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Isolation, Characterization, and Transplantation of Bone Marrow-Derived Hepatocyte Stem Cells

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Recently it was shown that a population of cells in the bone marrow-expressing hematopoietic stem cell antigens could differentiate into hepatocytes. However, explicitly committed hepatocyte progenitors, which exhibit highly differentiated liver functions, immediately upon isolation, have not yet been isolated from bone marrow. After studying common antigens on blast-like cells in fetal and adult regenerating cholestatic rat livers and human regenerating and malignant livers, we hypothesized that beta-2-microglobulin-negative (β_2m^-) cells might represent dedifferentiated hepatocytes and/or their progenitors. Utilizing a two-step magnetic bead cell-sorting procedure, we show that in bone marrow from rat and human, β_2m^-/Thy-1$^+$ cells consistently express liver-specific genes and functions. After intraportal infusion into rat livers, bone marrow-derived hepatocyte stem cells (BDHSC) integrated with hepatic cell plates and differentiated into mature hepatocytes. In a culture system simulating liver regeneration and containing cholestatic serum, these cells differentiated into mature hepatocytes and metabolized ammonia into urea. This differentiation was dependent on a yet nondescript humoral signal existing in the cholestatic serum. Transmission electron microscopy and three-dimensional digital reconstruction confirmed hepatocyte ultrastructure of cultured BDHSC. © 2001 Academic Press

Key Words: **stem cells; liver stem cells; hepatocyte; hepatocyte progenitors; bone marrow; cell differentiation; liver regeneration.**

Facultative progenitor cells like oval cells (OC) exist in the liver and can be induced to proliferate following hepatic injury, when growth of mature hepatocytes is suppressed (1). However, the nature, origin and role of OC and other putative liver cell progenitors in liver development and repair remain unclear.

For many years, OC were believed to originate from cells present in the canals of Herring or from blast-like cells located near the bile ducts (2, 3). Recently, results of cross-sex and cross-strain bone marrow (BM) and whole liver transplantation experiments indicated that the BM might be a source of hepatocyte progenitors (4–6). Further evidence for the existence of bone marrow-derived hepatocyte stem cells (BDHSC) comes from a recent study by Lagasse *et al.* (7), who showed that transplantation of adult BM cells into fumarylacetoacetate hydrolase (FAH)-deficient mice, animals with a fatal congenital tyrosinemia type I, rescued the mice and restored their liver biochemical functions.

Although various hematopoietic stem cells were found to repopulate injured livers, previous attempts at isolation of hepatocyte progenitors, both from bone marrow and liver, resulted in a mixture of hematopoietic and potential liver progenitor cells because they all share common cell surface receptors and antigens, including hematopoietic stem cell markers CD34, Thy-1, c-Kit, flt-3, and all known oval cell antigens (7–11).

Alterations in major histocompatibility complex (MHC), human leukocyte antigen (HLA) and β_2^- microglobulin (β_2m) expression are manifested in many types of cancer cells, as well as in diseased livers where regeneration and dedifferentiation are found (3, 12–16). Recently, while studying the expression of β_2m and MHC and HLA antigens in tissue specimens obtained from human hepatic malignancies and regenerating livers, we identified blast-like cells that stained negative for β_2m (data not published). This finding was intriguing because β_2m is the least polymorphic and the most conserved immune molecule in nature. It is expressed on all nucleated cells and the inner cell mass of the pre-implantation blastula, which is the site where totipotent embryonic stem cells are found, is the

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only place during morulation where a small population of β_2m-negative cells exists (17). Furthermore, adult human and rat spermatozoa do not express β_2m and absence of its expression in various cancer cells is viewed as indirect evidence that transformed cells originate from stem cells (18–20). Based on these data we hypothesized that lack of β_2m expression might represent a common antigenic denominator of dedifferentiated hepatocytes and/or their progenitors.

We herein report that in the β_2m^- fraction of BM cells from rodents and humans, a population of Thy-1^+ cells exists that express hepatocyte-specific markers and exhibit liver specific functions. These BDHSC and their more mature descendents are also found in normal and diseased rat and human livers. Moreover, after intraportal infusion into rat livers, BDHSC integrated with hepatic cell plates and differentiated into mature hepatocytes.

MATERIALS AND METHODS

Chemicals for cell harvest, culture and magnetic bead cell sorting (MACS) were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM) and bovine calf serum (BCS) were from Omega Scientific, Inc. (Tarzana, CA). Epidermal growth factor (EGF) and ITX mixture were from Gibco (Gaithersburg, MD), interleukin 3 (IL-3) and hepatocyte growth factor (HGF) from R & D Systems (Minneapolis, MN), fetal bovine serum (FBS) from Hyclone Laboratories, Inc. (Logan, UT), and Percoll from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Regular culture dishes and Matrigel were purchased from Becton–Dickinson CBP (Bedford, MA) and dual-chamber Transwell dishes from Corning Costar Corporation (Cambridge, MA). For immunohistochemistry, DAB substrate kit, blocking antibodies (mouse, rabbit, goat) and secondary antibodies (Universal Elite and goat IgG ABC kits) were purchased from Vector Laboratories, Inc. (Burlingame, CA). Antibodies against albumin were from Accurate Chemical & Scientific Corp. (Westbury, NY) and Dako (Carpinteria, CA), against Thy-1, c-Kit, CD34, CD38, CEBP/α, IL-3R, flt-3, and alpha-fetoprotein (αFP) from Santa Cruz Biotechnology (Santa Cruz, CA), against CK8, CK18, and CK19 from Sigma, against CAM-5.2 from Biogenex (San Ramon, CA), and against rat macrophages from Pharmingen (San Diego, CA). HepG2 and Hep3B cells were purchased from American Type Culture Collection (Rockville, MD).

Surgical Animal Models

Inbred Lewis and outbred Sprague–Dawley (SD) and D'Agouti rats were housed in a climate-controlled (21°C) room under a 12-h light–dark cycle and were given tap water and Rodent Chow 5001 (Ralston Purina, St. Louis, MO) *ad libitum*. The animals received humane care according to the guidelines prepared by the National Institute of Health, U.S.A. All operations were performed under general (methoxyflurane) anesthesia using sterile surgical technique.

Induction of cholestasis. The common bile duct (CBD) was transected between the two ligatures.

Transplantation of BDHSC. D'Agouti female rat livers ($n = 9$) were harvested and transplanted in the female inbred Lewis rat recipients ($n = 9$) with full revascularization, as described previously (21). Each transplanted liver was seeded via portal vein with male inbred Lewis rat BDHSC (5×10^4 cells/liver). Rats were maintained on a daily subtherapeutic dose of cyclosporine A (Novartis, Switzerland; 0.25–0.5 mg/kg im) to allow for mild injury of the transplanted liver due to immune rejection. Rats were evaluated every 8 h and sacrificed when they appeared moribund. Since the BDHSC were negative for MHC C3 antigen and both recipients and liver donors were C3-positive, BDHSC were detected in transplanted livers by immunohistochemistry.

Isolation of Liver Cells

(a) Livers of newborn ($n = 12$) and 4-day-old ($n = 6$) SD rat pups were removed three at a time, washed in 0.9% NaCl, minced and shaken in a warm (38°C) water bath initially in ethylenediaminetetraacetic acid (EDTA) and then in calcium-enriched 0.05% collagenase type IV solution. The dispersed cells were suspended in ice-cold DMEM/10% FBS, filtered through a 100-μm mesh, suspended in DMEM/10% FBS and placed on ice. Their viability was greater than 95% (trypan blue exclusion test).

(b) Liver cells from normal 24-day-old ($n = 6$) and adult SD rat ($n = 9$; 250–300 g) were harvested by an in situ two-step EDTA/collagenase portal vein perfusion method (22).

(c) Liver cells from adult cholestatic (CBD ligated) SD rats ($n = 18$) were harvested at days 7, 10, and 21 after surgery by an *in situ* two-step EDTA/collagenase retrograde hepatic vein perfusion method (22).

After enrichment through a Percoll density gradient, viability of the cells (b, c) was greater than 90%, as determined by trypan blue exclusion test.

(d) Human liver cells were harvested from tissue biopsies obtained from patients with fulminant hepatitis, acute alcoholic hepatitis, primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC) and human hepatocellular carcinoma (HCC). Digestion of tissue was achieved via microperfusion with calcium-enriched 0.05% collagenase type IV solution. Cells were washed three times in PBS, cyto-spun on glass slides and immediately subjected to immunohistochemical analysis.

From all source materials, a two-step magnetic bead cell sorting (MACS) technique (see below) was used to isolate the BDHSC.

Isolation of Bone Marrow Cells

Rat BM cells were obtained by flushing femurs. The femurs were accessed through laparotomy to avoid contamination and to increase the cell yield. Cells were suspended in DMEM/10% FBS and maintained at 4°C until further use. Human BM cells, from six different individuals, were obtained from the National Disease Research Interchange (Philadelphia, PA). The BDHSC were isolated from these source materials by a D/MACS technique (see below).

Magnetic Bead Cell Sorting (MACS)

The columns, MACS buffer and anti-mouse supermagnetic microbeads (50 nm in diameter) were from Miltenyi (Militenyi Biotec, Auburn, CA). The procedure involved two steps. In the first step, the cells (e.g., liver cell suspension, BM cells) previously labeled with mouse monoclonal anti-β_2m antibodies were incubated with anti-mouse magnetic beads and then passed though a depletion column placed in a magnet. Because the column matrix created a high-gradient magnetic field, labeled cells were retained in the column while β_2m^- cells passed freely through the magnetic field. In the second step, β_2m^- cells were passed through the selection column designed to retain cells expressing specific marker (e.g., Thy-1, IL-3R, c-Kit, flt-3, CD34). Sodium azide (0.01%) was added to the MACS buffer to prevent capping of surface antigen-antibody complexes. When BM cells were processed, the initial step involved destruction of red blood cells using a lysis buffer. At the end of the procedure all sorted cells were washed once with isotonic saline solution and thrice with serum-free PBS to prevent nonspecific albumin staining. Viability of the cells was consistently over 98%, as determined by trypan

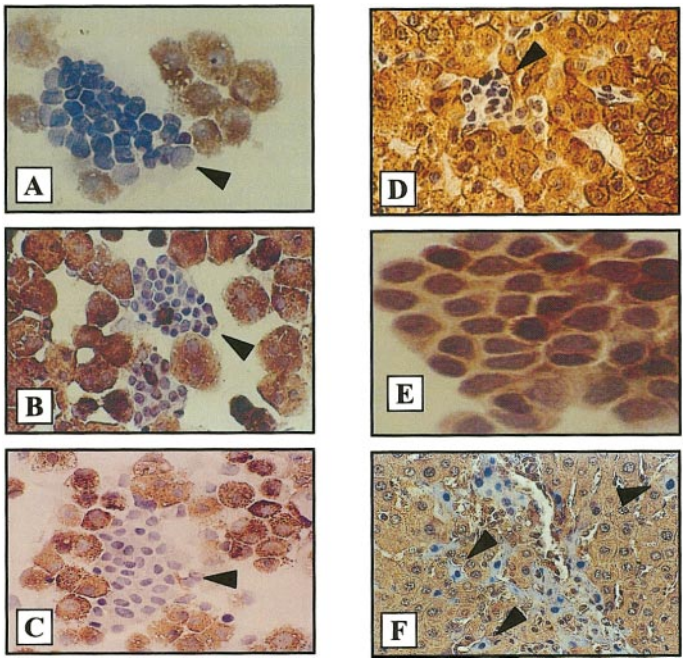

FIG. 1. Immunohistochemical staining for β_2m and Thy-1. All pictures were viewed through a bright-field microscope. Brown cells are positive for DAB and represent either β_2m- or Thy-1-expressing cells. Blue cells are β_2m-negative (arrow). (A) Liver cells isolated from 24-day-old rat. (B) Liver cells isolated from cholestatic rat liver after 10 days. (C) Liver cells isolated from cholestatic rat liver after 21 days. Notice the oval shape and a larger size of β_2m^- cells in C compared to smaller, dense cells in B. This may represent "maturation" process that β_2m^- cells undergo between day 10 and day 21 after induction of cholestasis. (D) Whole liver section obtained from human patient with primary sclerosing cholangitis. (E) Liver cells isolated from cholestatic rat liver after 21 days. Brown cells are positive for DAB and represent Thy-1-expressing cells. Staining was performed on the same specimen as in C (magnification ×400). (F) Whole liver section obtained from cholestatic rat liver after 21 days. It appears that β_2m^- cells emanate from a portal triad.

blue exclusion. The various fractions were cytospun on glass slides, fixed and stained for albumin, as described in Tables 1 and 2.

Culture of β_2m^-/Thy-1$^+$ Cells

β_2m^-/Thy-1$^+$ cells were suspended in DMEM/5% FBS and antibiotics, plated at densities of 4.5×10^5 cells/ml onto 35- or 60-mm dishes coated with type I rat tail collagen and placed in a humidified, 5% CO_2:95% air incubator at 37°C. After 2–3 h, the medium was replaced with DMEM enriched with 10% FBS, 20 mM Hepes, 10 mM nicotinamide, 1 mM ascorbic acid 2-phosphate, 10^{-7} M dexamethasone, 1 mg/ml galactose, 30 μg/ml proline, ITS mixture, 10 ng/ml EGF and antibiotics. Medium was replaced every other day. In addition to the basal medium, 1% DMSO was used from day four onwards. These culture conditions are known to facilitate survival and clonal growth of small hepatocytes (24).

TABLE 1

Incidence of β_2m^-/Thy-1$^+$ Cells in Newborn, Young, Adult, and Cholestatic Rat Livers

Source	β_2m^-/Thy-1$^+$ cells (%)
Newborn rats (n = 12)	8.0 ± 2.0
4-Day-old rats (n = 6)	8.0 ± 2.0
24-Day-old rats (n = 6)	1.0 ± 0.5
Adult normal rats (n = 6)	0.01 ± 0.001
Cholestatic rats (n = 8)	4.5 ± 1.5

Note. A two-step magnetic bead cell-sorting technique was performed to isolate the β2m$^-$ fraction of cells from whole cell suspensions.

TABLE 2

Albumin Expression by Different Fractions of β2m$^-$ Cells Harvested from Cholestatic Rat Livers

Liver (β_2m-negative fraction)	Albumin-positive cells (%)
Thy-1 (+)	100
Thy-1 (−)	<1
IL-3 (+)	5.0 ± 2.2
c-Kit (+)	15 ± 4.3
flt-3 (+)	5.0 ± 2.3
CD-34 (+)	<1

Note. A two-step magnetic bead cell-sorting technique was performed on whole liver cell suspensions. It involved (i) depletion of β2m$^+$ cells and (ii) selection of cells positive for various hematopoietic stem cell markers. The final cell isolates were immunostained for albumin.

Coculture of BDHSC and Hepatocytes

BDHSC and hepatocytes isolated from cholestatic rat livers were cocultured in Transwell dual chambers. The chambers were separated by a semipermeable (0.4 μm) polyterafluoroethylene (PTFE) membrane. The top chamber was coated with collagen type I and seeded with 50,000 hepatocytes/cm^2. The lower chamber was coated with Matrigel (25 μg/cm^2) and seeded with BDHSC. HGF (25 ng/ml) and autologous cholestatic serum (5%) collected from the liver donor were added to the culture media. Dexamethasone and IL-3 were added to the media after 72 h to enhance differentiation and proliferation of cells (23). Control dishes contained normal primary rat hepatocytes, BM cells only, and BDHSC only. Coculture of BDHSC and normal autologous hepatocytes and serum was carried out, as

TABLE 3

Albumin Expression by Different Fractions of β2m$^-$ Cells Harvested from Rat Bone Marrow

Bone marrow	Albumin-positive cells (%)
β_2m-Negative fraction	25 ± 5.7
Thy-1 (+)	100
Thy-1 (−)	<1
IL3 (+)	95 ± 2.3
c-Kit (+)	<5 ± 0.6
flt-3 (+)	<5 ± 4.5
CD-34 (+)	<1

Note. A two-step magnetic bead cell-sorting technique was performed on whole bone marrow cell suspensions. It involved (i) depletion of β2m$^+$ cells and (ii) selection of cells positive for various hematopoietic stem cell markers. The final cell isolates were immunostained for albumin.

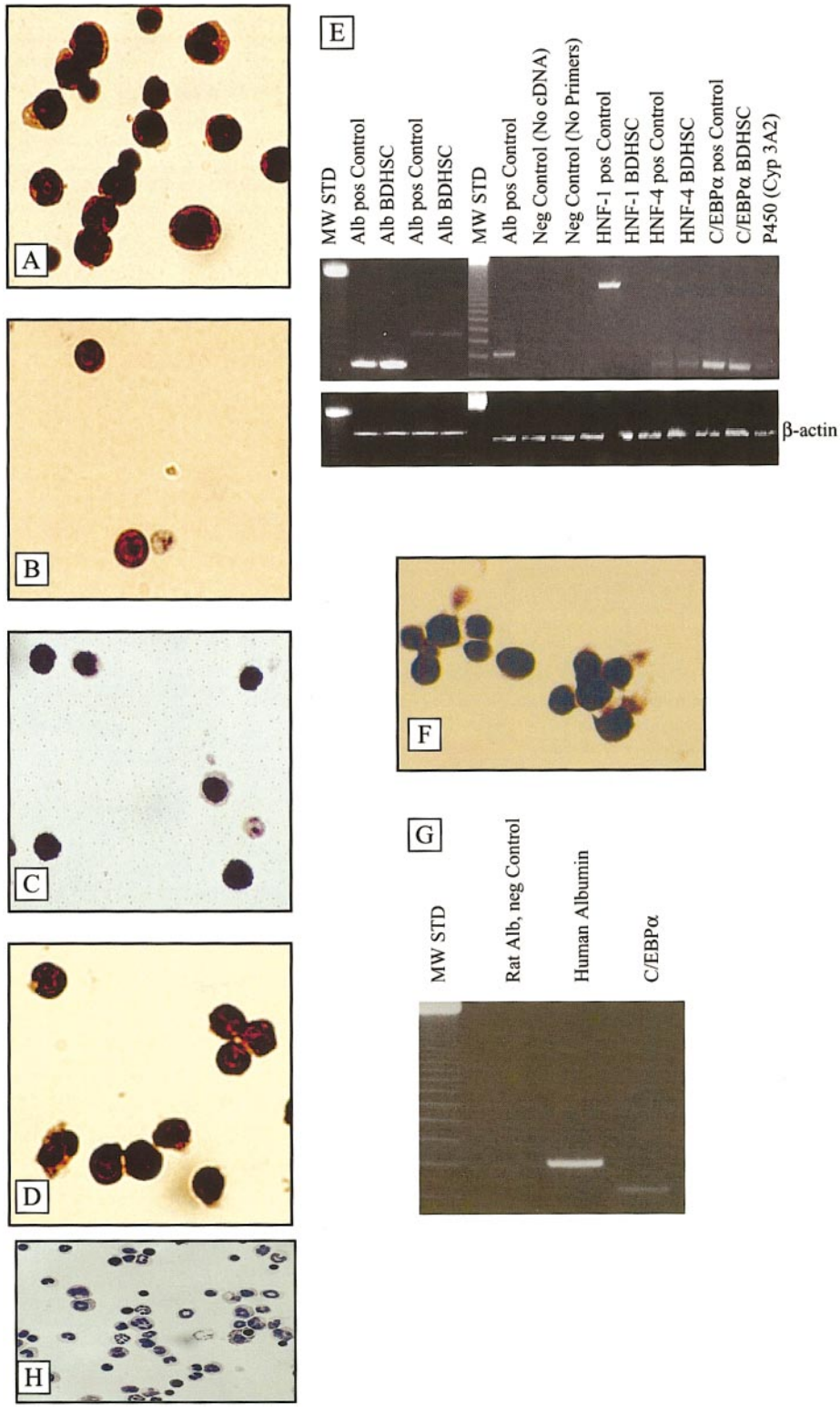

FIG. 2. Characterization of BDHSC harvested from the BM of cholestatic rats. Immunohistochemical staining for albumin (A), αFP (B), CK8 (C), and C/EBPα (D). (E) On RT-PCR, BDHSC were found to express albumin, C/EBPα, p450 (Cyp3A2) and HNF-4, but not HNF-1. Lanes 2 and 3 represent positive control for rat albumin mRNA and albumin expression by BDHSC, respectively, as determined using highly specific nested primers. Lanes 4 and 5 represent positive control for rat albumin mRNA and albumin expression by BMDHSC, respectively, as determined using regular rat albumin primers. (H) Rat whole BM cells did not express albumin. (F and G) Human BDHSC expressed albumin and C/EBPα at both the protein (F; immunocytochemistry) and mRNA (G; RT-PCR) levels.

well. In all groups, the chambers were separated after 3, 5, or 7 days. After placing the cells in fresh serum-free media, they were exposed to 1.5 mM NH_3 for 8 h. Urea levels were measured by an enzymatic colorimetric test (Boehringer Mannheim, R-Biopharm, Marshall, MI) (24).

Immunohistochemistry

Immunohistochemistry and immunofluorescence staining were performed using the ABC method (25) on (a) serial 5-μm thick sections from paraffin embedded tissue that were mounted on superfrost/plus slides, (b) cell suspensions (after cyto-centrifugation at 1000 rpm for 5 min), and (c) cells taken directly from culture dishes. All cell specimens were fixed in Glyoxx. Incubation with the various primary antibodies was done at 37°C for 2 h in a humidified chamber. Blocking sera and biotinylated secondary antibodies were matched with the primary antibody. DAB and Gill-I hematoxylin were used to visualize the immunoperoxidase and to counterstain, respectively. Semi-quantitative analyses, on stained slides, were done independently by two different investigators. Each staining was repeated three times and 20 random fields were examined. All numerical data were computed using Jandel Scientific Solutions 1.0 software (Jandel Scientific, San Rafael, CA). When indicated, estimated liver cell mass represented 4% body weight, i.e., the liver in a 250-g rat contained 9×10^8 hepatocytes.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

RT-PCR procedures were carried out on primary rat and human BDHSC and rat BDHSC after seven days in culture. Briefly, the cells were sorted into RT-PCR buffer containing RNase inhibitor and RT-PCR was carried out using the QIAGEN OneStep RT-PCR kit (QIAGEN, Valencia, CA) (26). Forty cycles of amplification were performed on a standard Perkin–Elmer 9600 thermal cycler platform (Norwalk, CT) (27). The primers used included C/EBPα, HNF-1, HNF-4 (28), p450 (CYP4A1; 29), rat albumin 1 (30), rat albumin 2 (nested primers): F-5′-tcggagacaagttatgcgcc-3′ and R-5′-gcagctattgagggcagatc-3′ (outside primers) and F-5′-gatgacaaccccaacctgcc-3′ and R-5′-gcattccaacaggtcgccgt-3′ (inside primers), human albumin (31), and β-actin: F-5′-gccccacgtgaaaagatgac-3′ and R-5′-agccaccaatcca cacagagta-3′.

Confocal Microscopy

Culture dishes were washed three times with normal saline, 2% saponin/PBS and PBS (pH 7.2) to remove Matrigel and then fixed with Glyoxx/0.1% Triton X-100. Indirect immunofluorescence staining for albumin (Accurate Chemical & Scientific, Westbury, NY) with secondary FITC anti-rabbit antibody (Dako, Glostre, Denmark) and for CAM-5.2 (Biogenex, San Ramon, CA) with secondary R-phycoerythrin antibody (Vector, Burlington, CA) was performed (32). To visualize the coexistence of albumin and CAM-5.2, three-dimensional digital reconstruction images were obtained using Leica TCS/SP confocal microscope (Heidelberg, Germany) and IMARIS software (Bitplane AG, Zurich, Switzerland).

Electron Microscopy

Culture dishes were washed with PBS (pH 7.5), fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer for 48 h, post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in a Zeiss EM 9-A Electron Microscope (Germany).

Statistical Analysis

Results were analyzed by ANOVA and Fisher's exact test, when deemed appropriate (Jandel Statistical Software, version 1.0). Data are shown as means ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

We examined a variety of tissues and cells in suspension for the expression of β_2m, hematopoietic stem cell markers (Thy-1, flt-3, CD 34, c-Kit, CD38) and liver associated markers (albumin, αFP, CK8, CK18, and CK19). Human tissue specimens, each from three different individuals, included surgical or diagnostic biopsies from patients with hepatocellular carcinoma and liver diseases known to be associated with hepatic regenerative response (primary sclerosing cholangitis, primary biliary cirrhosis, alcoholic hepatitis and fulminant hepatic failure). Additionally, fetal livers from abortion products and malignant cell lines HepG2 and Hep3B (14, 33) were studied. Rat specimens included livers obtained from newborn pups and adult SD rats that underwent CBD ligation to induce cholestasis and liver injury resulting in a regenerative response.

A significant finding in all source materials was the presence of small (5–8 μm) blast-like cells with high nuclear to cytoplasmic ratio containing only a few organelles. These cells, in contrast to all other nucleated cells, did not express β_2m (Figs. 1A–1F). Consequently, we performed magnetic bead cell sorting to isolate these β_2m^- cells from whole cell suspensions obtained from the livers of newborn ($n = 12$), 4-day-old ($n = 6$), 24-day-old ($n = 6$), normal adult ($n = 6$), and adult cholestatic ($n = 8$) rats. We found that the livers of newborn and 4-day-old rats contained significantly more β_2m^- cells than 24-day-old rat livers; adult rat livers contained very few β_2m^- cells. Remarkably, cholestatic rat livers contained 400× more β_2m^- cells than adult normal rat livers (Table 1). The blast-like cells were frequently in small clusters. They were seen near the portal triads and then reappeared as more mature β_2m^- cells distally in zone-1 and proximally in zone-2 of the hepatic acini. Characteristically, they were never observed in zone-3. During this pseudostreaming (Fig. 1F), the cells differentiated into mature hepatocytes, exhibited increase in cytoplasmic granularity, lost expression of αFP and maintained expression of albumin.

To assess what hematopoietic stem cell markers these β_2m^- cells might express, we used MACS to isolate them from whole liver cell preparations of cholestatic rats and analyzed them for the expression of Thy-1, flt-3, CD34, c-Kit and CD38. We found that Thy-1 was expressed by 99.0 ± 0.2% of the cells, flt-3 by 5 ± 0.2% of the cells and the remaining markers were expressed by less than 1% of the cells (data not shown). These results prompted us to develop a two-step MACS technique, where depletion and positive selection columns are used in sequence. Using this method, we isolated from cholestatic rat livers a population of β_2m^-/Thy-1^+ cells and demonstrated that they expressed albumin (Table 2) and other hepatocyte-specific markers like αFP, CK8, and CK18, but not

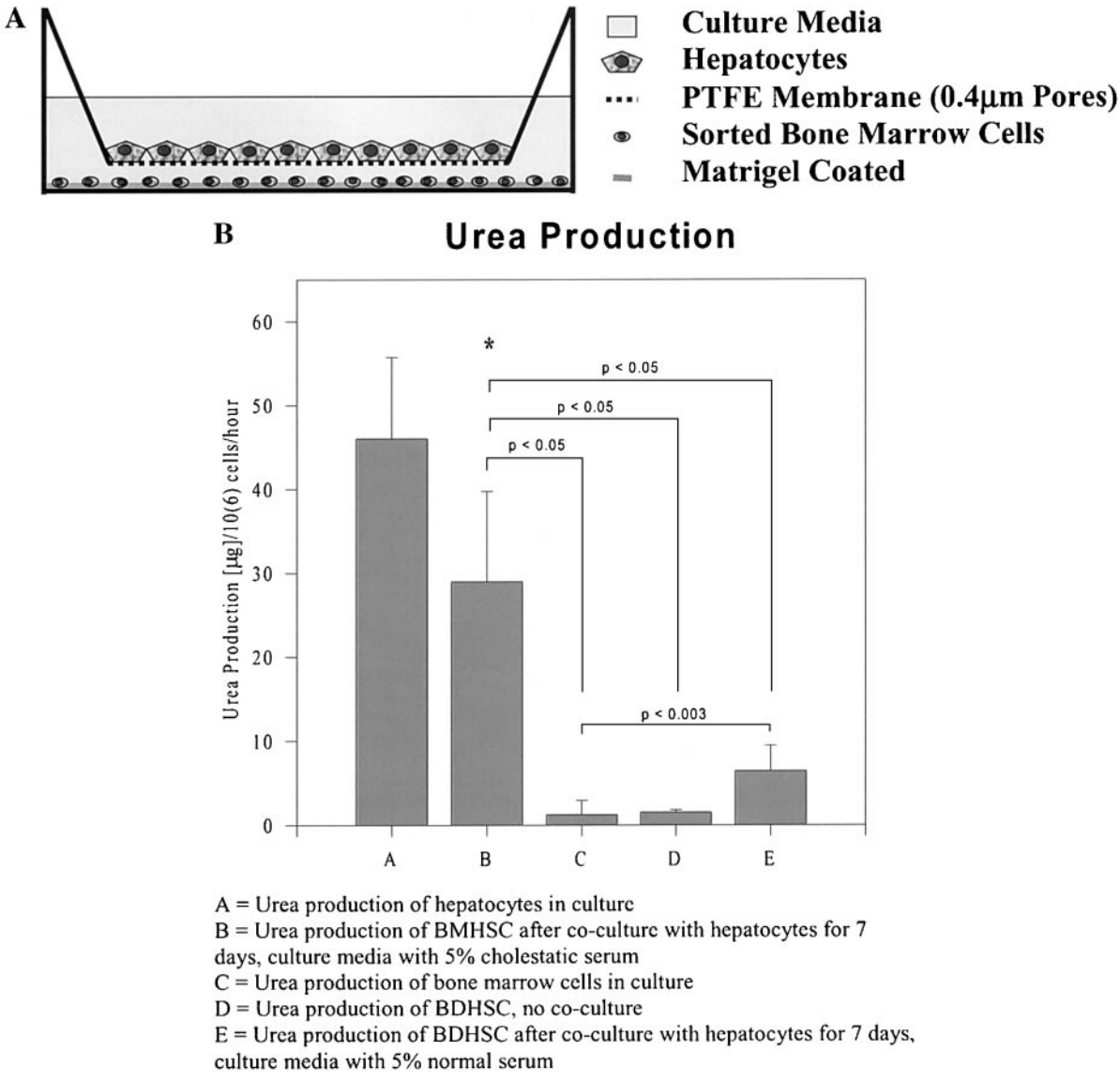

FIG. 3. BDHSC in culture. (A) The cells were cultured in a dual chamber with intervening semipermeable PTFE membrane. The top chamber contained hepatocytes isolated from cholestatic rat livers. The bottom chamber contained β_2m^-/Thy-1^+ cells isolated from autologous BM. The cells were cultured in hormonally defined DMEM enriched with 5% "toxic" (cholestatic) serum obtained from the same cell donor. Control cultures contained normal rat hepatocytes only (column A), rat BM cells only (column C), BDHSC only (column D), and BDHSC and normal hepatocytes in the presence of normal autologous rat serum (column E).

CK19 (data not shown). These results suggested that β_2m^-/Thy-1^+ cells could be hepatocyte progenitors. The site of their origin, however, was unclear.

In view of the data reported by Petersen *et al.* (4) and others (5, 6), we decided to examine whether we may be able to directly isolate β_2m^-/Thy-1^+ cells from the rat bone marrow. Using a two-step MACS method, we found that the β_2m^- cells do exist in the BM and that among six different subpopulations of β_2m^- cells (CD34^+, c-Kit$^+$, flt-3^+, IL-3^+, Thy-1^-, and Thy-1^+), virtually 100% of those expressing Thy-1 and 95% of those expressing IL-3 were albumin positive; in the remaining cell fractions, very few cells expressed albumin (Table 3). Based on these results, we concluded that the β_2m^-/Thy-1^+ BM cells might be hepatocyte stem cells. We named them bone marrow-derived hepatocyte stem cells (BDHSC).

To characterize the BDHSC, nine SD rats underwent CBD ligation and their BM was harvested after 10 days. The β_2m^-/Thy-1^+ cells were isolated, spun onto slides and immunostained for albumin, αFP, CK8, CK18, CK19, and C/EBPα. We found that albumin, αFP, CK8 and C/EBPα were expressed by 99.9 $\pm$ 0.01, 75 $\pm$ 16.3, 95 $\pm$ 3.0, and 100% of the BDHSC, respectively (Figs. 2A–2D). Additional cells were immunostained (Figs. 2A–2D) and also sorted directly into cell

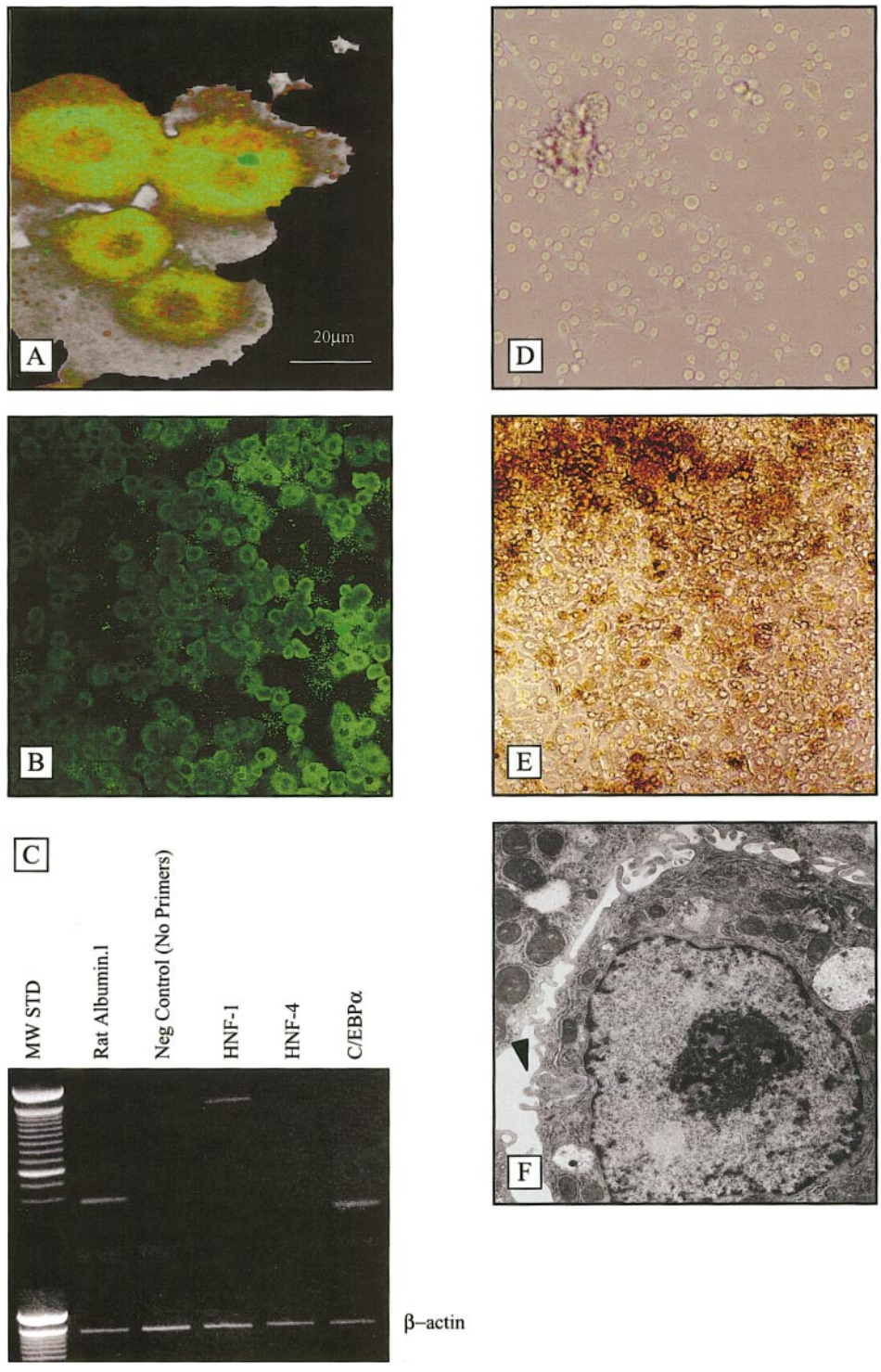

FIG. 4. BDHSC in culture. morphologic and genetic characterization. (A) 3D digital reconstruction of cells after 7 days in culture. The red and the green signal (immunofluorescence) represent staining for CAM5.2 and albumin, respectively. (B) Staining for albumin (green) of BDHSC cells after 7 days in culture. (C) Expression of albumin, C/EBPα, HNF-1, but not HNF-4, by the BDHSC cells after 7 days in culture (RT-PCR). (D) BDHSC cells after 12 h in culture; note the blast-like morphology of the cells. (E) After 7 days in culture, BDHSC assumed the hepatocytic morphology, which was confirmed by transmission electron microscopy (F; arrowhead points to the microvilli).

lysis buffer and analyzed using one-step RT-PCR. We found that at the mRNA level, the BDHSC expressed albumin, C/EBPα, p450 (Cyp3A2), HNF-4 but not HNF-1 (Fig. 2E). Quantitatively, the β_2m^- fraction obtained from normal rat BM (n = 7), represented 0.45 ± 0.60% of the total BM cell mass while the same

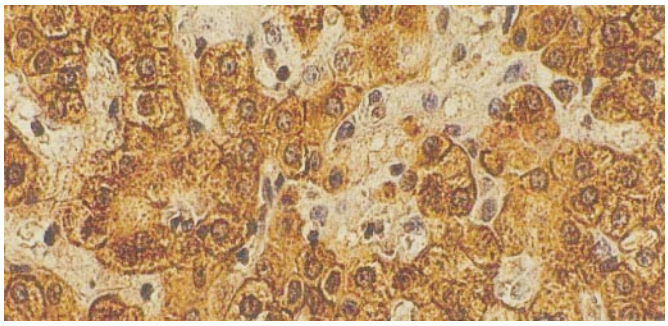

FIG. 5. BDHSC cells *in vivo*. After intraportal infusion, BDHSC integrate with hepatic cell plates. Brown represents positive DAB signal for the C3 antigen. The transplanted BDHSC do not express the C3 antigen while the native liver cells express it throughout.

cell fraction obtained from the BM of cholestatic animals represented 3.66 ± 0.1%. Of these fractions, β_2m^-/Thy-1^+ represented 1.9 ± 1.6 and 5.5 ± 2.3% of the total nonerythroid BM cell mass, respectively. In the subsequent studies, the β_2m^-/Thy-1^+ cells were extracted from six normal human BM samples. Again, all of the β_2m^-/Thy-1^+ cell samples expressed albumin and its transcription factor C/EBPα both at the protein (Fig. 2F; C/EBPα) and mRNA (Fig. 2G) levels. To assure the accuracy of these studies, we used four different β_2m, three albumin and two Thy-1 antibodies.

To further establish the hepatic nature of BDHSC, we designed a cell culture system that reproduces the regenerative response/signal induced by cholestasis *in vivo* (Fig. 3A). It consisted of two chambers separated by a semi-permeable membrane. The top chamber contained hepatocytes isolated from cholestatic rat livers. The bottom chamber contained autologous BDHSC. The modified media contained 5% autologous serum, i.e., cholestatic serum obtained from the same animal and thought to contain humoral factors that have a role in recruiting putative liver stem cells from BM to the injured liver and inducing them to proliferate and differentiate. The chambers were separated and filled with fresh serum-free media after 3, 5, or 7 days. Each cell culture was exposed to 1.5 mM NH_3 for 8 h. Data from 18 different assays using 6 different BDHSC isolates demonstrated that after coculture with autologous cholestatic hepatocytes and serum, BDHSC produced 29 μg of urea per 10^6 cells/h in comparison to 46 μg of urea produced by autologous hepatocytes (Figs. 3A and 3B). In contrast, neither rat BM cells nor BDHSC metabolized NH_3 when cultured alone (Figs. 3C and 3D). Normal BDHSC cocultured with normal autologous hepatocytes and serum did express ureagenesis, although to a much lesser degree when compared to BDHSC exposed to cholestatic serum (Fig. 3E). Although macrophages can synthesize urea *in vitro*, they need L-arginine as a substrate, a substance that was not present in our culture media. Additionally, BDHSC stained negative with monoclonal antibody specific for rat macrophages (data not shown). Based on these data we concluded that BDHSC express ureagenesis *in vitro* and that they require yet undefined humoral factor(s), possibly contained in cholestatic sera, to differentiate into functioning hepatocytes ($P < 0.05$).

Further characterization of BDHSC was performed using double immunofluorescence and three-dimensional confocal microscopy. We demonstrated that the cells express hepatocyte-specific markers albumin and CAM5.2 (34) (Figs. 4A and 4B). Additionally, utilizing one step RT-PCR, we confirmed that the BDHSC shown on Figs. 4A and 4B express albumin and its transcription factor C/EBPα (Fig. 4C). Interestingly, after certain time in culture, the cells showed *de novo* HNF-1 expression while losing HNF-4 (Fig. 4C).

After culturing BDHSC for seven days, each dish contained several aggregates of cells, which morphologically resembled hepatocytes (Figs. 4D–4E). The hepatocyte ultrastructure was confirmed using transmission electron microscopy (Fig. 4F). While some BDHSC transformed into hepatocytes, the remaining cells did not change their morphology and underwent extensive proliferation. Cultures were initially established with 5×10^4 cells per dish. On day 7, expansion to 1.5–2.0 $\times$ 10^6 cells/dish represented five to six cell divisions, or about one cell cycle per 24 h. In control dishes of BDHSC that were cocultured with autologous hepatocytes obtained from normal animals, similar cellular aggregates and proliferative responses were not observed.

Finally, we examined whether BDHSC can differentiate into mature hepatocytes *in vivo*. To facilitate BDHSC engraftment and, possibly, proliferation, the cells were seeded into rat livers undergoing a mild rejection. Accordingly, D'Agouti female rat livers were harvested and transplanted across the strain in the female inbred Lewis rat recipients. Each transplanted liver was seeded via portal vein with male inbred Lewis rat BDHSC (5×10^4 cells/liver). Rats were then maintained on a daily subtherapeutic dose of cyclosporine A (0.25–0.5 mg/kg im). We found evidence that BDHSC integrated with hepatic cell plates and differentiated into mature hepatocytes (Fig. 5).

The observations made in this study demonstrate that adult BM (human, rat) contains a population of cells with stem cell properties and that after exposure to appropriate stimuli these cells can differentiate into functioning hepatocytes. The necessity of the interaction between hepatocytes and BDHSC, likely via a humoral factor, was demonstrated by the lack of ureagenesis when BDHSC were maintained in culture alone (Fig. 3D). These findings resemble, to certain degree, embryogenesis during which interaction of committed endodermal cells with mesenchymal components of the primitive liver appears to be critical for proper cell lineage development and differentiation. Early liver development coincides with the expression

of a subset of hepatocyte-enriched transcription factors like HNF-1 and HNF-4 (35, 36). These transcription factors are expressed prior to the emergence of committed liver cells and their expression, in particular the C/EBPα, is crucial in the determination and characterization of early liver cells. Hepatoblasts that express albumin, αFP, HNF-1 and HNF-4 represent the earliest committed liver cells in the developing embryo. In this study, BDHSC resembled phenotypically hepatoblasts of the fetal liver (Fig. 1). Although BDHSC did not express HNF-1 early on, following culture with a putative humoral factor(s) (possibly contained in cholestatic serum) they differentiated into hepatocytes and, possibly, cholangiocytes, as judged by the expression of αFP and CK19 as well as *de novo* expression of HNF-1.

In summary, this is the first report to isolate liver stem cells directly from the bone marrow (rat, human). Using β_2m and Thy-1 as cell markers, we found that blast like β_2m^-/Thy-1^+ cells exist in the BM and in injured (regenerating) and malignant livers in rats and humans. Since the cells expressed albumin and other hepatocyte-specific markers (HNF-1, HNF-4, C/EBPα, CK8, etc.), we named them bone marrow derived hepatocyte stem cells. Based on these findings, we have developed a two-step magnetic bead cell sorting procedure for the isolation and purification of these cells. In culture, BDHSC proliferated and upon appropriate stimulation (e.g., exposure to cholestatic serum), they differentiated into hepatocytes and synthesized urea. Additionally, we found that BDHSC have the capability to repopulate regenerating livers *in vivo.* These findings open new horizons in the field of liver stem cell research and suggest that the BM can be the source of autologous liver stem cells. If this technology could be transferred to the clinical setting, it might have a significant therapeutic impact on the care of patients with liver failure of various etiologies. Further studies are needed to elucidate the nature of the humoral signal(s) that activate these BDHSC *in vivo* and initiate their differentiation and migration to the injured liver.

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