Dako Denmark A/S) or Alexa Fluor 488 anti-rabbit IgG and Texas Red anti-mouse IgG (Molecular Probes, Leiden, The Netherlands, http://probes.invitrogen.com) were used as secondary Ab. Confocal microscopy analysis was performed using a Zeiss LSM 5 Pascal Model Confocal Microscope (Carl Zeiss International, Jena, Germany, http://www.zeiss.com). Ethidium bromide or Hoechst 33258 dye (Sigma) was added for nuclear staining.

In Vitro Culture in Rotary Cell Culture System

HLSCs and hMSCs were incubated under a condition of microgravity in the Rotary Cell Culture System (RCCS; Synthecon Incorporated, Houston, TX, http://www.synthecon.com), as reported previously [36, 37]. RCCS maintains the metabolic functions of hepatocytes. In this system, cells were placed in 10-ml so-called vessels that horizontally rotate about their axis at 8–10 rotations per minute. Cells were cultured in RCCS at a concentration of 200,000 per milliliter in the presence of α-MEM/EBM 3:1, 12 mM Hepes, 2% FCS with or without hepatocyte growth factor (HGF) (10 ng/ml; Sigma), and fibroblast growth factor 4 (FGF4) (10 ng/ml; Sigma); hMSCs were cultured in RCCS in α-MEM under the same conditions. The same quantity of cells was plated in two flasks coated with Matrigel in a volume of 5 ml of α-MEM/EBM 3:1, 12 mM Hepes, 2% FCS with or without HGF (10 ng/ml) and FGF4 (10 ng/ml).

After 15 days of culture, we evaluated the activity of cytochrome P450 enzymes that are markers of mature hepatocytes [38]. Cells were washed with PBS and then left overnight at 37°C at 5% CO_2 with 5-chloromethylfluorescein diethyl ether (Molecular Probes) [39], which is a cytochrome P450 substrate. This substrate yields a product with a fluorescence emission, making this substrate useful for the direct measurement of cytochrome P450 enzymatic activity by flow cytometric analysis.

In Vitro Albumin Secretion and Urea Production

After 15 days, we evaluated the production of urea and secretion of albumin into the supernatant of HLSCs and hMSCs under all culture conditions described above. Production of urea was determined by a Beckman Synchron CX9 clinical system (GMI, Inc., Ramsey, MN, http://www.gmi-inc.com). For this purpose, culture medium was completely changed every 2 days. Secretion of albumin was determined by nephelometry (Dade Behring Diagnostic, Marburg, Germany, http://ir.dadebehring.com); in this case, cells were cultured without FCS. When needed, HGF and FGF4 were added into the media every 3 days.

In Vitro Differentiation into Insulin-Producing Cells

Differentiation into pancreatic insulin-producing cells was obtained, as described in [40]. Briefly, cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% FCS for a month, and then for the last 5–7 days, 10 mM nicotinamide (Sigma) was added. Three-dimensional cell clusters that formed were stained with anti-insulin and anti-Glut2 antibodies for immunofluorescence studies. Moreover, these clusters were stained with the zinc-chelating agent dithizone (Sigma), which is specific for the insulin-containing granules [41].

In Vitro Osteogenic, Adipogenic, and Endothelial Differentiation

For osteogenic differentiation, cells were cultured in α-MEM that was supplemented with 10% FCS, 10% HS, 100 U/ml penicillin, 100 μg/ml streptomycin, 12 mM L-glutamine, 20 mM β-glycerol phosphate, 50 ng/ml thyroxine, 1 nM dexamethasone and 0.5 μM ascorbate 2-phosphate (all from Sigma) [32]. The medium was changed two times per week for 3 weeks. To evaluate the differentiation, cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature and stained with alizarin red, pH 4.1 (Sigma) for 20 minutes at room temperature.

For adipogenic differentiation, cells were cultured in α-MEM that was supplemented with 10% FCS, 10% HS, 100 U/ml penicillin, 100 μg/ml streptomycin, 12 mM L-glutamine, 5 μg/ml insulin, 50 μM indomethacin, 1×10^{-6} M dexamethasone, and 0.5 μM 3-isobutyl-1-methylxanthine (all from Sigma) [32]. The medium was changed two times per week for 3 weeks. To evaluate the differentiation, cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature and stained with 0.5% oil red O (Sigma) in methanol (Sigma) for 20 minutes at room temperature. Endothelial cell differentiation was obtained by culturing HLSCs in EBM (Cambrex) with vascular endothelial growth factor (VEGF; 10 ng/ml; Sigma) for 10 days, as described previously [42].

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from 3×10^6 HLSCs from different clones using TRIzol reagent (Invitrogen) according to the manufacturer's instructions; 2 μg of total RNA were treated with DNase (Invitrogen), reverse-transcribed using oligo(dT) primers and 15 U of reverse transcriptase enzyme (Eppendorf AG, Hamburg, Germany, http://www.eppendorf.com). Polymerase chain reaction (PCR) was performed as follows: 94°C, 15 seconds; 55–60°C, 1 minute; and 72°C, 1 minute for 40 cycles. Primer sequences for human albumin, AFP, and CK18 are published [43]. Human liver total RNA (BD Biosciences) and human tubular total RNA [44] were used, respectively, as positive and negative control.

In Vivo Experimental Model of Liver Acute Injury

Pathogen-free male severe-combined immunodeficient (SCID) mice, 6–8 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME, http://www.jax.org). All animals

were fasted overnight before *N*-acetyl-*p*-aminophen (Sigma) treatment. *N*-Acetyl-*p*-aminophen was dissolved in warm saline (15 mg/ml). Animals received an intraperitoneal injection of 150 mg/kg *N*-acetyl-*p*-aminophen or, as control, vehicle alone [45]. After 3 hours, mice were injected intravenously with HLSCs (2×10^5) or vehicle alone. After 7 or 30 days, the animals were killed by cervical dislocation, and the livers were excised, rinsed in saline, and placed in 10% phosphate-buffered formalin to be used in immunohistochemical analysis. Six animals were used in every experimental condition.

Histology

Mouse livers were formalin-fixed and paraffin-embedded before sectioning. Sections from paraffin-embedded blocks were collected onto poly(L-lysine)-coated slides. Endogenous peroxidase activity was blocked with 6% H_2O_2 for 8 minutes at room temperature. Goat anti-mouse β_2-microglobulin or rabbit anti-human HLA class I polyclonal antibodies (Santa Cruz Biotechnology) were applied to slides overnight at 4°C. Horseradish peroxidase-labeled anti-rabbit or anti-goat Envision polymers (Dako Denmark A/S) were incubated for 30 minutes. The reaction product was developed using 3,3-diaminobenzidine. Omission of the primary antibody or substitution with unrelated rabbit or goat IgG served as negative control. Mayer's hemalum solution (Merck, Darmstadt, Germany, http://www.merckbiosciences.co.uk) was used for nuclear counterstaining.

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) was performed using the Vysion kit for the detection of human chromosome 17 (Vysis Inc., Downers Grove, IL, http://www.vysis.com). SG CEP 17 DNA probe hybridizes to the centromere (band region 17p11.1–q11.1, locus D17Z1) of human chromosome 17. In situ hybridization was performed on 5-μm sections according to the manufacturer's guidelines. In brief, the sections were deparaffinized, dehydrated in 100% ethanol, and dried at 45°C–50°C for 2–5 minutes. Slides were then subjected to protease digestion for 10–20 minutes at 38°C, denatured (72°C for 5 minutes), and hybridized (37°C) with prewarmed probes (CEP17 Spectrum Green; Vysis Inc.) overnight (16–18 hours) in HYBrite hybridization system (Vysis Inc.). They were then washed with post-hybridization wash buffer (2× SSC, 0.3% Nonidet P-40) at 72°C and counterstained with 4,6-diamidino-2-phenylindole, mounted, maintained in darkness for 15 minutes at 4°C, and then observed with a confocal microscope.

Electron Microscopy

Cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.4) as previously described [46]. Samples were postfixed in 2.5% glutaraldehyde, dehydrated in alcohol, dried, and coated with gold by sputter coating. The specimens were examined in a scanning Jeol T300 electron microscope. Images were obtained via secondary electron at a working distance of 15–25 mm and at an accelerating voltage of 20–25 kV [46].

Table 1. Immunophenotype of undifferentiated HLSCs

Markers	FACS analysis (% of positive cells: mean ± SD)
Isotypic control	0
CD34	0
CD45	0
CD14	0
CD73	100
CD29	100
CD44	100
CD117	0
CD90	80 ± 10
CD146	18 ± 11
CD133	0
CD105	20 ± 12
	Immunofluorescence analysis (% of positive cells: mean ± SD)
Albumin	100
AFP	100
CK8	11 ± 7
CK18	15 ± 8
CK19	0
Vimentin	100
Nestin	100
α-SMA	0
NCAM	0
STRO-1	0

Cytofluorometric or immunofluorescence analysis of different cell antigens was performed on 24 different clones of HLSCs and results are expressed as mean ± SD of positive cells.
Abbreviations: AFP, α-fetoprotein; CK, cytokeratin; HLSC, human liver stem cell; α-SMA: α -smooth muscle actin; NCAM, neural cell adhesion molecule.

RESULTS

Isolation and Characterization of HLSCs from Adult Human Liver

HLSC lines were obtained by culturing under stringent conditions hepatocytes obtained from eight different normal human liver preparations, including four preparations from fresh liver tissue and four from cryopreserved hepatocytes. Hepatocytes seeded at a density of $1.0–1.5 \times 10^5$ viable cells per cm^2 onto collagen-coated culture plates were cultured in hepatocyte cell culture medium for 2 weeks. After 2 weeks, the large majority of hepatocytes were dead. Surviving cells were removed, plated in limiting dilution, and cultured in α-MEM/EBM supplemented with 10% FCS and 10% HS. Individual attached cells were observed after three additional weeks of culture. Single clones were subcultured, expanded, and analyzed when they approached confluence. Twenty-four different cell clones were obtained and kept in culture in undifferentiating medium for 6 months without undergoing senescence. These data indicate that single progenitor cells of the adult liver have the ability to self-renew. HLSCs were unable to grow in α-MEM plus 10% FCS and 10% HS, the commonly used culture medium for MSCs [32].

HLSCs showed the presence of several stem cell markers and some liver tissue-specific markers by fluorescence-activated cell sorting (FACS), immunofluorescence, and reverse transcription-PCR analysis (Table 1). As shown in Figure 1A, all

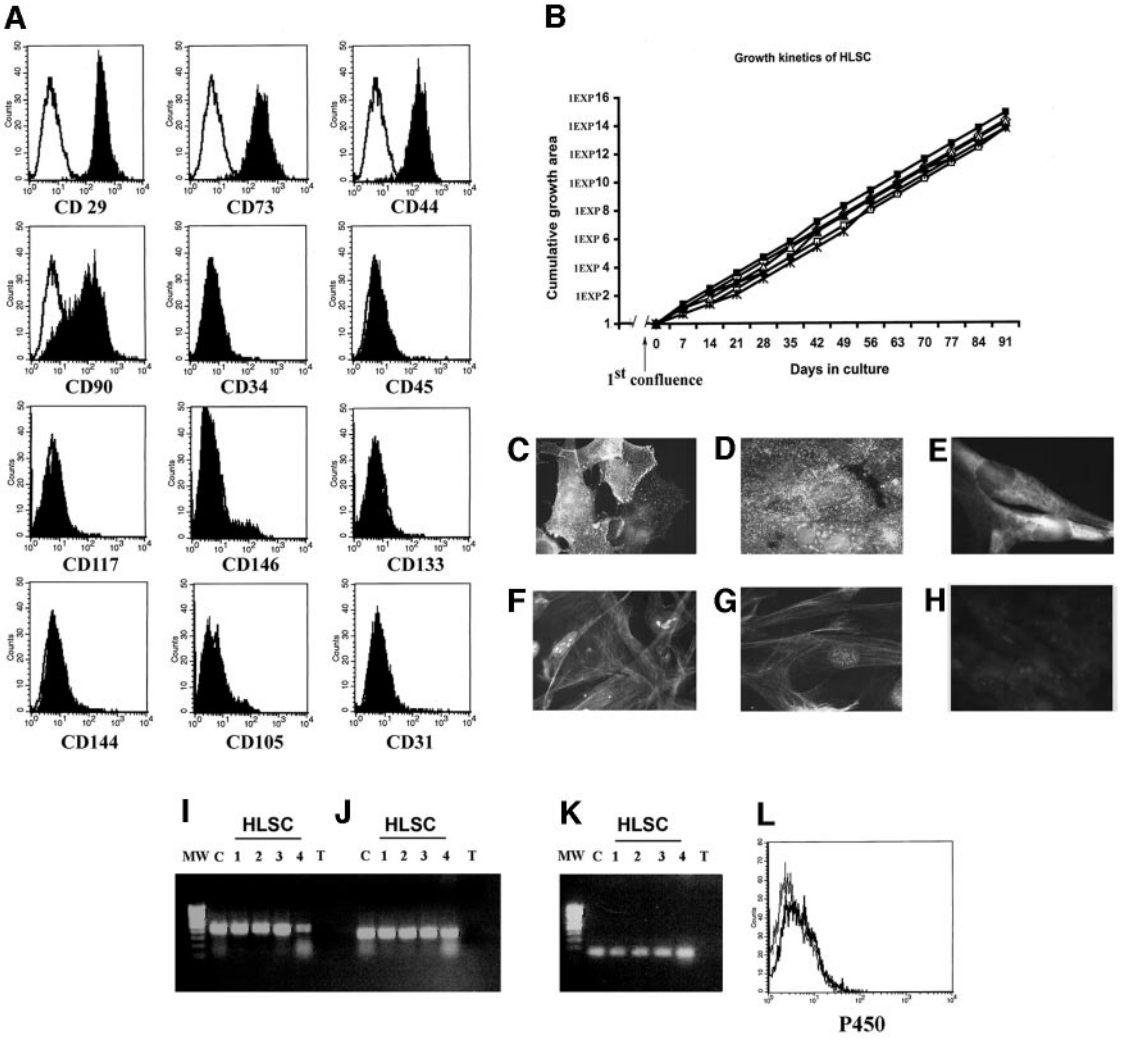

Figure 1. Characterization of HLSCs. (A)**:** Representative flow cytometric analysis of a HLSC clone (black histograms; white histograms represent isotypic controls). Cells were labeled with fluorescein isothiocyanate- or phycoerythrin-conjugated antibody. Virtually all HLSCs were positive for CD29, CD73, CD44, and CD90 and negative for CD34, CD45, CD117, CD133, CD144, and CD31. All HLSC clones were examined by flow cytometric analysis with similar results. In the HLSC clone shown in the figure, 10% of the cells were positive for CD146 and CD105. The positivity for CD146 and CD105 varied from clone to clone (CD146, range 7%–29%; CD105, range 8%–32%). **(B):** Growth kinetics of HLSCs. The growth area occupied by a primary HLSC culture, corresponding to 25 cm^2, was assumed as 1. Growth area is represented as a multiple of the area occupied by a confluent primary culture, arbitrarily set to 1 (see Materials and Methods). Each curve indicates one of the six different HLSC clones studied. **(C–H):** Representative immunofluorescence micrographs of HLSCs stained with antibodies against albumin **(C)**, α-fetoprotein **(D)**, CK18 **(E)**, vimentin **(F)**, nestin **(G)**, or CK19 **(H)**. The nuclei of the cells were stained with ethidium bromide. Original magnification, ×400. **(I–K):** Representative micrographs of reverse transcription-polymerase chain reaction of four different clones of HLSCs (**[I, J, K]**, lanes 1–4) showing mRNA expression for CK18 (**[I]**, lane 1–4), α-fetoprotein (**[J]**, lanes 1–4), and albumin (**[K]**, lanes 1–4); total mRNA from human liver was used as positive control (C) and total mRNA from human renal tubular cells was used as negative control (T). **(L):** Representative flow cytometric analysis of HLSCs showing the absence of cytochrome P450 enzymatic activity (black line) in cells maintained in nondifferentiation conditions (gray line, isotopic controls). All HLSC clones were examined with similar results. Abbreviations: C, positive control; HLSC, human liver stem cell; MW, molecular weight; T, negative control.

HLSC clones were positive for the mesenchymal stem cell markers CD73, CD90, CD29, and CD44 and negative for the hematopoietic stem markers CD45, CD34, CD117 (c-kit), and CD133. The positivity for CD146 and CD105 varied in different clones (Table 1). All HLSC clones were also negative for CD31 and CD144 (vascular endothelial [VE]-cadherin). In addition, HLSCs were positive for the liver tissue-specific proteins such as AFP (Table 1; Fig. 1D, 1J), which is considered a marker of hepatocyte precursors, and human albumin (Table 1; Fig. 1C, 1K) and for CK18 and CK8 (Table 1; Figs. 1E, 1I, 2J). Nestin and vimentin were also positive in 100% of cells of different clones (Fig. 1F, 1G; Table 1). In contrast, CK19, a marker of oval cells (Fig. 1H), as well as cytochrome P450, a marker of mature hepatocytes (Fig. 1L), was negative. In addition, HLSCs were negative for α-SMA, NCAM, and STRO-1 (Table 1).

The results indicate that cloned HLSCs expressed markers of both MSCs and immature liver cells. The morphology and the absence of CD34, c-kit (Fig. 1A; Table 1), and CK19 (Fig. 1H; Table 1) indicated that HLSCs represent a population of liver precursors different from oval cells.

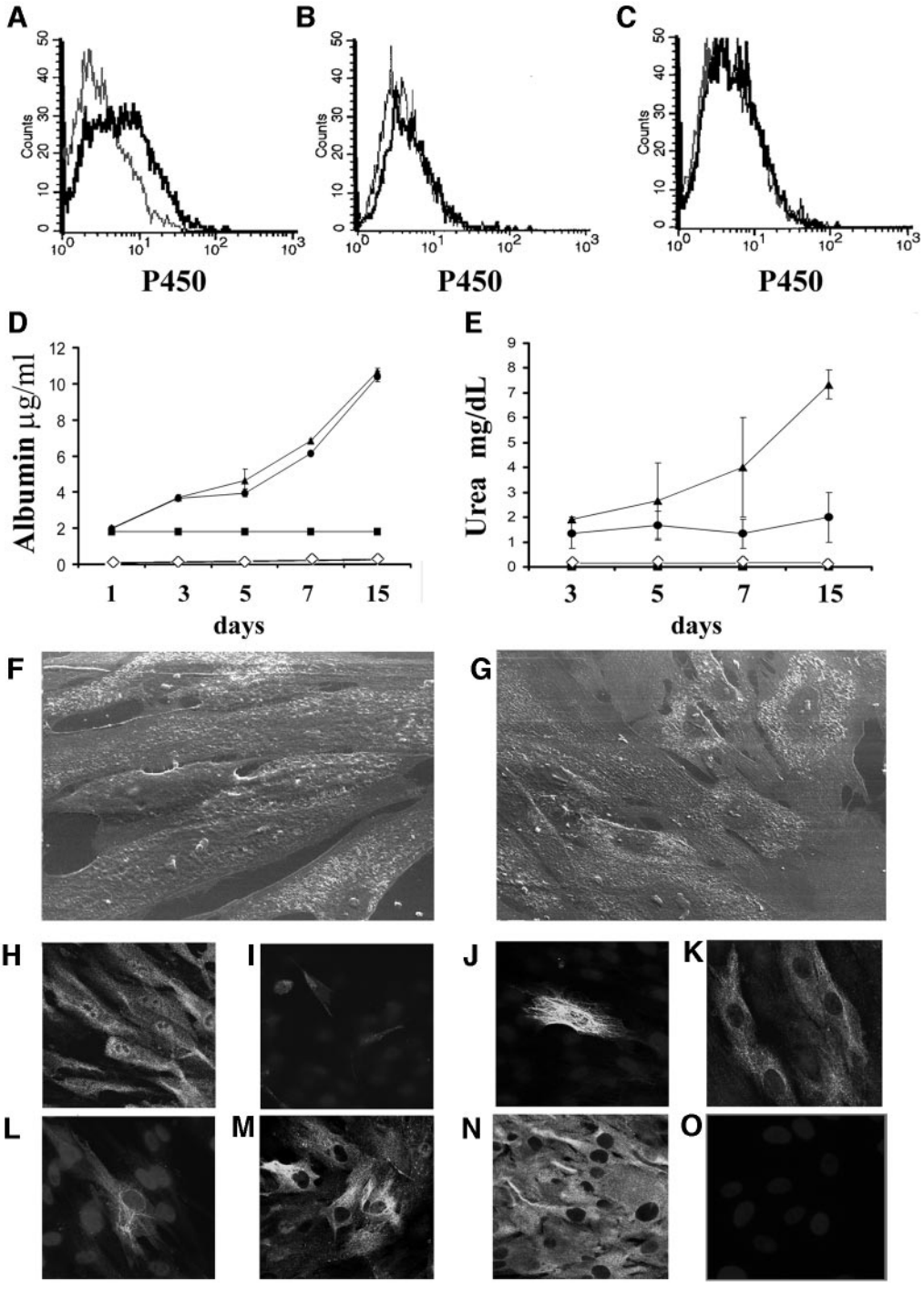

Figure 2. Differentiation of human liver stem cells (HLSCs) into mature hepatocytes. **(A–C):** Representative flow cytometric analysis showing the presence of cytochrome P450 enzymatic activity in 2×10^6 HLSCs (**[A]**, black line) cultured for 15 days in the presence of 10 ng/ml HGF and FGF-4 under microgravity conditions. In 2×10^6 hMSCs cultured in the same condition there was no enzymatic activity (**[B]**, black line). In HLSCs cultured in adhesion with HGF and FGF-4 there also was no enzymatic activity (**[C]**, black line). Gray lines indicate the isotypic controls. **(D, E):** 2×10^6 HLSCs were also cultured without sera in the presence of 10 ng/ml HGF and FGF-4 in microgravity conditions (▲) or in adhesion (●). Cells were also cultured under microgravity conditions as well as in adhesion in the absence of growth factors (■). Also, 2×10^6 hMSCs were cultured without sera in the presence of 10 ng/ml HGF and FGF-4 in microgravity conditions (◇). The production of albumin **(D)** and urea **(E)** in the supernatant was evaluated. **(F, G):** Representative scanning electron micrographs of HLSCs showing a change in the morphology from elongated **(F)** to cuboid cells **(G)** in the presence of 10 ng/ml HGF and FGF-4. **(H–O):** Representative immunofluorescence staining for AFP **(H, I)**, CK8 **(J, K)**, CK18 **(L, M)**, and human albumin **(N)** of HLSCs before **(H, J, L)** and after **(I, K, M)** differentiation induced by HGF and FGF-4. **(O)** shows staining for isotypic control. Nuclear staining was performed with Hoechst dye 33258. Original magnification: **(E, F)**, ×1,200; **(G–N)**, ×600.

Table 2 Number of HLSC colonies observed in CFU-F assay per 0.5×10^6 viable hepatocytes from six different preparations

Preparations	CFU-F number
Cryopreserved	
1	8
2	4
3	6
Fresh	5
1	
2	3
3	6
Mean ± SD	5.33 ± 1.75

Abbreviation: CFU-F, colony-forming unit-fibroblast.

HLSC Growth Kinetic and Frequency

The growth curves of six different HLSC clones are shown in Figure 1B. Confluence of the primary culture was usually reached 25–30 days after the beginning of the hepatocyte cultures (confluence is indicated by 1 in the *y*-axis). The interval between passages varied among clones from 3 to 5 days until passage 5, and from then on, was established every 3 days. We have not observed replicative senescence in HLSC cultures. Clones were cryopreserved by the end of this study.

To estimate the frequency of HLSCs in liver, we used a colony forming unit-fibroblast assay test [33]. Counting of colonies was performed after a period of 20 days from the beginning of hepatocyte cultures. Colonies with at least five cells were considered. The frequency of HLSCs in adult human livers was estimated as 5.33 ± 1.75 HLSCs per 0.5×10^6 hepatocytes (mean ± SD of six different preparations), which corresponds to one HLSC per 94,000 hepatocytes (Table 2). No differences between the frequency and growth kinetics of HLSC clones derived from cryopreserved and fresh hepatocytes were observed.

In Vitro Hepatocyte Differentiation of HLSCs

To obtain differentiation in human mature hepatocytes, HLSCs were cultured both under adhesion and microgravity conditions in media supplemented or not with HGF and FGF4. As shown in Figure 2, the expression of functional cytochrome P450 was obtained only in HLSCs cultured for 15 days under microgravity conditions in the presence of HGF and FGF4. FACS analysis indicated that cells acquired positivity for cytochrome P450 activity in this condition (Fig. 2A). HGF and FGF4 also promoted the synthesis and release of albumin and urea by HLSCs (Fig. 2D, 2E). The production of urea occurred only when HLSCs were cultured under microgravity conditions (Fig. 2E). Under the same experimental conditions, hMSCs derived from bone marrow did not acquire positivity for cytochrome P450 activity (Fig. 2B) or produce a significant amount of albumin and urea (Fig. 2D, 2E). At variance with this finding, albumin was synthesized and released by HLSCs cultured both under adhesion and microgravity conditions (Fig. 2D). Moreover, HGF and FGF4 induced change in the morphology from elongated to cuboid cells (Fig. 2F, 2G) and a reduction of AFP expression from 100% undifferentiated to 18 ± 5% differentiated cells (Fig. 2H, 2I), and an increase in CK8 (Fig. 2J, 2K) and CK18 (Fig. 2L, 2M) expression from 11 ± 7% and 15 ± 8% undifferentiated to 60 ± 13% and 92 ± 7% differentiated cells, respectively. The cells maintained the expression of albumin (Fig. 2N). Similar results were obtained with all HLSC clones.

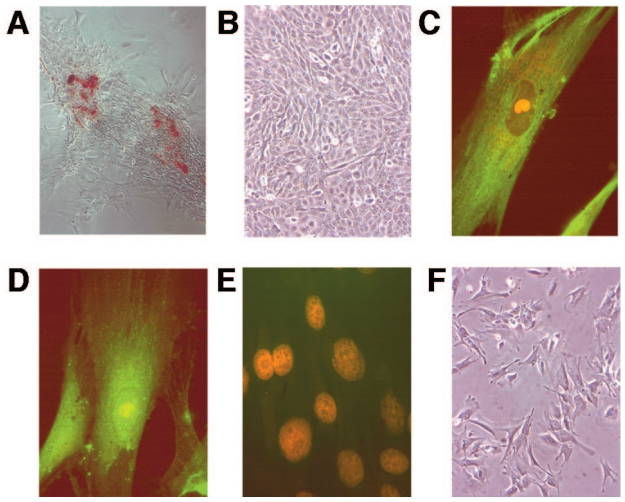

Figure 3. Osteogenic differentiation of human liver stem cells (HLSCs). Representative micrographs showing osteogenic but not adipogenic differentiation of HLSCs are shown. **(A, B):** Positive staining for calcium deposits using Alizarin Red was detectable in HLSCs cultured for 21 days in osteogenic differentiation medium **(A)** but not in α-MEM/EBM **(B)**. **(C–E):** Representative micrographs showing immunofluorescence staining of HLSCs expressing the osteogenic differentiation markers osteopontin **(C)** and osteocalcin **(D)**. In experiments using isotypic control Abs, no fluorescent signal was detected; nuclei were stained with ethidium bromide **(E)**. **(F):** HLSCs cultured for 21 days in the presence of adipogenic differentiation medium and stained with Oil Red O showed no adipogenic differentiation. Original magnification: **(A, B, F)**, ×250; **(C–E)**, ×400. Three different cell clones were studied with similar results.

In Vitro Osteogenic, Adipogenic, and Endothelial Differentiation of HLSCs

HLSCs cultured for 3 weeks in osteogenic differentiation medium exhibited deposits of calcium (Fig. 3A) and expression of osteocalcin and osteopontin (Fig. 3C, 3D), indicating an osteogenic differentiation. Moreover, the cells became negative for albumin, AFP, and CK18 (data not shown). At variance with hMSCs, which were able to undergo adipogenic differentiation (not shown), HLSCs maintained with adipogenic differentiation medium for 3 weeks did not stain positively for lipid droplets (Fig. 3F).

When cultured in EBM supplemented with VEGF, HLSCs expressed the endothelial markers CD31, CD34, kinase insert domain receptor (KDR or VEGFR-2), CD144 (VE-cadherin), and von Willebrand factor, which were negative under undifferentiated conditions (Fig. 4A, 4B), indicating an endothelial differentiation. Under these conditions, the expression of CD105 and CD146 was enhanced with respect to undifferentiated cells. During endothelial differentiation, albumin, AFP, and CK18 were lost (Fig. 4C–4E).

In Vitro Differentiation of HLSCs in Islet-Like Structures

When HLSCs were cultured in DMEM with high glucose content (23 mM) for a month followed by 5–7 days of culture in the presence of 10 mM nicotinamide, cells from the elongated morphology (Fig. 5A, 5G) began to form small spheroid cell clusters on

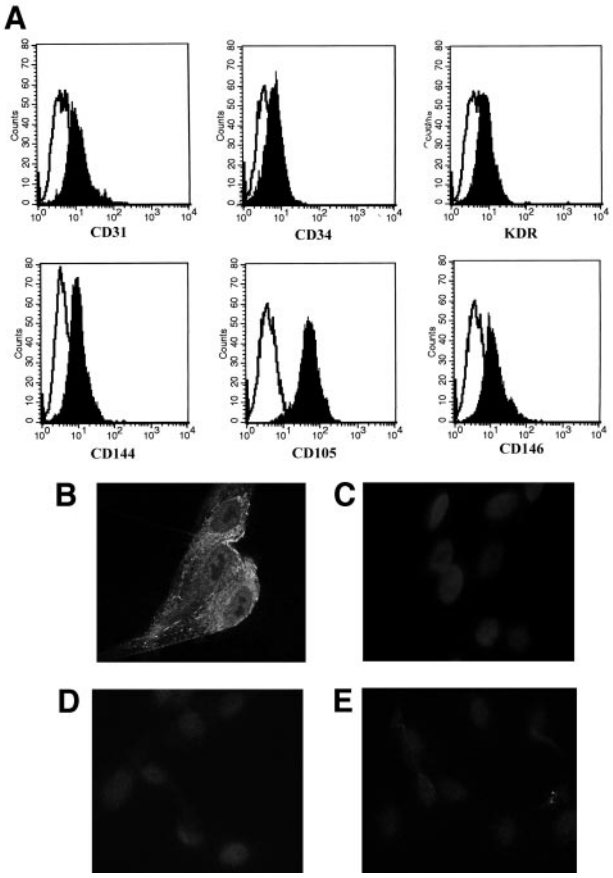

Figure 4. Endothelial differentiation of human liver stem cells (HLSCs). **(A):** Representative cytofluorometric analysis of HLSCs after 10 days of culture in the presence of VEGF. Endothelial differentiated cells acquired the expression of the endothelial markers CD31, CD34, KDR, CD144, CD105, and CD146. **(B–E):** Representative immunofluorescence micrographs of endothelial markers in HLSCs cultured in endothelial differentiation medium. von Willebrand factor expression was detected in endothelial differentiated cells **(B)**, which were negative for AFP **(C)**, CK18 **(D)**, and albumin **(E)**. Nuclear staining was performed with Hoechst dye 33258. Original magnification: ×630. Ten cell clones were studied with similar results.

top of the confluent cell monolayer, morphologically resembling pancreatic islets (Fig. 5B, 5H). These three-dimensional cell clusters were positively stained for human insulin and Glut2 (Fig. 5D, 5E), which is a glucose transporter that has been suggested to function as a glucose sensor in pancreatic β-cells [40]. Moreover, HLSCs under this differentiating condition were positively stained with the zinc-chelating agent dithizone (Fig. 5C), which is specific for insulin-containing granules [41]. These results suggest that HLSCs differentiated into islet-like structures.

Liver Regeneration Capacity of HLSCs in SCID Mice

To investigate the self-renewal property of HLSCs in vivo, HLSCs were injected into SCID mice with acute liver injury induced by the analgesic drug *N*-acetyl-*p*-aminophen, which causes hepatotoxicity and hepatocyte necrosis in the centrilobular areas [47]. Seven days after the induction of liver injury, only a few HLSCs were detected by their positivity

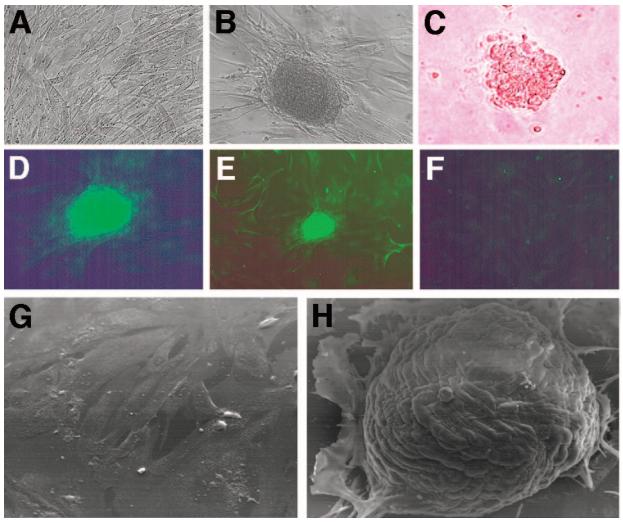

Figure 5. Differentiation of human liver stem cells (HLSCs) into islet-like structures. Representative micrographs of HLSCs after 37 days of culture in high-glucose Dulbecco's modified Eagle's medium (DMEM) and nicotinamide are shown. **(A, B):** Representative phase contrast micrographs of HLSCs cultured in α-minimum essential medium/endothelial cell basal medium-1 **(A)** or in islet differentiating medium **(B)** showing formation of cell aggregates (islet-like structures) from the cell monolayer. **(C):** Representative micrographs of HLSCs stained with the Zn-chelating agent dithizone to show the cell aggregates. **(D–F):** Representative immunofluorescence micrographs of HLSCs differentiated in islet-like structures. Expression of insulin **(D)** and Glut2 **(E)** was detected in islet-like structures. In experiments using isotypic control Abs, no fluorescent signal was detected **(F)**. **(G, H):** Representative micrographs of scanning electron microscopy showing HLSCs cultured in nondifferentiating medium **(G)** or in high-glucose DMEM and nicotinamide **(H)**, in which cell clusters forming islet-like structures are visible. Original magnification: **(A–F)**, ×150; **(G, H)**, ×1,200. Ten cell clones were studied with similar results.

for human HLA class I antigen (Fig. 6A, 6B), and the expression of human chromosome 17 (not shown) was observed within the injured parenchyma. After 30 days, areas negative for the expression of mouse β_2-microglobulin were observed (Fig. 6C). In parallel, several clusters of human HLA class I antigen-positive cells (Fig. 6D, 6E) expressing human chromosome 17 (Fig. 6F) were detectable, suggesting a contribution of HLSCs in liver regeneration. No HLSC localization was observed in mice without liver damage that were injected with HLSCs (data not shown) or in mice with liver damage that were injected with vehicle alone (Fig. 6G, 6H).

Discussion

In the present study we identified and characterized normal adult human liver stem cells with multiple differentiating capabilities distinct from those of oval stem cells. HLSCs expressed several mesenchymal but not hematopoietic stem cell markers and albumin, AFP, and CK18, indicating a partial commitment to the hepatic lineage.

The presence of hepatic stem cells in the adult mammalian liver remains controversial, mainly because of the absence of specific markers and the achievement of regeneration after acute injury through proliferation of adult hepatocytes [1–3]. It has

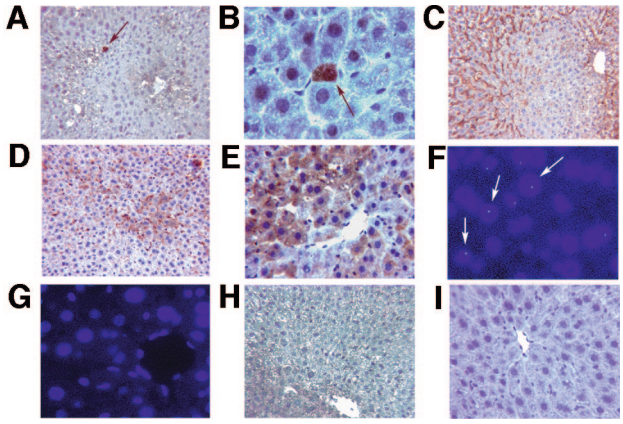

Figure 6. Regenerative potential of human liver stem cells (HLSCs) in a model of acute liver injury in severe-combined immunodeficient (SCID) mice. Representative micrographs showing localization of HLSCs in the liver parenchyma of SCID mice with acute liver injury induced by *N*-acetyl-*p*-aminophen and injected with HLSCs are displayed. **(A, B):** Immunoperoxidase staining of human HLA class I antigen in the liver of SCID mice 7 days after the induction of liver damage. Only few positive cells (arrows) were detectable within the parenchyma, which shows evident degenerative alterations. **(C)** shows areas of liver parenchyma negative for the mouse β_2-microglobulin antigen. **(D–F)** show the presence of clusters of HLSCs detected by immunoperoxidase staining of human HLA class I antigen **(D, E)** and by FISH detection of human chromosome 17 ((**F**), arrows) 30 days after the induction of liver damage. **(G, H):** Absence of positive staining for human HLA class I antigen or human chromosome 17 in control animals treated with *N*-acetyl-*p*-aminophen and injected with vehicle alone. **(I):** Representative negative staining of immunoperoxidase controls in which the primary antibody was replaced with an irrelevant antiserum. Original magnification: **(A, C, D, H)**, ×150; **(E, I)**, ×250; **(F, G)**, ×630. Six animals were used in every experimental group.

been shown in models of liver repopulation experiments based on hepatocyte transplantation that under highly selective conditions, hepatocytes acquire properties of liver stem cells [48–53]. Whether this property is due to the dedifferentiation of mature hepatocytes or to the expansion of an immature progenitor population, present in very low number in the adult liver, remains to be determined.

The results of the present study indicate the presence in human adult liver of a cell population able to survive in stringent culture conditions in which mature hepatocytes undergo cell death. A characteristic of stem cells is to survive toxic and hypoxic stimuli because of their low cell cycling [54]. Exploiting this property, stem cells have been isolated in several normal tissues [55–59]. HLSC clones isolated from the adult human liver had the capacity of self-renewal in nondifferentiating conditions, another characteristic of stem cells. The lack of hematopoietic stem markers CD45, CD34, CD117, and CD133 suggests that HLSCs did not derive from circulating hematopoietic cells.

HLSCs expressed several MSC markers, including CD73, CD90, CD29, CD44, and nestin, considered a stem cell marker [60, 61]. It has been shown recently in mice that long-term MSC cultures could be established from virtually all postnatal organs irrespective of their embryonic origin [62]. The nature and localization of MSCs in vivo is scarcely understood because of the absence of specific markers and the low number of these cells. Because the majority of cell markers are specific in a given context, the alternative strategy used to determine their histochemical localization has been the systematic isolation of MSCs from different organs and tissues [63]. Based on the expression of α-SMA and similarities to pericytes, it has been suggested that MSCs extend to different organs through the perivascular compartment [64, 65]. HLSCs, however, differed from MSCs in several phenotypic and functional properties. HLSCs did not express α-SMA and expressed liver tissue-specific proteins such as AFP, which is considered a marker of hepatocyte precursors, and human albumin, suggesting a partial commitment to hepatocyte lineage. Moreover, HLSCs were unable to grow in the commonly used mesenchymal stem cell culture medium and were unable to undergo differentiation into adipocytes, characteristic of MSCs [32, 66, 67]. On the other hand, HLSCs, similarly to MSCs, underwent endothelial and osteogenic differentiation. One possible explanation for this is that HLSCs may represent a mesenchymal population modified by the influence of the local environment, reflecting the importance of the niche in establishing the phenotype of MSCs [68]. It is known that MSCs in appropriate culture conditions may differentiate into hepatocytes [69]. However, when we compared the ability of hMSCs and HLSCs in the same culture condition to differentiate into mature hepatocytes, based on production of albumin and urea and cytochrome P450 activity, hMSCs failed to differentiate. In contrast, HLSCs lost the stem cell markers and acquired functional and phenotypic markers of mature hepatocytes.

In the liver, the presence of a so-called "oval cell population" with stem cell characteristics has been described in rodents [2, 5–7]. Oval cells are a heterogeneous population of cells that can be induced to proliferate under different pathophysiological conditions [2, 4, 7]. These cells express one or more hematopoietic stem cell markers such as CD34, c-kit, and Sca-1 and are bipotent, that is, capable of differentiating into hepatocytes and bile duct cells [8, 12–15]. Recently, stem-like cells have been identified in human hepatoblastoma and in the regenerative structures in patients with extensive chronic liver injury or massive hepatic necrosis with morphological and histochemical characteristics of oval cells [8, 14, 23–29]. The HLSC population that we isolated from adult normal liver did not express the oval cell markers CD34, c-kit, and CK19 and, at variance with oval cells, were pluripotent, suggesting that they were a distinct stem cell-like population. Indeed, in the present study, we show the ability of HLSCs to differentiate into epithelial, osteogenic, and endothelial cells when cultured in appropriate media. In addition, HLSCs differentiated into both mature hepatocytes and insulin-producing islet-like cells. The presence and the phenotype of a population of human hepatic progenitors have been recently demonstrated [70]. HLSCs that we describe herein differed from this population by the absence of NCAM, c-kit, and CK19 and by the presence of AFP. Finally, the self-renewal property of HLSCs was supported by their clonogenicity and by the in vivo experiments using a model of acute liver injury in SCID mice in which HLSCs contributed to the regeneration of the liver parenchyma.

CONCLUSION

We have proven that a HLSC population expressing markers of mesenchymal but not of hematopoietic stem cells is present in the adult human liver. HLSCs were clonogenic and expressed some markers of immature hepatocytes. However, this population did not express oval cell markers and, at variance with oval cells that are bipotent, was able to undergo multiple differentiations including epithelial, endothelial, osteogenic, and islet-like differentiation. In addition, HLSCs contributed to regeneration of the liver parenchyma in SCID mice. In conclusion, we identified in the adult human liver a cell population that fulfils criteria for stem cell definition such as the capacity for self-renewal and multipotent differentiation.

ACKNOWLEDGMENTS

This work was supported by Consorzio Nazionale per la Ricerca Sulle Cellule Staminali (CONSTEM), Progetto di Ricerca Integrato per la Riparazione Cellulare delle Malattie del Fegato Invalidanti by Fresenius Medical Care Deutschland, and by the European Foundation for the Study of Diabetes.

DISCLOSURES

M.B.H., B.B., and G.C. are named inventors on a related patent application on liver stem cells. C.T. has served as an officer or member of the Board of Fresenius Medical Care (Deutschland) within the last 2 years. S.B. indicates a financial interest in Fresenius Medical Care.

REFERENCES

1 Alison MR, Vig P, Russo F et al. Hepatic stem cells: from inside and outside the liver? Cell Prolif 2004;37:1–21.

2 Fausto N. Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. Hepatology 2004;39:1477–1487.

3 Shafritz DA, Oertel M, Menthena A et al. Liver stem cells and prospects for liver reconstitution by transplanted cells. Hepatology 2006;43(suppl 1):89–99.

4 Sell S. Heterogeneity and plasticity of hepatocyte lineage cells. Hepatology 2001;33:738–750.

5 Evarts RP, Nagy P, Marsden E et al. A precursor-product relationship exists between oval cells and hepatocytes in rat liver. Carcinogenesis 1987;8:1737–1740.

6 Evarts RP, Nagy P, Nakatsukasa H et al. In vivo differentiation of rat liver oval cells into hepatocytes. Cancer Res 1989;49:1541–1547.

7 Newsome PN, Hussain MA, Theise ND. Hepatic oval cells: helping redefine a paradigm in stem cell biology. Curr Top Dev Biol 2004;61:1–28.

8 Peterson B, Grossbard B, Hatch H et al. Mouse A6 positive hepatic oval cells also express several hematopoietic stem cell markers. Hepatology 2003;37:632–640.

9 Van Eyken P, Sciot R, Desmet V. Intrahepatic bile duct development in the rat: a cytokeratin-immunohistochemical study. Lab Invest 1988;59:52–59.

10 Van Eyken P, Sciot R, Callea F. The development of the intrahepatic bile ducts in man: a keratin-immunohistochemical study. Hepatology 1988;8:1586–1595.

11 Shah KD, Gerber NA. Development of intrahepatic bile ducts in humans: immunohistochemical study using monoclonal cytokeratin antibody. Arch Pathol Lab Med 1989;113:1135–1138.

12 Fujio K, Evarts RP, Hu Z et al. Expression of stem cell factor and its receptor, c-kit, during liver regeneration from putative stem cells in the adult rat. Lab Invest 1994;704:511–516.

13 Matsusaka S, Tsujimura T, Toyosaka A et al. Role of c-kit receptor tyrosine kinase in development of oval cells in the rat 2-acetylaminofluorene/partial hepatectomy model. Hepatology 1999;29:670–676.

14 Crosby HA, Kelly DA, Strain AJ. Human hepatic stem-like cells isolated using c-kit or CD34 can differentiate into biliary epithelium. Gastroenterology 2001;120:534–544.

15 Peterson BE, Goff JP, Greenberger JS et al. Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat. Hepatology 1998;27:433–445.

16 Wang X, Foster M, Al-Dhalimy M et al. The origin and liver repopulating capacity of murine oval cells. Proc Natl Acad Sci U S A 2003;100:11881–11888.

17 Menthena A, Deb N, Oertel M et al. Bone marrow progenitors are not the source of expanding oval cells in injured liver. STEM CELLS 2004;22:1049–1061.

18 Gordon GJ, Coleman WB, Hixson DC et al. Liver regeneration in rats with retrorsine-induced hepatocellular injury proceeds through a novel cellular response. Am J Pathol 2000;156:607–619.

19 Gordon GJ, Coleman WB, Grisham JW. Temporal analysis of hepatocyte differentiation by small hepatocyte-like progenitor cells during liver regeneration in retrorsine-exposed rats. Am J Pathol 2000;157:771–786.

20 Vig P, Russo FP, Edwards RJ et al. The sources of parenchymal regeneration after chronic hepatocellular liver injury in mice. Hepatology 2006;43:316–324.

21 Popper H, Kent G, Stein R. Ductular cell reaction in the liver in hepatic injury. J Mt Sinai Hosp 1957;24:551–556.

22 Popper H. The relation of mesenchymal cell products to hepatic epithelial systems. Prog Liver Dis 1990;9:27–38.

23 Roskams T, De Vos R, Van Eyken P et al. Hepatic OV-6 expression in human liver disease and rat experiments: evidence for hepatic progenitor cells in man. J Hepatol 1998;29:455–463.

24 Tan J, Hytiroglou P, Wieczorek R et al. Immunohistochemical evidence for hepatic progenitor cells in liver diseases. Liver 2002;22:365–373.

25 Theise ND, Saxena R, Portmann BC et al. The canals of Hering and hepatic stem cells in human. Hepatology 1999;30:1425–1433.

26 Lemmer ER, Shepard EG, Blakolmer K et al. Isolation from human fetal liver of cells co-expressing CD34 haematopoietic stem cell and CAM 5.2 pancytokeratin markers. J Hepatol 1998;29:450–454.

27 Selden C, Chalmers SA, Jones C et al. Epithelial colonies cultured from human explanted liver in subacute hepatic failure exhibit hepatocyte, biliary epithelial, and stem cell phenotypic markers. STEM CELLS 2003;21:624–631.

28 Mahieu-Caputo D, Allain J-E, Branger J et al. Repopulation of athymic mouse liver by cryopreserved early human fetal hepatoblasts. Hum Gene Ther 2004;15:1219–1228.

29 Stamp L, Crosby HA, Hawes SM et al. A novel cell-surface marker found on human embryonic hepatoblasts and a subpopulation of hepatic biliary epithelial cells. STEM CELLS 2005;23:103–112.

30 Malhi H, Irani AN, Gagandeep S et al. Isolation of human progenitor liver epithelial cells with extensive replication capacity and differentiation into mature hepatocytes. J Cell Sci 2002;115:2679–2688.

31 Lazaro CA, Croager EJ, Mitchell C et al. Establishment, characterization, and long-term maintenance of cultures of human fetal hepatocytes. Hepatology 2003;38:1095–1106.

32 Pittinger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–147.

33 Da Silva Meirelles L, Nardi NB. Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. Br J Haematol 2003;123:702–711.

34 Herrera MB, Bussolanti B, Bruno S et al. Mesenchymal stem cells contribute to the renal repair of acute tubular epithelial injury. Int J Mol Med 2004;14:1035–1041.

35 Bussolati B, Mariano F, Biancone L et al. Interleukin-12 is synthesized by mesangial cells and stimulates platelet-activating factor synthesis, cytoskeletal reorganization, and cell shape change. Am J Pathol 1999; 154:623–632.

36 Khaoustov VI, Darlington GJ, Soriano HE et al. Induction of three dimensional assembly of human liver cells by simulated microgravity. In Vitro Cell Dev Biol 1999;35:501–509.

37 Yoffe B, Darlington GJ, Soriano HE et al. Cultures of human liver cells in simulated microgravity environment. Adv Space Res 1999;24:829–836.

38 Lavon N, Benvenisty N. Study of hepatocyte differentiation using embryonic stem cells. J Cell Biochem 2005;96:1193–1202.

39 White IN, Green ML, Legg RF. Fluorescence-activated sorting of rat hepatocytes based on their mixed function oxidase activities towards diethoxyfluorescein. Biochem J 1987;247:23–28.

40 Yang L, Li S, Hatch H et al. In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci U S A 2002;99:8078–8073.

41 Shiroi A, Yoshikawa M, Yokota H et al. Identification of insulin-producing cells derived from embryonic stem cells by zinc-chelating dithizone. STEM CELLS 2002;20:284–292.

42 Rafii S, Lyden D, Benezra R. Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? Nat Rev Cancer 2002;2: 826–835.

43 Schwartz RE, Reyes M, Koodie L et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest 2002;109:1291–1302.

44 Conaldi PG, Biancone L, Bottelli A et al. HIV-1 kills renal tubular cells by a mechanism that involves fas and caspase-3 activation. J Clin Invest 1998;102:2041–2049.

45 Knight TR, Ho YS, Farhood A et al. Peroxynitrite is a critical mediator of acetaminophen hepatotoxicity in murine livers: protection by glutathione. J Pharmacol Exp Ther 2002;303:468–475.

46 Camussi G, Kerjaschki D, Gonda M et al. Expression and modulation of surface antigens in cultured rat glomerular visceral epithelial cells. J Histochem Cytochem 1989;37:1675–1687.

47 Thomas SHL. Paracetamol (acetaminophen) poisoning. Pharmacol Ther 1993;60:91–120.

48 Laconi E, Oren R, Mukhopadhyay D et al. Long term, near total liver replacement by transplantation of isolated hepatocytes. Am J Pathol 1998;153:319–329.

49 Witek RP, Fisher SH, Petersen BE. Monocrotaline, an alternative to retrorsine-based hepatocyte transplantation in rodents. Cell Transplant 2005;14:41–47.

50 Guha C, Sharma A, Gupta S et al. Amelioration of radiation-induced liver damage in partially hepatectomized rats by hepatocytes transplantation. Cancer Res 1999;59:5871–5874.

51 Malhi H, Gorla GR, Irani AN et al. Cell transplantation after oxidative hepatic preconditioning with radiation and ischemia-reperfusion leads to extensive liver repopulation. Proc Natl Acad Sci U S A 2002;99:13114–13119.

52 Mignon A, Guidotti JE, Mitchell C et al. Selective repopulation of normal mouse liver by Fas/CD-95 resistant hepatocytes. Nat Med 1998; 4:1185–1188.

53 Yuan RH, Ogawa A, Ogawa E et al. p27Kip1 inactivation provides a proliferative advantage to transplanted hepatocytes in DPPIV/Rag2 double knockout mice after repeated host liver injury. Cell Transplant 2003;12:907–919.

54 Oliver JA, Maarouf O, Cheema FH et al. The renal papilla is a niche for adult kidney stem cells. J Clin Invest 2004;114:795–804.

55 Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 1992;255:1707–1710.

56 Tropepe V, Coles BL, Chiasson BJ et al. Retinal stem cells in the adult mammalian eye. Science 2000;287:2032–2036.

57 Jackson KA, Mi T, Goodell MA. Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proc Natl Acad Sci U S A 1999; 96:14482–14486.

58 Toma JG, Akhavan M, Fernandes KJ et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 2001;3: 778–784.

59 Bussolati B, Bruno S, Grange C et al. Isolation of renal progenitor cells from adult human kidney. Am J Pathol 2005;166:545–555.

60 El-Helou V, Dupuis J, Proulx C et al. Resident nestin+ neural-like cells and fibers are detected in normal and damaged rat myocardium. Hypertension 2005;46:1219–1225.

61 Mayer EJ, Carter DA, Ren Y et al. Neural progenitor cells from postmortem adult human retina. Br J Ophthalmol 2005;102–106.

62 Da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 2006;119:2204–2213.

63 Zuk PA, Zhu M, Mizuno H et al. Multilineage cells from human adipose tissue: Implication for cell-based therapies. Tissue Eng 2001;7:211–228.

64 Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 2003; 18:696–704.

65 Farrington-Rock C, Crofts NG, Doherty MG et al. Chondrogenic and adipogenic potential of microvascular pericytes. Circulation 2004;110: 2226–2232.

66 Barry F, Boynton R, Murphy M et al. The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human mesenchymal stem cells. Biochem Biophys Res Commun 2001;289:519–524.

67 Reyes M, Lund T, Lenvik T et al. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 2001;98: 2615–2625.

68 Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: stem cells and their niche. Cell 2004;116:769–778.

69 Lee KD, Kuo TK, Whang-Peng J et al. In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology 2004;40:1275–1284.

70 Schmelzer E, Wauthier E, Reid LM. The phenotypes of pluripotent human hepatic progenitors. STEM CELLS 2006;24:1852–1858.