Isolation and Characterization of a Novel Population of Progenitor Cells from Unmanipulated Rat Liver

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Widespread use of liver transplantation in the treatment of hepatic diseases is restricted by the limited availability of donated organs. One potential solution to this problem would be isolation and propagation of liver progenitor cells or stem cells. Here, we report on the isolation of a novel progenitor cell population from unmanipulated (that is, no prior exposure to chemicals and no injury) adult rat liver. Rat liver cells were cultured following a protocol developed in our laboratory to generate a unique progenitor cell population called liver-derived progenitor cells (LDPCs). LDPCs were analyzed by fluorescence-activated cell sorting, real-time polymerase chain reaction (RT-PCR), immunostaining and microarray gene expression. LDPCs were also differentiated into hepatocytes and biliary epithelium *in vitro* and examined for mature hepatic markers and urea and albumin production. These analyses showed that, LDPCs expressed stem cell markers such as cluster domain (CD)45, CD34, c-kit, and Thy 1, similar to hematopoietic stem cells, as well as endodermal/hepatic markers such as hepatocyte nuclear factor (HNF)3 β , hematopoietically-expressed homeobox gene-1, c-met, and transthyretin. LDPCs were negative for OV-6, cytokeratins (CKs), albumin, and HNF1 α . The microarray gene expression profile demonstrated that they showed some similarities to known liver progenitor/stem cells such as oval cells. In addition, LDPCs differentiated into functional hepatocytes *in vitro* as shown by albumin expression and urea production. In conclusion, LDPCs are a population of unique liver progenitors that can be generated from unmanipulated adult liver, which makes them potentially useful for clinical applications, especially for cell transplantation in the treatment of liver diseases. *Liver Transpl* 14:333-345, 2008. © 2008 AASLD.

Received July 4, 2007; accepted September 30, 2007.

Diseases of the liver are common causes of morbidity and mortality in the world.¹ Despite the high incidence of liver diseases that result in liver dysfunction and failure, current medical therapies are limited to supportive care, rather than curative approaches, with the possible exception of liver transplantation.

Liver transplantation is considered to be the standard treatment for end-stage liver disease.² Unfortunately, its extensive application is restricted by the limited availability of donor organs. In addition, liver trans-

plantation is associated with significant morbidity and mortality. As most liver disorders result from hepatocyte dysfunction, there has been great interest in transplantation of isolated hepatocytes. However, their clinical application is also dependent on the availability of good quality donor livers.

To overcome the problem of limited donor organs and to make hepatocytes available for other applications, several approaches to isolate and propagate liver stem cells or progenitor cells have been developed.^{3,4} It is

This article contains Supplementary Material available at <http://www.mrw.interscience.wiley.com/suppmat/1527-6465/suppmat>.
Abbreviations: AAF, acetaminofluorene; Ab, antibody; AFP, alpha-fetoprotein; BM, bone marrow; CD, cluster domain; CK, cytokeratin; ELISA, enzyme-linked immunoassay; FBS, fetal bovine serum; hex, hematopoietically-expressed homeobox gene; HNF, hepatocyte nuclear factor; LDPC, liver-derived progenitor cell; mRNA, messenger RNA; QRT-PCR, quantitative real-time polymerase chain reaction; RNA, ribonucleic acid; RT-PCR, real-time polymerase chain reaction.

Supported by the National Institutes of Health (U19 DK61244 and R01 HL073221) and by an award from the Alpha 1-Antitrypsin Foundation (to C.M.V.).

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DOI 10.1002/lt.21380

Published online in Wiley InterScience (www.interscience.wiley.com).

well known that the liver is capable of regenerating itself,^{4,5} chiefly because mature hepatocytes themselves can undergo multiple cell divisions *in vivo*. Under circumstances in which hepatocytes are unable to divide, or do so to a limited degree, such as in alcoholic liver disease, chronic cholestatic diseases, or hepatitis induced by viral infection, liver progenitors start to proliferate and differentiate into mature hepatocytes.³⁻⁵

A number of different liver progenitor/stem cell populations derived from adult animals have been described. Among these, the best known is a population of stem cells called oval cells. Oval cells proliferate *in vivo* following liver damage when the ability of hepatocytes to divide is inhibited.^{6,7} Oval cells are thought to be localized in biliary ductules (canal of Hering) in normal adult liver and have also been identified during liver embryonic development.⁸ Oval cells are bipotential and give rise to both hepatocytes and biliary ductal cells.^{9,10} Recent studies have identified many cell-surface markers for oval cells both in rodents and humans, including the hematopoietic markers Thy1.1, cluster domain (CD)34, Flt3-receptor, and c-kit.¹¹⁻¹⁴ Oval cells also express alpha-fetoprotein (AFP), cytokeratin (CK)19, and γ -glutamyl-transferase and stain positive with the monoclonal antibodies; OC.2, OV-6, and BD1.¹⁵ Until recently, it was believed that oval cells were exclusively derived from biliary ductal cells. However, this notion has been challenged by a number of studies suggesting that they may also be derived from bone marrow (BM).¹⁶⁻¹⁹ Therefore, it appears that oval cells can be obtained from multiple sources depending on the nature and severity of the liver injury as well as the methods used to isolate them.^{20,21} As oval cells are commonly isolated from liver following treatment with carcinogenic agents such as 3,5-diethoxycarbonyl-1,4-dihydrocollidine,¹⁷ or from animals on a choline-deficient diets and treated with the hepatocarcinogenic agent N-2-acetylaminofluorene (AAF),^{22,23} cells isolated by these protocols are often transformed, precluding their use for clinical transplantation.²⁴⁻²⁶

Here, we demonstrate that a population of progenitor cells exhibiting similarities to oval cells and hematopoietic stem cells can be isolated from noninjured liver of adult rats. These cells were characterized by RNA and protein analysis and by *in vitro* differentiation studies.

MATERIALS AND METHODS

Isolation of Liver Cells

Liver cells from Sprague Dawley rats weighing 150 to 300 g (Jackson Labs, Bar Harbor, ME) were harvested by a modified 2-step *in situ* collagenase perfusion technique as previously described.²⁷ After digestion by collagenase, a single-cell suspension was obtained by filtering the liver cells through a 100- μ m sterile nylon gauze (Sigma-Aldrich, St. Louis, MO). The cells were then washed in phosphate buffered saline and centrifuged (50g for 3 minutes) twice at room temperature prior to culture. The cells were not subjected to any fractionation (parenchymal versus nonparenchymal).

Liver-Derived Progenitor Cell (LDPC) Culture Conditions

Freshly isolated rat liver cells with viability between 50% and 90% were cultured in a medium consisting of 75% Dulbecco's modified Eagle's medium (DMEM-LG; Cellgro, Herndon, VA) + 25% MCDB 201 (Sigma-Aldrich) supplemented with 9% rat serum (Equitech, Kerville, TX), 1% fetal bovine serum (FBS; Hyclone, Logan, UT), 1 mg/mL of bovine serum albumin (BSA; Sigma), 100 μ M of β -mercaptoethanol (Gibco, Carlsbad, CA), 25 mM of 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES; Cellgro), 5 mM of Nicotinamide (Sigma), and penicillin (100 μ g/mL)/streptomycin (100 μ g/mL; Invitrogen, Carlsbad, CA) on Type I collagen (Upstate Biotechnology, Danvers, MA) coated tissue culture plates or dishes at a density of 5×10^4 cells/mL and 1×10^4 cells/cm² at 37°C in 7% CO₂. The seeding density was 33% lower for younger rats that weighed less than 200 g. The medium was changed by 80% on days 2 and 4. Subsequently, no media changes were made and LDPCs were allowed to appear and proliferate for the following 2 to 3 weeks. Between days 15 and 21, LDPCs were harvested for various studies. Karyotyping on LDPCs was done following standard protocols in our laboratory.

LDPC Clonal Isolation

LDPCs from bulk cultures were plated onto 96-well plates at a density of 1 cell/well in the conditioned media (100%) obtained from bulk cultures. After 3 to 4 weeks, 10% of the wells had colonies of LDPCs consisting of 100 to 10,000 cells.

Immunohistochemistry

Immunocytochemical analysis of the cells was performed by using the streptavidin-biotin-peroxidase method or the streptavidin-biotin alkaline phosphatase method (Dako, Glostrup, Denmark) after fixation with 4% paraformaldehyde. Mouse monoclonal antibodies (Abs) were against CK7 (Dako; 1:75), CK18 (Cymbus Biotechnology, Chandlers Ford, UK; 1:10), CK19 (Maine Biotechnology Services, Portland, OR; 1:75), desmin (ICN, 1:12, Costa Mesa, CA), and OV6 (gift from Dr. S. Sell, University of Texas Health Science Center, Houston, TX; 1:50). Rabbit antisera were against hepatocyte nuclear factor (HNF)1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:50) and albumin (ICN/Cappel, Aurora, CO; 1:500). Goat antiserum was against HNF-3 β (Santa Cruz Biotechnology; 1:50). Anti-mouse and anti-rabbit biotinylated secondary Abs were from Amersham Pharmacia (Uppsala, Sweden) and diluted 1:300. Anti-goat biotinylated secondary Ab was from Vector Laboratories (Burlingame, CA), and diluted 1:300. Color development was with the DAB+ Substrate-Chromogen System (Dako) or with the New Fuchsin Substrate System (Dako).

Flow Cytometry

For flow cytometric analysis, LDPCs, fresh BM, or fresh rat liver cells were stained sequentially with primary Abs: anti-rat granulocyte clone RP-1, anti-rat CD45R clone OX-33, anti-rat CD45 clone OX-1, and anti-rat CD3 clone 1F4, and immunofluorescent secondary Abs and analyzed using a FACSCalibur (all from Becton Dickinson Pharmingen, San Diego, CA).

Real-Time Polymerase Chain Reaction (RT-PCR) and Quantitative RT-PCR (QRT-PCR)

RNA was extracted from 3×10^5 to 3×10^6 LDPCs. messenger RNA (mRNA) was reverse transcribed and complementary DNA (cDNA) underwent 40 rounds of amplification (ABI-Prism 7700; PerkinElmer/Applied Biosystems, Foster City, CA) as follows: 40 cycles of a 2-step PCR (95°C for 15 seconds, 60°C for 60 seconds) after initial denaturation (95°C for 10 minutes) with 2 μ L of DNA solution, 1 $\times$ TaqMan SYBR Green Universal Mix PCR reaction buffer (Applied Biosystems). Primers used for amplification were obtained from various references.^{13,14,28-31} mRNA levels were normalized using GAPDH as housekeeping gene and compared with mRNA levels in fresh rat liver cells, rat BM, whole pancreas, pyloric stomach, and duodenum. Rat thyroid total RNA was obtained from Clontech (Mountain View, CA).

Western Blot

Protein lysates, obtained from LDPCs, rat liver cells, or rat BM were separated on 8.0% polyacrylamide electrophoresis gels (Sigma-Aldrich). Gels were transferred to membrane blots, which were incubated with Abs against CK18 (Cymbus Biotechnology, Chandlers Ford, UK; 1:100), CK19 (Maine Biotechnology Services/Portland, ME; 1:200), CD34 (Santa Cruz Biotechnology; 1:250), CD117 (Santa Cruz Biotechnology; 1:250). Blots were washed and incubated with a goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody (1:10,000 and 1:20,000 dilution; Amersham Biosciences, Piscataway, NJ). Bands were visualized by means of an enhanced chemiluminescence detection system (Amersham Biosciences).

Liver Differentiation from LDPCs

LDPCs were harvested from the cultures between days 15 and 21 by tapping the dishes gently to avoid contaminating the samples with adherent cells. Cells were plated on collagen-coated tissue culture plates at a density of 1×10^5 cells/cm² in expansion medium supplemented with 10% FBS (instead of 1% FBS and 9% rat serum), 20 ng/mL of hepatocyte growth factor (HGF; R&D Systems, Minneapolis, MN), and 100 nM of dexamethasone. The medium was changed on days 7 and 14. Cultures were analyzed between days 21 and 42.

Urea Production Assay

Urea concentrations were determined by colorimetric assay (Sigma-Aldrich) per manufacturer's instructions. Briefly, 100 μ L of culture supernatant or media was added to a cuvette; then 0.5 mL of a urease solution was added and incubated for 20 minutes; 1 mL of phenol nitroprusside followed by 1 mL of alkaline hypochlorite was then added. A total of 5 mL of water was added to the cuvette and the sample was mixed and allowed to incubate for 30 minutes before the absorbance reading was examined. Rat hepatocytes grown in monolayer were used as positive controls and fresh culture medium used as negative control. As the assay measures ammonia metabolized from urea, samples were assessed before and after urease addition. No urea or ammonia was detected in culture medium alone.

Albumin Enzyme-Linked Immunoassay (ELISA)

Rat albumin concentrations were determined by a competitive enzyme linked immunoassay (ELISA) described previously for human and mouse albumin measurements with substitution of human or mouse albumin and anti-human or anti-mouse albumin Abs for the rat components where appropriate. Peroxidase conjugated anti-human albumin and reference human albumin were from Cappel. Peroxidase-conjugated and affinity-purified anti-mouse albumin and reference mouse albumin were from Bethyl Laboratories (Montgomery, TX). To ensure specificity of the ELISA, human, mouse, and rat Abs were incubated for 2 hours at 37°C with 3% bovine serum albumin in distilled water (dH₂O).

Processing of RNA Samples and Oligonucleotide Microarray Analysis

Total cellular RNA was isolated from LDPCs and rat liver cells using the PicoPure RNA Isolation Kit (Arcturus, Mountain View, CA) per the manufacturer's instructions. A total of 1 million LDPCs and hepatocytes were placed directly into 100 μ L extraction buffer provided with the PicoPure RNA Isolation Kit prior to RNA isolation. Labeled complementary RNA (cRNA) was generated by one round of linear amplification using the RiboAmp OA RNA Amplification Kit (Arcturus) followed by labeling with the Enzo Bioarray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instructions. Samples were hybridized to Affymetrix Rat 230 2.0 chips (Santa Clara, CA), washed and scanned at the University of Minnesota Affymetrix Microarray Core Facility as described in the Affymetrix GeneChip Expression Analysis Technical Manual. Oligonucleotide microarray data analysis. Affymetrix Rat 230 2.0 arrays were processed using GeneData Refiner software (Basel, Switzerland) to assess overall quality. Feature intensities for each chip were condensed into a single intensity value per gene using algorithms in the Microarray Analysis Suite, the Affymetrix Statistical Algorithm (MAS 5.0).

Figure 1. Emergence of liver-derived progenitor cells (LDPCs) in primary liver cultures. By day 10 the majority of hepatocytes die out and an entirely distinct population of cells appears. These cells, LDPCs, rapidly repopulate the cultures forming very dense colonies by day 21. At this point LDPCs constitute >95% of the cells, the rest are fibroblast-like cells while no hepatocytes remain in the cultures.

Expression data was analyzed using GeneData's Expressionist and Microsoft Excel (Redmond, WA). Differential gene expression for comparison of LDPCs versus hepatocytes was defined by using a paired Student *t* test with a threshold of $P < 0.05$, and a paired fold change was used to rank gene lists. Differentially expressed genes were classified according to their respective gene pathways and gene ontologies when available by using the web-based Affymetrix NetAffx analysis tool (Affymetrix) and the National Institutes of Allergy and Infectious Disease Database for Annotation, Visualization, and Integrated Discovery analysis tool (<http://apps1.niaid.nih.gov/david>).

Microarray QRT-PCR Confirmation

Labeled cRNA was reverse-transcribed to generate cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. QRT-PCR was performed using the ABI-Prism 7000 Sequence Detection System (Applied Biosystems), as described earlier.

Colony-Forming Assay and Stromal Cell Cocultures

LDPCs and total rat BM were plated in methylcellulose medium (M3234; Stem Cell Technologies, Seattle, WA) supplemented with 20 ng/mL recombinant stem cell factor, 10 ng/mL interleukin-3, 10 ng/mL mouse inter-

leukin-6 (all from PeproTech, Rocky Hill, NJ), and 3 U/mL human erythropoietin (Amgen Inc., Thousand Oaks, CA). Total rat BM and LDPCs were plated at 1000, 5000, and 10,000 cells/mL. All cultures were incubated at 37°C and 5% CO₂. Colonies were counted between day 10 and 14. For stromal cell cocultures, LDPCs were plated on top of irradiated stromal cells, AFT024,³² UG26-1B6, and EL08-1D2, and cultured at 37°C with 5% CO₂ for 7 to 14 days. Cells were then analyzed for hematopoietic lineage markers by flow cytometry.

Animal Transplantations

RAG2 γ c immunodeficient mice from Taconic (Hudson, NY) were cotransplanted with lineage+ BM from green fluorescent protein-positive C57Bl/6 mice and either LDPCs or rat BM. All protocols involving mice were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and all animals received humane care. Recipient mice were irradiated at 750 cGy prior to transplantation. Then, 1×10^6 BM mononuclear cells from Sprague-Dawley rat or 1×10^6 LDPCs were mixed with 1×10^5 lineage+ mouse BM cells and injected via the tail vein. After 2 months, peripheral blood cells were obtained, and after 4 months, BM was obtained, and examined for rat hematopoietic markers by flow cytometry.

Figure 2. (A) Flow cytometric analysis of liver-derived progenitor cells (LDPCs): Antibodies (Abs) against ckit, Thy-1, CD45, RT1A, RT1B, granulocyte, or control immunoglobulin G (IgG). Cells were analyzed by flow cytometry. Plots show isotype control IgG staining profile (dark solid blue) versus specific Ab staining profile (thick green line). Each analysis shown is 1 representative example from a total of 10 analyses. Values on the x-axis indicate intensity log. LDPCs express c-kit, Thy-1, CD45, and RT1A but not RT1B or granulocyte markers. c-kit, Thy-1, and CD45 are expressed classically on hematopoietic cells and some oval cell populations. LDPCs express RT1A, an equivalent of Class I majorhistocompatibility antigen (MHC) while they do not express RT1B, an equivalent of Class II MHC. In addition, they do not express a granulocyte marker. (B) Flow cytometric analysis of freshly isolated single-cell preparation from liver.

RESULTS

Generation of LDPCs from Primary Adult Liver Cells

When liver cells were plated, they quickly attached to the dishes and formed monolayer cultures with a confluency of 10 to 20% within 48 hours. Following media change on day 4, the large hepatic epithelial cells gradually died and by day 10 approximately 10% of the cells remained alive (Fig. 1). Between day 7 and day 10, a population of cells emerged that were much smaller than the original hepatocytes (average size 5-10 μm in diameter), with round to oval morphology. Over the next 7 to 14 days, the number of the small, round cells

increased rapidly, reaching an average maximum density of 1 to 2×10^4 cells/ cm^2 , representing 2 to 4 LDPCs generated per liver cell plated (range, $0.5\text{-}3 \times 10^4$ cells/ cm^2). At this density, they formed tightly packed, round cell clusters. Such cultures also contained 1 to 5% fibroblast-like cells that were not present in the cultures immediately after plating hepatocytes. Generation of LDPCs is highly reproducible ($n > 50$) provided that the actual viable cell density in culture at 24 hours is optimal ($5\text{-}10 \times 10^3$ cells/ cm^2). Attempts at expanding LDPCs beyond day 21, however, resulted in no further increase in their number (that is, LDPCs were generated each time from a fresh liver harvest and not passaged).

Figure 3. Because of high autofluorescence, immunohistochemical analysis (IHC) was done on liver-derived progenitor cells (LDPCs). They stain positive for hepatocyte nuclear factor (HNF)-3 β . LDPCs are negative for oval cell marker OV-6, stellate cell marker desmin, and hepatocyte marker CK-18 and albumin. They also stain negative for biliary epithelial markers CK-7 and 19 (not shown).

Flow Cytometric and Karyotypic Analyses of LDPCs

Standard karyotypic analysis of LDPCs demonstrated a normal karyotype ($n = 5$; data not shown). Flow cytometric analysis showed that LDPCs were CD45, c-kit, Thy-1 (dim), and RT1A positive while they were negative for RT1B, granulocytic (Fig. 2A), CD3 and erythroid markers (data not shown). Flow cytometric analysis of fresh liver samples failed to show a population of cells with similar phenotype ($n = 3$) (Fig. 2B).

Immunohistochemistry on LDPCs

We used immunohistochemistry to further characterize the phenotype of LDPCs. They were negative for CK18, albumin, OV-6 (Fig. 3A), CK7, CK8, CK19, desmin, and HNF-1 α (not shown). LDPCs were positive for HNF-3 β (Fig. 3A) and GATA-4 (not shown), whereas staining for AFP was inconclusive. In contrast, the mature control hepatocytes were CK7, CK19, desmin, and OV-6 negative while positive for albumin, HNF-3 β , GATA-4, and HNF-1 α , whereas biliary ductal cells stained positive for CK19 and OV-6 (data not shown). The small number of fibroblast-like cells found adherent to the plates stained positive for desmin, but were negative for CK7, CK18, CK19, OV-6, albumin, HNF-3 β , GATA-4, and HNF-1 α (not shown).

Western Blot and RT-PCR Analyses

Western blot confirmed that CD34 and c-kit proteins were present in LDPCs whereas CK18 and CK19 were absent ($n = 5$) (Fig. 4). QRT-PCR analysis confirmed our immunohistochemical and flow cytometric data (Table 1). Transcripts for c-Kit, Thy1, and CD34 were expressed in LDPCs at levels between 1 and 2.5% that of whole BM, while

no expression of these markers could be detected in fresh liver samples. LDPCs expressed moderate levels of transcripts for transthyretin and very low levels for AFP. In addition, LDPCs expressed high levels of c-met mRNA relative to hepatocytes and CXCR4 mRNA relative to BM. LDPCs did not have any appreciable levels of transcripts for CK18, CK19, Albumin, and HNF-1. QRT-PCR was also used to examine expression of genes associated with other endodermally-derived tissues such as thyroid transcription factor-1, expressed in thyroid tissue; gastrin, expressed in pyloric stomach; secretin, expressed in the duodenum; caudal related homologs 1 and 2 (CDX-1 and CDX-2), expressed in the duodenum; musashi, expressed in the liver; hematopoietically-expressed homolog gene (HEX1), expressed in the liver; and pancreatitis-associated protein, expressed in the pancreas (Table 2). LDPCs did not express thyroid transcription factor-1, secretin, gastrin, and CDX-1 but expressed musashi and HEX1 at 1200% and 6% relative to hepatocytes. LDPCs also expressed CDX2 and pancreatitis-associated protein-1 at low levels while hepatocytes did not express either of these markers. As some have suggested that oval cells may be BM-derived and LDPCs express hematopoietic cell surface markers, we used QRT-PCR to examine expression of genes known to be important in hematopoiesis such as Gata1, Gata2, Tal1, Lmo2, Pu.1, Mll1, and Runx1. LDPCs did not express Gata1 but expressed Gata2 and Tal1 at very low levels and expressed Lmo2, Pu.1, and Runx1 at high levels (Table 1). LDPCs have remained phenotypically stable for up to 8 weeks when cultured in medium that was partially (75%) changed with fresh medium once a week (data not shown).

Clonal Generation of LDPCs

LDPCs from 2-week-old cultures were subcloned at 1 cell/well ($n = 3$ isolations) in conditioned media (100%)

Figure 4. Protein lysates were transferred to Immuno-Blot polyvinylidene fluoride (PVDF) membrane, and incubated overnight with antibodies (Abs) against CK19, CK18, CD34, C-kit, or β -actin. Liver-derived progenitor cells (LDPCs); (-), negative control; BM, rat bone marrow; Hep, rat hepatocytes. Rat BM and rat hepatocyte used as control.

TABLE 1. mRNA Expression Profile of LDPCs for Various Stem Cell and Endodermal/Hepatic Markers: Quantitative RT-PCR Analysis of LDPCs

	LDPCs (+/-)	Hepatocytes (+/-)	BM (+/-)	Cycle Number	Relative Expression of Hepatocytes	LDPCs Relative Expression of BM
CD45	+	-	+	31	NT	3% (BM)
CKit	+	-	+	33	NE (Hep)	2% (BM)
CD34	+	-	+	29	NE (Hep)	2% (BM)
Thy1	+	-	+	29	NE (Hep)	2% (BM)
CXCR4	+	-	+	24	NE (Hep)	13% (BM)
Gata1	-	-	+	38	NT	NE in LDPC
Gata2	+	NT	+	30	NT	<1%
Tal1	+	NT	+	33	NT	<1%
Lmo2	+	NT	+	20	NT	2%
Pu.1	+	NT	+	20	NT	3%
Mll1	+	NT	+	21	NT	<1%
Runx1	+	NT	+	24	NT	2%
CK19	-	-	-	34	1% (Liver)	NE (BM)
CK18	-	+	-	33	2% (Hep)	NE (BM)
AFP	+/-	+	-	33	0.1% (Hep)	NE (BM)
Alb	-	+	-	35	0% (Hep)	NE (BM)
TTR	+	+	-	25	0.01% (Hep)	NE (BM)
HNF-1	-	+	-	34	0% (Hep)	NE (BM)
HNF3 β	+	+	-	28	3% (Hep)	NE (BM)
cMet	+	+	-	21	112% (Hep)	NE (BM)

NOTE: LDPCs were harvested and subjected to quantitative RT-PCR with the SYBR green method for mRNAs as indicated. The mRNA levels were normalized with rat glyceraldehyde 3-phosphate dehydrogenase as a housekeeping gene. + or - indicates whether the sample was positive or negative for the respective marker. Cycle number is the PCR cycle at which the PCR product reached a certain specified fluorescence intensity, which was kept constant for all samples. The background level of the assay was around 34 to 40 cycles, depending on the specific primer. The relative expression values represent LDPC mRNAs relative to hepatocytes or BM where indicated. For c-kit, CD34, Thy-1, CXCR4, Gata2, Tal1, Lmo2, Pu.1, and Runx1, relative expression numbers are relative to BM as they are not expressed by hepatocytes. Likewise, CK19, CK18, AFP, albumin, TTR, HNF-1, HNF-3 β , and cMET are relative to hepatocytes as they are not expressed by BM. Data in this table are representative examples of 7 RT-PCR analyses.

Abbreviations: AFP, alpha-fetoprotein; BM, bone marrow; CK, cytokeratin; LDPC, liver-derived progenitor cell; mRNA, messenger RNA; NE, not expressed; NT, not tested; PCR, polymerase chain reaction; RT-PCR, real-time polymerase chain reaction; CXCR4, chemokine receptor 4; Gata, globin transcription factor; Tal1, T-cell acute leukemia 1; Lmo2, LIM domain only 2; Pu.1, putative oncogene Spi-1; Mll1, mixed lineage leukemia-1; Runx1, runt-related transcription factor 1; TTR, transthyretin; cMet, mesenchymal epithelial transition factor.

obtained from bulk cultures. After 3 to 4 weeks, we could detect colonies of 100-10,000 LDPCs in 10% of the wells. By RT-PCR, clonal LDPCs were CD45, c-kit, CD34, Thy-1 CXCR4, AFP, cMet, HNF-3 β , musashi, hex, and CDX2 mRNA positive, but did not express CK18, CK19, or albumin transcripts identical to LDPCs from bulk cultures (not shown).

Microarray Analysis of LDPC

In order to better characterize and compare LDPC with other known populations of hepatocyte progenitor or stem cells, we evaluated the transcriptome of LDPCs in comparison with that of the liver cells that gave rise to LDPCs using Affymetrix gene arrays. The fidelity of our microarray results was confirmed using QRT-PCR (data not shown). The expressed gene profile of 5 different LDPC isolations was highly consistent (R^2 value >0.90)

but differed significantly from that of the fresh hepatocytes used to initiate the cultures (R^2 value ~0.71) (Fig. 5A and B). We identified 4636 total probe sets that were differentially expressed between LDPCs and hepatocytes (paired Student *t* test; $P < 0.05$; data not shown) of which 1211 gene transcripts were greater than 1.5-fold differentially expressed. Of these, 577 transcripts were more highly expressed in LDPCs and 634 in hepatocytes (Supplemental Table 1). Compared with hepatocytes, LDPCs did not express, or expressed at much lower levels, genes encoding for hepatic metabolic and secretory functions, such as albumin, transthyretin, and transferrin; enzymes associated with glycogen production and cholesterol and fatty acid production, and genes associated with the detoxifying function of hepatocytes, such as the cytochrome 250 2A-2E family of genes. Of the 577 transcripts expressed more highly in LDPCs, more than 50% of genes were unclassified and

TABLE 2. mRNA Expression Profile of LDPCs for Various Stem Cell and Endodermal/Hepatic Markers: Expression of Some Endodermal/Gut Markers in LDPCs

	Hepatocytes (+/-)	Duod/PS (+/-)	LDPCs (+/-)	LDPCs	
				Relative Expression of Hepatocytes	Relative Expression of Duod/PS
TTF1	-	-/-	-	NE	NE/NE
Secretin	-	+/-	-	NE	NE/NT
Gastrin	-	-/+	-	NE	NT/NE
CDX1	-	+ /NT	-	NE	NE/NE
CDX2	-	+ /NT	+/-	NE	10%/NT
Musashi	+	NT/NT	+	1200% (Hep)	NE/NE
PAP	-	-/-	+/-	NE (Hep)	0.1% (Panc)
Hex1	+	NT/NT	+	6%	NT/NT

NOTE: + or - indicates whether the sample was positive or negative for the respective marker. The relative expression values represent LDPC mRNAs relative to hepatocytes, Duod, PS, or thyroid where indicated. For TTF-1, thyroid tissue was used as a control. For Secretin, CDX1, and CDX2, relative expression numbers are relative to Duod as they are not expressed by hepatocytes. Likewise, Musashi and HEX1 are relative to hepatocytes as they are not expressed by Duod. For Gastrin, relative expression numbers are relative to PS. Data in this table are representative examples of 7 RT-PCR analyses.

Abbreviations: Duod, duodenum; LDPC, liver-derived progenitor cell; mRNA, messenger RNA; NE, not expressed; NT, not tested; PAP, pancreatitis-associated protein; PS, pyloric stomach; RT-PCR, real-time polymerase chain reaction; CDX, caudal homeobox transcription factor.

lacked functional annotation (Fig. 5C). Of the 236 annotated genes, there were 211 unique gene transcripts, 49 of which encode for genes known to be important in liver regeneration, which represents a disproportionate enrichment (Supplemental Table 2).

We also compared the genes more highly expressed in LDPCs than hepatocytes with published gene expression data on epithelial stem and progenitor cells from liver or the gastrointestinal tract (Supplemental Table 3). LDPC gene expression is very similar to other liver and epithelial progenitor cell populations. Epithelial membrane protein 1, found to be highly expressed in all other epithelial progenitor cell populations was highly expressed in LDPC.³³ Cimica et al.,³⁴ who used serial analysis of gene expression to compare cells from animals that underwent partial hepatectomy + 2-AAF versus sham laparotomy + 2-AAF administration, described 27 genes that were more highly expressed and 14 genes that were expressed at lower levels in oval cells compared to normal liver cells. Six of 27 more highly expressed and 6/14 genes less expressed in oval cells were also more highly or significantly less expressed, respectively, in LDPCs compared with hepatocytes.³⁴ An additional 14 genes more highly expressed in LDPC were common gene family members of genes found in oval cells in the Cimica study.³⁴ Comparing LDPCs with BM-derived hepatic stem cells described by Wang et al.³⁵ indicates that 8 genes and 13 family members are in common between LDPCs and BM-derived hepatic stem cells. Further validation that LDPCs represent a population of early progenitors comes from comparison with the transcriptome of small intestinal epithelial progenitors and gastric progenitors.^{32,36} A total of 22% of LDPC genes had identity with the small intestinal

epithelial progenitors data set, which increased to 29% if we also include genes from the same family.

Differentiation of LDPCs into Hepatocytes and Ductal Cells

LDPCs collected from cultures by gentle tapping (in order to avoid any attached fibroblast-like or epithelial cells) were cultured on Type I collagen in 10% fetal bovine serum and HGF for 21 to 42 days. A total of 10 to 20% of the cells differentiated into hepatocytes during the first 3 to 4 weeks but with longer culture period (up to 6 weeks) this percentage reached 60 to 70% (Fig. 6A). The differentiated cells stained positive for albumin, CK-18, and HNF-1 α , consistent with hepatocyte differentiation (Fig. 6B). In some differentiation cultures, LDPCs also formed lumen-like structures that were surrounded by CK19 positive cells, a marker of biliary duct epithelium, albeit at a lower frequency (Fig. 6B). Additionally, LDPCs reproducibly formed three dimensional duct-like structures when cultured in Matrigel (data not shown). LDPCs themselves did not produce urea or albumin in vitro. When LDPCs were cultured for 4 weeks in hepatogenic differentiation media, urea and albumin were secreted in the supernatant (Fig. 7A and B).

Hematopoietic Potential of LDPCs

As LDPCs express multiple markers commonly found on hematopoietic progenitors, we investigated whether LDPCs have hematopoietic potential in vitro by methylcellulose colony forming assay, and coculture with stromal cell lines shown to support hematopoietic cultures.^{37,38} In all experiments, LDPCs fail to differentiate

Correlation Analysis of LDPC Microarray

Figure 5. Microarray analysis of 5 separately isolated liver-derived progenitor cells (LDPCs) and hepatocyte samples. (A) Log-log plot comparing representative LDPC and hepatocyte samples. (B) Correlation ratios of LDPCs and hepatocyte samples. (C) Gene ontology analysis of more highly expressed genes in LDPC and hepatocytes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

into hematopoietic lineages as demonstrated by lack of erythroid, granulocytic, and lymphoid lineage markers. We also transplanted LDPCs and rat BM in Rag2/ γ_c null mice to determine their *in vivo* hematopoietic potential. Flow cytometric analysis of peripheral blood and BM did not demonstrate LDPC-derived blood cells ($n = 15$ animals) whereas control animals transplanted with rat BM showed evidence of rat hematopoiesis by flow cytometry ($n = 10$ animals; Supplemental Fig. 1).

DISCUSSION

Here we report isolation and characterization of a novel population of progenitor cells derived from uninjured adult rat liver, termed LDPCs or liver derived progenitor cells. The method we used to isolate LDPCs requires no chemicals or toxins in contrast to commonly used protocols for oval cell isolation^{13,17,39} which frequently result in chromosomal abnormalities in oval cells precluding their clinical application.

LDPCs are, like oval cells,^{13,17,39-41} HNF-3 β , CD45,

CD34, c-kit, and Thy-1 positive. In contrast to oval cells, LDPCs are negative for OV-6, CK7, and CK19 and express virtually no AFP. Clonally-derived LDPCs also exhibit an identical profile. Thus, the phenotype of LDPCs shows some similarities to that of oval cells. Like oval cells, LDPCs differentiate into both hepatocytes and ductal cells *in vitro* suggesting that LDPCs are bipotent progenitors, although without clonal differentiation experiments a definitive conclusion can not be drawn regarding their bipotentiality. Such studies are currently underway.

As LDPCs express CD34, CD45, c-Kit, Runx1, and Lmo2, markers traditionally associated with hematopoietic cells, we wanted to exclude the possibility of a hematopoietic origin (not BM origin) and investigated this possibility vigorously by *in vitro* and *in vivo* experiments. In these studies, LDPCs did not demonstrate any hematopoietic potential. The results of these studies, along with PCR and microarray data which show expression of a number of endodermal and hepatic

Figure 6. (A) In hepatic differentiation medium, liver-derived progenitor cells (LDPCs) undergo a series of morphological changes. Between days 5 and 7, fibroblast-like cells appear in the culture, then around day 14 small clusters of hepatocytes emerge from these cells, followed by expansion of the clusters. At day 28, 10 to 20% of the cells have hepatocyte morphology, the rest are fibroblast-like cells and few LDPCs. (B) On day 28, the hepatocyte clusters were analyzed by immunohistochemistry analysis (IHC). The clusters were positive for cytokeratin (CK)-18 albumin and hepatocyte nuclear factor (HNF)-1 α , confirming that they were hepatocytes. They were negative for CK-7 and CK-19 (not shown). In some cultures, LDPCs formed lumen-like structures where surrounding cells were positive for CK-19, a biliary ductal marker, in a perinuclear fashion (far right).

markers by LDPCs, strongly suggest that LDPCs do not have a hematopoietic origin. This is in line with the recent observation by many groups that such presumed hematopoietic stem cell specific markers are not uniquely expressed by hematopoietic cells but by many other stem cells, progenitor cells and differentiated cells including oval cells.

Microarray analysis of LDPCs further supports the notion that LDPCs represent a population of hepatic progenitors, as the transcriptome of LDPCs is highly similar to that of oval cells. Many of the genes found to be highly expressed in LDPCs are also expressed in other hepatic stem cells, such as oval cells, during liver regeneration. For instance, Annexin A1 and A2, 2 of the most differentially expressed genes, are up-regulated during liver regeneration and hepatocyte proliferation.⁴² Other genes important in oval cell proliferation and liver regeneration such as genes influenced by IFN- γ stimulation (gp91phox, caspase 1, urokinase plasminogen activator, IL-18, and IL-1 β) are abundantly expressed in LDPCs. In addition, LDPCs express many genes also identified in oval cells and other hepatic stem cell population whose role are uncertain; glutathione-s-transferase, pi isoform, 5-HT receptor, toll-like receptors 2 and 4, complement component 3

(C3A), complement component receptor 1 (C5A receptor), follistatin, vimentin, and connective tissue growth factor.⁴³⁻⁴⁹ Another interesting feature of LDPCs is the high level expression of CXCR4 which is an important chemokine receptor involved in homing of stem cells following their transplantation in vivo.⁵⁰ In contrast, mature hepatocytes do not express any CXCR4 which suggests that LDPCs, having also a much smaller size, might be better suited for cell transplantation purposes than mature hepatocytes.

To put LDPCs in perspective, it might be helpful to compare them briefly with some of the other stem/progenitor cell populations described in the literature. These cell populations can be divided into 2 main groups; those derived from fetal liver and those from post-natal liver. Relatively few groups have succeeded in isolating hepatic progenitors from noninjured post-natal liver. Mitaka et al.⁵¹ isolated cells, termed small hepatocytes, that can clonally proliferate to form large colonies of small cells that subsequently can be induced to differentiate to hepatocytes.⁵² Unlike LDPCs, small hepatocytes express hepatocyte specific genes such as albumin, CK8, CK18, and connexin 32.⁵³ Nagai et al.⁵⁴ also isolated cells from adult rat liver called HSL cells that, like LDPCs, are propagated on type I colla-

Figure 7. Liver-derived progenitor cells (LDPCs) do not produce urea whereas hepatocytes derived from LDPCs produce urea. Because the assay measures ammonia metabolized from urea, samples were assessed before and after urease addition. No urea or ammonia was detected in culture medium alone (A) LDPC-derived hepatocytes produce albumin (B). Studies were done in triplicate and repeated twice. Due to the nature of culture conditions, data for LDPC reflect the levels in 2 week old media, whereas data for primary hepatocytes were obtained from 24 hour old cultures.

gen, do not express albumin, CK18 and CK19, but unlike LDPCs, they clearly have an epithelial morphology and express high levels of AFP. Tateno et al.⁵⁵ examined another cell type that also grows in clusters and expresses some mature hepatocyte markers such as CK8, CK18 and albumin but unlike small hepatocytes also express AFP. More recently, Fougere-Deschatrette et al.⁵⁶ reported a clonal cell line derived from adult mouse liver, termed bipotential adult mouse liver cells, which is capable of differentiating into both hepatic and biliary lineages. These cells, in contrast to LDPCs, are CK19 positive and express number of markers found in mature hepatocytes.

Among the adult stem cell populations described in the literature, perhaps the one that is most closely related to LDPCs has been reported in rats treated with allyl alcohol by Sell et al.²⁸ In the periportal regenerative zones, these investigators found several population of cells which appeared in response to injury. One population called "periportal small stem cells" were negative for CK, AFP, OV-6 and desmin similar to LDPCs but they did not express CD45 (LCA) in contrast to LDPCs. Because of the nature of the experiment; however,

these cells could not be extensively examined for the expression of other stem cell markers, which makes it difficult to fully compare them with LDPCs. This difficulty exists for most of the adult liver progenitors described in the literature. Nevertheless, based on the available information, it is clear that LDPCs represent a different and possibly a less differentiated cell type than those described above.

There are also a number of embryonal/fetal liver progenitors described in the literature.

Suzuki et al.^{29,30} isolated hepatic stem cells from the c-Met⁺ CD49f^{+/low} c-kit⁻ CD45⁻ TERR119⁻ fetal liver fraction. These cells possess the ability to differentiate into hepatocytes, biliary duct, pancreatic ductal cells, and the intestinal epithelium suggesting that they may be endodermal stem cells. Similar cells could also be isolated from fetal pancreas and liver of newborn mice.^{29,30} In addition to phenotypic differences between these cells and LDPCs, currently we have no evidence that LDPCs have extrahepatic potential. Another fetal progenitor population was described by Lazaro et al.³¹ Termed human fetal hepatocytes, these cells were positive for stem cell markers CD34 and Thy1 but in contrast to LDPCs they expressed OV6, CK18, CK19 and AFP. More recently, Dan et al.⁵⁷ described a new progenitor population derived from human fetal liver which were positive for a number of stem cell markers similar to LDPCs but unlike LDPCs they were CK18 and CK19 positive and they were capable of mesenchymal differentiation. Obviously the major distinction of LDPCs from these fetal cell populations is their presence in the liver of post-natal and older adult animals which is potentially a clinical advantage especially in cell transplantation for liver diseases.

The origin of LDPCs, at this time, is not clear. The absence of CK expression, which is present in virtually all adult liver stem/progenitor cells previously described, indicate that LDPCs are nonepithelial progenitors and probably more primitive than oval cells. As such they may exist in the nonparenchymal fraction of the liver cells or they may originate from an extrahepatic site. Even though they are cultured out of uninjured adult liver in a relatively short period of time in large numbers suggesting that they reside in the liver, we can not rule out the possibility of an extrahepatic origin such as BM without lineage-tracing studies. We are currently planning studies that will conclusively show their origin and animal studies designed to test their hepatic potential.

We conclude that LDPCs are a novel population of liver progenitors with some similarities to oval cells and hematopoietic stem cells. LDPCs can be generated highly reproducibly from unmanipulated and uninjured adult liver which makes them potentially useful for clinical applications as well as research purposes.

ACKNOWLEDGMENTS

We thank Scott Soltis for help with hepatocyte isolation and Jingbo Du and Todd Lenvik for their assistance with the microarray setup and analysis. We also thank

Dr. Stewart Sell for supplying OV-1 and OV-6 antibodies, and Dr. Clifford Steer for his help with the revision of the manuscript.

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