In Focus

Defined Conditions for Development of Functional Hepatic Cells from Human Embryonic Stem Cells

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ABSTRACT

Human embryonic stem (hES) cells provide an important means to evaluate specific soluble and cell-bound stimuli that regulate development of specific cell lineages. Here, we examined specific cytokines and extracellular matrix (ECM) proteins that support differentiation of hES cells to hepatocytes. Tests of several different conditions determined that addition of fibroblast growth factor (FGF)-4 and hepatocyte growth factor in completely serum-free cultures of hES cell-derived embryoid bodies subsequently allowed to attach to type I collagen-coated dishes led to maximal differentiation into cells, not only with the morphologic and phenotypic characteristics of hepatocytes but also the functional characteristics. Expression of common hepatic transcription factors including HNF-3 β , HNF-1, and GATA-4 were all significantly induced under these conditions. Hepatocyte function was demonstrated by multiple complementary criteria: production of urea and albumin, phenobarbital-induced cytochrome P450 expression, and uptake of indocyanine green. These hES cell-derived hepatocytes will serve as a resource to understand normal human hepatocyte development and for applications such as cell replacement therapies and screening of pharmacologic drugs.

INTRODUCTION

LIVER FAILURE AS A RESULT of congenital and acquired diseases is directly responsible for over 40,000 deaths annually in the United States (1). Liver transplantation remains the most successful treatment for many cases of end-stage liver disease (2–4). However, the efficacy of liver transplantation is limited by the shortage of available donor organs, risk of rejection, infections, and other complications caused by the lifelong immunosuppression. As whole liver transplantation is most commonly performed to correct hepatocyte dysfunction (4), there has been considerable interest in hepatocyte

transplantation for the treatment of acute and chronic liver failure, as well as inherited metabolic disorders (5,6). Hepatocyte transplantation has several advantages over whole liver transplantation: lower morbidity, treatment of several recipients from a single organ, cryopreservation of cells (7), and potentially less immunogenicity (8). Unfortunately, hepatocyte transplantation remains experimental because this approach is limited by a paucity of donor organs and by a marked donor-to-donor variability in the quality of hepatocytes.

Hepatic stem cells and their progeny are considered one promising new approach to treat patients with end-stage liver diseases (9). Isolation of oval cells and other

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liver-specific progenitor cells in animal models commonly requires the *in vivo* use of toxins and other chemicals, thus precluding their use in the clinical setting (10). Others have immortalized primary hepatocytes using viral oncogenes to allow long-term hepatocyte expansion *in vitro*, although this often results in karyotypic changes (11,12). Consequently, these different cell therapies are not yet suitable for human clinical transplantation.

Human embryonic stem (hES) cells may serve as an ideal source of cells for hepatocyte transplantation. ES cells, derived from the inner cell mass of preimplantation blastocysts, can be cultured essentially indefinitely without senescence or evidence of differentiation (13,14). When placed in the proper environment, derivatives of all three embryonic germ layers—ectoderm, endoderm, and mesoderm—can be demonstrated (13,15). ES cells provide an optimal model to analyze both cellular and molecular events in the earliest stages of development. To date, human ES cells have been used to study differentiation into multiple cell lineages including endothelial cells (16), neurons (17), hematopoietic cells (18), and cardiomyocytes (19). A limited number of studies have evaluated development of hepatocytes or “hepatocyte-like cells” from hES cells. One used only nonspecific stimuli to derive “hepatocyte-like cells” and only limited phenotypic and functional data was described (20). Another study used more defined conditions, although the required characterization of the cells and conditions was limited (21). Here, we systematically analyzed specific cytokines (individually and in combination) and extracellular matrix (ECM) proteins to improve derivation and characterization of hepatocytes from hES cells. Importantly, we demonstrate hepatocyte development can occur efficiently in completely serum-free media with defined cytokines as a means to avoid the nonspecific effects of unknown serum proteins. Moreover, results presented thoroughly characterize the phenotype, gene expression, and function of hES cell-derived hepatocytes.

MATERIALS AND METHODS

Chemicals and cytokines

Basic fibroblast growth factor (bFGF), FGF-4, and hepatocyte growth factor (HGF) (R&D Systems, Minneapolis, Minnesota), Oncostatin M (OSM), acidic (a)FGF, and FGF7 (Research Diagnostics, Flanders, New Jersey), and sodium butyrate (Sigma, St. Louis, MO) were all reconstituted and used per manufacturers' instructions.

Antibodies

The antibodies (Abs) used were against HNF-3 β , HNF-1, asialoglycoprotein receptor I, and β -actin (Santa

Cruz Biotechnology), CK18 (clone CY-90, Sigma), and albumin (Bethyl Laboratory Montgomery, Texas). Secondary Abs used were anti-goat IgG-Cy-3, anti-goat IgG-FITC, anti-mouse IgG-Cy-3, anti-mouse IgG-FITC, anti-rabbit-FITC, anti-rabbit Cy-3 anti-goat IgG-Cy-5, anti-mouse IgG-Cy-5, and anti-rabbit Cy-3 (all from Jackson Immuno-Research Laboratories, West Grove, PA). Secondary Abs used for immunoblots were horseradish peroxidase (HRP)-linked donkey anti-mouse and HRP-linked donkey anti-rabbit (Amersham, Arlington Heights, IL) and HRP-linked donkey anti-goat (Sigma).

hES culture

The hES cell lines H1 and H9 were maintained as undifferentiated cells by co-culture with irradiated (7,000 rads) mouse embryonic fibroblast (MEF) cells in serum-free media as described (22). Cytogenetic analysis revealed that H9 cells were cytogenetically normal; however, some subclones of H1 cells displayed trisomy 12, as has been described elsewhere (23). Studies done with karyotypically abnormal H1 cells were repeated with cytogenetically normal H1 cells with identical results.

Embryoid body formation from hES cells

Embryoid bodies (EBs) were derived from intact hES cell colonies using dispase (Sigma) as previously described (24,25). Colonies formed into EBs when resuspended in Stemline serum-free medium (Sigma) or Dulbecco's modified Eagle medium (DMEM) (Invitrogen) and transferred to ultralow-binding six-well plates (Corning). The next day, medium was replaced with serum-free (SF) basal medium (Sigma) with or without addition of fetal bovine serum (FBS; Hyclone) or defined cytokines, and EBs allowed to develop for 7–14 days.

Hepatocyte differentiation

Day-7 EBs maintained in suspension were allowed to attach to Type I collagen (8 μ g/cm²) (26), fibronectin (50 μ g/cm²) (26), laminin (2 μ g/cm²) (26), Matrigel (35 μ g/cm²) (26), or an uncoated culture dish in either SF basal medium, SF basal medium supplements with indicated cytokines and 10% FBS-containing medium. Medium was changed every 2 days, and collected medium was flash frozen and kept at -80°C for future analysis.

Total RNA isolation and quantitative RT-PCR

RNA was extracted from a minimum of 1×10^5 hESCs, hEBs, hES-derived hepatocytes, MEFs, human hepatocytes, or HEPG2 cells. mRNA was reverse transcribed and cDNA was amplified as previously described (27). mRNA levels were normalized using β -actin or 18S

as housekeeping genes. mRNA from human adult liver RNA (Clontech Laboratories, Palo Alto, CA) was used as control when appropriate. Primers used for amplification have already been described (27).

Immunoblot analysis

Immunoblot analyses were performed as described (27). Abs to human albumin, cytokeratin (CK) 18, and asialoglycoprotein receptor I (100 ng/ml) were diluted in 2.5% milk blocking buffer. Goat anti- β -actin was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Secondary Abs were HRP-linked donkey anti-mouse and HRP-linked donkey anti-rabbit (Amersham, Arlington Heights, Illinois) and HRP-linked donkey anti-goat (Sigma). Enhanced chemiluminescence (ECL) was performed according to manufacturer's instructions (Amersham).

Immunofluorescence

hES-derived cells were fixed with 4% paraformaldehyde (Sigma) for 10 min followed by incubation with 100 mM glycine for 15 min for all samples except for samples that were examined for cytoskeletal proteins, which were fixed with methanol (Sigma) for 4–6 min at -20°C . For nuclear and cytoplasmic ligands, cells were permeabilized with 0.1 Triton-X 100 (Sigma) for 10 min. Slides were blocked with 1% serum of the secondary Ab for 1 h at room temperature. Slides were then incubated overnight with primary Ab. Slides were washed with phosphate-buffered saline (PBS) and labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), Cy3 or Cy5-coupled anti-mouse, anti-goat, or anti-rabbit IgG. Cells were examined by fluorescence microscopy using a Zeiss Axiovert microscope (Carl Zeiss Inc., Thornwood, New York). For quantification, a 32×32 grid with $40\text{-}\mu\text{m}$ spacing was placed onto the slide. ImageJ computer software was used to randomly select and count 1 cell from within each grid box, for a total of 1,089 cells per grid, were counted and quantified as positive or negative based on immunofluorescent staining of specific proteins (28). Two areas were analyzed per slide and a total of five slides were analyzed per experiment to assess percentage of cells that expressed indicated proteins.

Urea assay

Urea concentrations were determined by colorimetric assay (Sigma) as previously described (27).

Albumin dot blots

Conditioned media from hES cells, HEPG2 cells, and ES-derived hepatocytes were filtered and frozen until

analysis. A total $5\text{ }\mu\text{l}$ of conditioned media was placed on Immuno-Blot PVDF membrane along with a control curve of human albumin. The membrane was blocked with milk for 1 h and incubated overnight with an HRP-conjugated human-specific Ab against albumin (Bethyl Laboratory and analyzed by ECL). Specificity of the Ab was initially determined by immunoblot.

Pentoxifyresorufin (PROD) assay

Measurement of cytochrome p450 activity in the presence or absence of pentobarbital was done as previously described (27).

Indocyanine green

A total of 25 mg of Indocyanine Green (ICG) (Sigma-Aldrich) was dissolved in 5 ml of ethanol and added to 20 ml of medium (1 mg/ml final concentration). The ICG solution was added to the cell cultures for 30 min at 37°C . After rinsing three times with PBS, the cellular uptake of ICG was examined with a stereomicroscope. After examination, PBS was removed and medium replaced. ICG was completely eliminated from the cells 6 h later.

RESULTS

Defined cytokines regulate hES cell differentiation to hepatic lineage

The hES cell lines H1 and H9 were used to evaluate conditions to support endoderm and hepatocyte development. EB formation was used as a means to promote initial endoderm differentiation. In a first series of studies, we used quantitative RT-PCR for genes known to be expressed in endoderm and hepatocytes: α -fetoprotein (AFP), CK19, HNF-3 β , and GATA-4 (29–31). Because undifferentiated hES cells express only low levels of these genes, we used this genetic analysis to allow us to evaluate most readily specific soluble proteins and extracellular matrices that best support hepatic development (Tables 1–4).

Hepatocyte differentiation from mouse (m) ES cells can be induced by serum alone (32–34). Therefore, we initially tested the ability of serum to enhance hepatocyte differentiation when added throughout a 21-day method of hES cell differentiation. This protocol allowed the hES cells to differentiate in suspension as EBs for 7 days, followed by attachment to type I collagen as extracellular matrix support for 14 days. EB development did not efficiently occur without serum during the first 7 days (data not shown) and consequently FBS was added to basal medium for the first 7 days. Under these conditions, transcripts of multiple hepatic genes were markedly increased

TABLE 1. SERUM INFLUENCES HEPATOCYTE TRANSCRIPT EXPRESSION

	<i>FBS→FBS</i>	<i>FBS→SF</i>
	<i>Relative expression ES cells</i>	<i>Relative expression ES cells</i>
AFP	34.21 ± 5.18 [•]	30.1 ± 1.81 [•]
Albumin	7.06 ± 1.09 ^{#,^}	8.03 ± 0.60 [#]
CK19	19.69 ± 3.93 [#]	17.32 ± 1.51 [#]
CK18	1.51 ± 0.36 [#]	1.95 ± 0.32 [#]
HNF3 β	1.04 ± 0.10 [•]	0.98 ± 0.14 [•]
HNF-1	6.2 ± 1.62 ^{#,^}	9.3 ± 0.837 [#]
GATA-4	1 ± 0.26 ^{*•}	1.1 ± 0.23 [•]

hES cells were differentiated for 7 days as EBs in FBS, and then plated for 14 days on type I collagen in basal medium supplemented with either no serum (SF) or FBS. Quantitative RT-PCR was performed for liver-specific transcripts, which were normalized to human actin or 18S as housekeeping genes. The expression in differentiated cells was compared to levels seen in either undifferentiated hES cells ([•] values) and for albumin, CK19, CK18, and HNF-1 relative to hEBs (day 2, [#] values), because they were not expressed in undifferentiated hES cells. Values are means of four RT-PCR analyses. Carat ([^]) values differ by a statistically significant level ($p < 0.05$) when comparing samples supplemented with FBS → FBS versus FBS → SF conditions.

(Table 1). Results were similar whether FBS was included for the entire 21 days of differentiation, or just the first 7 days prior to transfer to SF conditions for the subsequent 14 days (Table 1), although albumin and HNF-1 mRNA levels were increased when FBS was only used for the first 7 days of differentiation (Table 1).

We next wished to evaluate specific cytokines that may support hepatocyte development from hES cells. Cytokine signals that govern initial endoderm specification during gastrulation are not well understood, although those involved with differentiation of endoderm to liver are better characterized. FGF-4 is known to induce development of primitive and visceral endoderm (35). Transplantation of endoderm near cardiac mesoderm leads to hepatocyte differentiation and maturation, at least in part due to secreted factors such as aFGF and bFGF from cardiac mesoderm (30). In addition, bone morphogenetic protein-2 (BMP2) and BMP4 produced in the septum transversum (29), and oncostatin M (OSM) from hematopoietic progenitors (36) are required for hepatocyte differentiation and maturation, whereas HGF has been implicated indirectly in liver development and proliferation (37). Consequently, we tested the inductive role of these cytokines in formation of hES cell-derived hepatocytes. We also tested a nonspecific inducer of hepatocyte maturation, sodium butyrate. All studies were done using EBs grown in suspension with FBS for 7 days and then replated on type I collagen for an additional 14 days.

SF medium plus the specified cytokines were used throughout the last 14 days of culture. Addition of varying concentrations (0.01–10 mmol/L) of sodium butyrate alone did not lead to a significant increase in endoderm or hepatocyte transcript levels (data not shown). bFGF did not lead to a significant increase in endoderm or hepatocyte transcript levels compared to that of the serum-containing conditions (Table 2). Although aFGF and OSM did have a significant increase in two to three markers, the extent of this increased expression was lower than that of HGF or FGF-4. FGF4 alone induced expression of AFP, albumin, CK18, HNF-3 β , and HNF-1 mRNAs by 115-, 86-, 26-, 4-, and 6-fold, respectively (Table 2). HGF alone induced AFP, albumin, CK18, HNF-3 β , and HNF-1 expression by 186-, 86-, 26-, 4-, and 6-fold, respectively. Combined cultures with either aFGF, bFGF, or OSM added to either FGF-4 or HGF did not significantly increase hepatic gene expression over FGF-4 or HGF alone (data not shown). Hepatocyte development was most pronounced by treatment with a combination of FGF-4 plus HGF, which increases expression of these transcripts by 326-, 218-, 29-, 10-, and 7.9-fold, respectively (Table 2). The expression of endoderm or hepatocyte transcript levels compared well to that of HEPG2 and hepatocytes (Table 3). In addition, this expression was shown to be specifically induced by the combination of FGF-4/HGF plated on type I collagen as statistically significant increases in all marker expression was seen when compared to cells plated without ECM or in the absence of FGF-4 and HGF (Table 3). The combination of FGF-4/HGF and type I collagen increased the expression of various cytochrome P450 transcripts (transcripts typically expressed or by mature hepatocytes) by 104- to 526-fold (Table 3).

Specific ECM proteins influence hES cell differentiation to hepatic lineage

Early hepatic development is accompanied by laminin production and deposition (38). As hepatocyte precursors mature, they degrade and remove laminin and replace it with type I collagen (39). Laminin and collagen type I differ in their ability to bind cytokines, engage different adhesion receptors, and have differing physical stress properties (35,36). Consequently, we examined the effect different ECMs have on hepatocyte commitment (Table 4). When EBs were cultured on fibronectin or laminin, no significant upregulation of hepatocyte-specific transcripts were seen as compared to when no ECM was used at all. Culture of EBs on Matrigel induced a 157-, 5-, and 4-fold increase in mRNA for albumin, CK18, and HNF-3 β relative to hES cells or day-2 EBs. Likewise, replating EBs on type I collagen induced a 298-, 241-, 76-, 34-, 14-, 10-, and 6-fold increase, respectively, in AFP, albumin, CK19, CK18,

TABLE 2. SF MEDIUM PLUS CYTOKINES INCREASES HEPATOCYTE TRANSCRIPT EXPRESSION

	<i>aFGF</i>	<i>bFGF</i>	<i>OSM</i>	<i>FGF4</i>	<i>HGF</i>	<i>FGF4 + HGF</i>	<i>FBS→FBS</i>	<i>FBS→SF</i>
	<i>Relative expression human ES</i>	<i>Relative expression human ES</i>	<i>Relative expression human ES</i>	<i>Relative expression human ES</i>	<i>Relative expression human ES</i>	<i>Relative expression human ES</i>	<i>Relative expression human ES</i>	<i>Relative expression human ES</i>
AFP	38.6 ± 6.3* [●]	13.6 ± 5.7 [●]	19 ± 4.2 [●]	115 ± 91.2 [●]	186 ± 74.3* [●]	326 ± 46* [●]	31.68 ± 3.12 [●]	29.6 ± 1.73 [●]
Albumin	16.9 ± 8.2* [●]	0.22 ± 0.06 [#]	2.9 ± 0.5 [#]	86.5 ± 48.2* [●]	86 ± 14.7* [●]	218 ± 41* [●]	8.16 ± 2.23 [#]	11.07 ± 1.54 [#]
CK18	8.3 ± 3.8* [●]	1.4 ± 0.2 [#]	3.4 ± 0.74* [●]	26.3 ± 4.8* [●]	26.3 ± 6.1* [●]	29.8 ± 5.19* [●]	1.64 ± 0.28 [#]	2.3 ± 0.42 [#]
HNF3 β	4.9 ± 1.5* [●]	0.34 ± 0.2 [●]	2.1 ± 0.81* [●]	4.6 ± 3.2* [●]	3.6 ± 1.3* [●]	10.4 ± 1.54* [●]	0.96 ± 0.27 [●]	0.86 ± 0.16 [●]
HNF-1	2.3 ± 0.3 [#]	0.8 ± 0.7 [#]	1.5 ± 0.32 [#]	6.5 ± 1.8 [#]	7.0 ± 1.18 [#]	7.9 ± 1.01 [#]	5.7 ± 1.63 [#]	9.7 ± 0.89 [#]

hES cells were differentiated for 7 days as EBs in serum and then plated for 14 days on collagen type I in SF medium supplemented with FBS, aFGF, bFGF, OSM, HGF, and FGF4 alone or FGF-4 and HGF in combination. Quantitative RT-PCR was performed as described in Table 1. The expression in differentiated cells was compared to levels seen in undifferentiated hES cells ([●] values) and for albumin, CK18, and HNF-1 relative to hEBs (day 2, [#] values), because they were not expressed in undifferentiated hES cells. Values are means and standard deviations of 10 RT-PCR analyses of independent experiments. Starred (*) values are increased and differ by a statistically significant level ($p < 0.05$) when compared to FBS/SF samples.

TABLE 3. FGF-4 AND HGF PROMOTE MATURE HEPATOCYTE MARKER EXPRESSION

	Type I collagen FGF-4+HGF	Type I collagen FGF-4+HGF	Type I collagen FGF-4+HGF	Type I collagen SH→SF	No ECM FGF-4+HGF
	Relative expression human ES	Relative expression HEPG2	Relative expression human hepatocytes	Relative expression human ES	Relative expression human ES
AFP	274 ± 89*, ^{\$}	2.13 ± 1.8	1,986 ± 1321	29.80 ± 2.33	31.8 ± 4.03
Albumin	236 ± 52*, ^{*,^{\$}}	0.10 ± 0.03	0.01 ± 0.0022	13.9 ± 1.18 [#]	10.5 ± 1.52 [#]
CK19	64.8 ± 13.7*, ^{*,^{\$}}	11.26 ± 3.9	NT	21.97 ± 4.0 [#]	19.3 ± 3.60 [#]
CK18	37.1 ± 8.2*, ^{*,^{\$}}	5.24 ± 1.6	1.3 ± 0.3	2.9 ± 0.77 [#]	2.17 ± 0.19 [#]
HNF3β	12.7 ± 4.13*, ^{\$}	3.66 ± 2.3	0.07 ± 0.03	1.4 ± 0.41	0.99 ± 0.10
HNF-1	11.4 ± 1.42*, ^{*,^{\$}}	0.436 ± 0.2	0.02 ± 0.01	9.0 ± 1.4 [#]	4.9 ± 1.47 [#]
GATA-4	8.3 ± 2.8	7.6 ± 1.1	0.03 ± 0.01	NT	NT
CYP1A1	218 ± 34 [^] , ^{*,^{\$}}	0.83 ± 0.21	0.15 ± 0.03	1.26 ± 0.40 [^]	1.3 ± 0.63 [^]
CYP1A2	113 ± 19 [^] , ^{*,^{\$}}	2.15 ± 0.38	0.08 ± 0.02	1.3 ± 0.27 [^]	1.8 ± 0.45 [^]
CYP2B6	526 ± 37 [^] , ^{*,^{\$}}	1.85 ± 1.3	0.05 ± 0.01	12 ± 4.3 [^]	18 ± 7.4 [^]
CYP3A4	104 ± 21 [^] , ^{*,^{\$}}	1.36 ± 0.9	0.07 ± 0.02	4.5 ± 1.3 [^]	3.2 ± 1.9 [^]
ASPR1	—	0.13 ± 0.3	0.03 ± 0.01	—	—

hES cells were differentiated for 7 days as EBs in FBS and then plated for 14 days on collagen type I in SF medium supplemented with HGF and FGF4 alone or in combination. Quantitative RT-PCR was performed as described in Table 1. The expression in differentiated cells was compared to hES cells. Values are means and standard deviations of 10 RT-PCR analyses of independent experiments. The expression in differentiated cells was compared to levels seen in undifferentiated hES cells and for albumin, CK19, CK18, HNF-1, CYP1A1, CYP1A2, CYP2b6, and CYP3A4 relative to hEBs [day 2 indicated by #, day 7 indicated by (^)] because they were not expressed in undifferentiated hES cells. Starred (*) values are increased and differ by a statistically significant level ($p < 0.05$) when compared to FBS/SF samples plated on type I collagen. (\$) values are increased and differ by a statistically significant level ($p < 0.05$) when compared to FGF-4/HGF cultured samples plated on no ECM. NT, Not tested.

HNF-3β, HNF-1, and GATA-4 transcripts relative to hES cells or day-2 EBs. Therefore, only type I collagen and Matrigel increased hepatocyte development, and comparatively the increases with type I collagen are greater and encompass more of the tested genes.

Immunofluorescence analysis of human ES cell-derived hepatic cells

To characterize these putative hES cell-derived hepatic cells by immunofluorescent microscopy better, EBs were

TABLE 4. ECM INFLUENCES HEPATOCYTE TRANSCRIPT EXPRESSION

	Type I collagen	Fibronectin	Laminin	Matrigel	No ECM
	Relative expression human ES	Relative expression human ES	Relative expression human ES	Relative expression human ES	Relative expression human ES
AFP	298 ± 126 [•]	NT	NT	NT	32.4 ± 2.91 [•]
Albumin	241 ± 59*, [*]	0 [#]	2.25 ± 1.6 [#]	157.5 ± 43*, [*]	9.2 ± 1.78 [#]
CK19	76.5 ± 36.0*, [*]	7.82 ± 2.96 [#]	1.13 ± 0.37 [#]	1.13 ± 0.86 [#]	18.6 ± 2.43 [#]
CK18	34.3 ± 9.4*, [*]	0.84 ± 0.91 [#]	1.39 ± 0.89 [#]	5.13 ± 1.38*, [*]	1.93 ± 0.34 [#]
HNF3β	14.7 ± 2.38*, [*]	0.84 ± 0.35 [•]	1.23 ± 0.73 [•]	4.24 ± 1.6*, [*]	1.15 ± 0.26 [•]
HNF-1	10.6 ± 1.93*, [*]	0.01 ± 0.02 [#]	0.18 ± 0.11 [#]	0.02 ± 0.02 [#]	5.40 ± 1.37 [#]
GATA-4	6.72 ± 1.36*, [•]	NT	NT	NT	1.39 ± 0.48 [•]

hES cells were differentiated for 7 days as EBs in FBS and then plated for 14 days in SF medium supplemented with HGF and FGF4, on different ECMs. Quantitative RT-PCR was performed as described in Table 1. The expression in differentiated cells was compared to levels seen in undifferentiated hES cells (• values) and for albumin, CK19, CK18, and HNF-1 relative to hEBs (day 2, # values) because they were not expressed in undifferentiated hES cells. Values for type I collagen, fibronectin, laminin, and Matrigel are means and standard deviations of six analyses of independent experiments. Starred (*) values are increased and differ by a statistically significant level ($p < 0.05$) when compared to FBS/SF samples cultured on no extracellular matrix. NT, Not tested.

grown in suspension for 7 days and then cultured on collagen type I for 14 days in SF media plus FGF4/HGF. After these 21 days (d21), outgrowths around the initial area where the EBs attached were dissected out, made into a single-cell suspension, and replated onto type I collagen. Cells were then labeled with antibodies against HNF-3 β , CK18, albumin, and HNF-1 and analyzed by conventional fluorescent microscopy. Differentiated hES cells derived from these regions had varying morphology, but most often had an epithelioid appearance (Fig. 1). Undifferentiated hES cells did not express albumin, HNF-3 β , or HNF-1 (Fig. 1D). In contrast, approximately $2 \pm 0.4\%$ of total d21 hES-derived cells expressed albumin, CK18, HNF-3 β , and HNF-1 (Fig. 1) using a computer-assisted quantification system (27). Expression of these proteins was also tested by immunoblot analysis (Fig. 2). Undifferentiated hES cells did not express albumin or CK18 protein (data not shown) while d21 progeny expressed albumin, CK18, and HNF-1 (Fig. 2A–C). We also evaluated expression of asialoglycoprotein receptor 1 (ASPGR1), a receptor for terminal galactose sugars on proteins. Expression of ASPGR1 is both abundant and specific to mature hepatocytes (Fig. 2D) (40). Undifferentiated hES cells did not express ASPGR1 (data not shown) while day-21 progeny expressed ASPGR1 (Fig. 2D).

Differentiated hES cell-derived cells acquire functional activity typical of hepatocytes

We used four different complementary assays to determine whether populations of cells with morphologic and phenotypic characteristics of hepatocytes also had functional attributes of hepatocytes. As expected, undifferentiated hES cells did not produce urea. Low levels of urea production were detectable (Fig. 3) 14 days after initiation of EB formation. Within 14 days after replating day-7 EBs on collagen type I (21 days total in culture), urea production increased from 0.1 mg/dl to 4.23 mg/dl. Levels of urea produced by hES-derived hepatocytes were similar to those in monolayer cultures of primary rat hepatocytes (3.6–5.7 mg/dl) and greater than that of HEPG2 cells (data not shown). Urea production was also detected in cells differentiated on Matrigel and laminin, but not fibronectin (data not shown).

Undifferentiated hES cells and hEBs grown in suspension for 14 days did not secrete albumin. Following culture on collagen type I in the presence of FGF-4 and HGF, these cells produced albumin in a time-dependent manner (Fig. 3). As was seen for urea production, only hES cells differentiated on Type I collagen and Matrigel, but not fibronectin or laminin, produced albumin. Levels of albumin produced by hES-derived cells were equivalent

FIG. 1. Phenotype of hES cell-derived hepatocytes. Differentiated hES cells were stained for immunohistochemical localization of albumin-FITC and HNF-1-Cy3 (A), CK18-FITC (B), albumin-FITC and HNF-3 β -Cy3 (C), and albumin-FITC and HNF-1-Cy3 (D) on undifferentiated hES cells used as a negative control. Scale bar, 20 μ m.

FIG. 2. Immunoblot analysis of hES-derived hepatocytes. Lysates (800 ng) of cells were separated by SDS-PAGE, transferred to Immuno-Blot PVDF membrane, and incubated with Abs against HNF-1 (A), CK18 (B), albumin (C), ASPGR1 (D), and β -actin (E). Human hepatocytes were used as positive control.

lent to those seen in monolayer cultures of primary rat hepatocytes (6.4–14 μ g/ml per 3 days).

Fresh rat and human hepatocytes readily uptake the organic anion ICG, while undifferentiated human ES cells did not (data not shown). Fourteen days after replating EBs on type 1 collagen (21 days total in culture), we could detect ICG-positive clusters. These clusters were typically near the initial areas where EBs attached to the matrix and near areas of beating cells (presumed cardiomyocytes) (Fig. 4). This suggests that cardiac tissue present in these beating areas may contribute to hepatocyte development in vitro.

Finally, we assessed cytochrome P450 (CYP) activity in wells with human ES-derived hepatocytes using the PROD assay. No PROD activity was seen in undifferentiated hES cells (Fig. 5) or early EBs (data not shown). However, 21 days following culture (7 days as EB and 14 days cultured on Type I collagen) with FGF-4 and HGF, aggregates had PROD activity (Fig. 5), similar to that of primary rat hepatocyte aggregates on Matrigel. Many cell types have CYP activity, but CYP activity up-regulation by phenobarbital occurs solely in hepatocytes because of the presence of CYP2B6. Therefore, we also tested CYP activity in the presence or absence of phenobarbital. When aggregates were cultured for 96 h with phenobarbital, a 94% increase ($p < 0.05$) in PROD activity was seen, suggesting the presence of functional hepatocyte-specific CYP2B6 in hES cell-derived hepatocytes.

DISCUSSION

These studies aim to define specific conditions (i.e., ECM and soluble factors) that support hES cell development into cells with hepatocyte phenotype and function. Of note, several studies that examine development of cells of hepatic lineage from varying stem cell precursor populations describe the progeny as either hepatocytes or “hepatocyte-like” cells (10,20,34,38,41–44). On the basis of the multiple criteria outlined, we chose to refer to these cells differentiated from hES cells as hepatocytes, although acknowledging these cells may not be equivalent to freshly isolated hepatocytes in all respects. EBs derived from hES cells and cultured under hepatocyte differentiation conditions expressed primitive and mature endodermal and hepatocyte markers. We used quantitative RT-PCR as a screening tool to determine which ECM and cytokine combinations resulted in maximal up-regulation of genes typically expressed in hepatocytes. We tested the effect of aFGF, bFGF, FGF-4, HGF, and OSM, all thought to be important for hepatocyte differentiation during embryological development (45). Only FGF-4 and HGF when used alone and when used in combination significantly promoted hepatocyte differentiation. Surprisingly, aFGF and bFGF, thought to be important in hepatocyte specification of endoderm by cardiac mesoderm, did not significantly promote hepatocyte differentiation in this hES cell-based system. Because ICG staining was detected almost exclusively near areas of beating cells, cardiomyocytes present in the differentiating cultures may contribute to hepatocyte development, similar to the inductive process that occurs during normal mammalian ontogeny (30,33). Therefore, it is possible that aFGF and bFGF could be produced endogenously in these cultures at a level that precludes the beneficial effect of addition of these soluble factors.

Other signals known to influence differentiation to hepatocytes include ECM-derived signals. Because there are many ECMs present in liver development, we tested several matrices: type I collagen, fibronectin, laminin, and Matrigel. We found that type I collagen promoted the greatest degree of hepatocyte differentiation, as evaluated by quantitative RT-PCR. This result is consistent with the findings that differentiation of mES cells to the hepatocyte lineage occurs best on type I collagen (42).

Quantitative RT-PCR analysis demonstrate that hES cells when cultured on Type I collagen in the presence of FGF-4 and HGF differentiated into hepatocytes as evidenced by endoderm and hepatocyte marker expression on the same order of magnitude as that of HEPG2 and human hepatocytes. hES-derived hepatocytes expressed mature markers of hepatocyte expression such that of CYP2B6, CY3A4, and ASPGR1.

These quantitative RT-PCR analyses represent studies of an entire population of cells present at the different

HEPATOCYTES DERIVED FROM HUMAN ES CELLS

FIG. 3. hES cell-derived hepatocytes produce urea and albumin. Urea (**A**) and albumin (**B**) production by undifferentiated hES cells (day 0) or hEBs cultured on Type I collagen in SF medium with HGF and FGF4 (day 7 through 21). Studies were an analysis of nine independent experiments and bars represent standard deviation.

stages of differentiation. Therefore, immunofluorescence microscopy and functional studies were done to define functional hepatocyte attributes of the differentiated cells further at a single-cell level. Approximately 2% of the differentiated hES cell progeny labeled positive for albumin and CK18. We also used four independent functional assays to prove functional hepatocyte attributes of the differentiated cells. We demonstrate that hES-derived hepatocytes produce urea and albumin, contain pentobarbital-inducible CYP activity, and could take up ICG. Although urea production is characteristic of hepatocyte activity, kidney tubular epithelium can also produce urea. In contrast, albumin production is a relatively specific test for the presence and activity of hepatocytes. CYP, although found in hepatocytes, is also present in many other cell types. However, CYP2B6 activity in humans, especially its ability to be up-regulated by pentobarbital is considered hepatocyte specific (46). Presence of this form of CYP was shown by RT-PCR (Table 4). Together these functional tests suggest strongly that the cells generated on type 1 collagen in HGF- and FGF4-containing medium are of the hepatocyte lineage.

Several reports have described hepatocyte differentiation from mES cells (32–34). Hamazaki et al demonstrated that mES cells differentiate into hepatocyte-like cells on the basis of RT-PCR evaluation of hepatocyte markers (42). However, their study did not examine whether mRNA expression coincided with protein expression of these markers, or whether these cells had functional activity indicative of hepatocytes. Yin et al. used an AFP promoter-driven green fluorescent protein (GFP) construct to select AFP⁺ cells from EBs (47). Sorted cells engrafted in vivo and corrected apolipoprotein A (ApoE) and haptoglobin deficiency in ApoE^{-/-} or haptoglobin^{-/-} mice, suggesting that they had matured into functional hepatocytes in vivo. Another recent study demonstrated the ability of mouse ES cell-derived hepatocytes to engraft in a cirrhosis model (48). Although none of these studies examined hepatocyte function, Ishizaka et al. demonstrated urea and triacylglycerol production in HNF-3 β -transfected mES cells (43), while Yamada et al. showed ICG uptake in mES-derived hepatocytes (41).

In contrast, few studies have been published describing endoderm and hepatocyte differentiation from

hES cells. Rambhatla et al. used a combination of FBS and sodium butyrate to induce hepatocyte differentiation (20), but did not delineate specific cytokine and ECM conditions that support hepatocyte differentiation. Furthermore, analysis of these hepatocyte-like cells was limited to detection of liver-specific transcripts and proteins with little evidence of functional properties. In another study, Schuldiner et al. found treatment with NGF and HGF induced liver-specific transcript expression (49). Again, no further phenotypic or functional characterization was demonstrated. Another recent study did evaluate specific conditions to support hepatocyte development from hES cells, although FBS was used in all conditions (48). Here, we chose to examine specific cytokines and growth factors in completely serum-free media, to avoid variations between serum lots and the potential effects of undefined serum protein. Moreover, the ability to support hepatocyte development in a serum-free system may allow easier translation to clinical therapies by avoiding exposure of hES cells to known and unknown xenogenic proteins. Indeed, some serum proteins may lead to greater immunogenicity of hES cells (50).

Previously, we have published studies in which we differentiated postnatal bone marrow-derived multipotent adult progenitor cells (MAPC) into hepatocyte-like cells (27,51,52). This now allows us to compare and contrast hepatocyte differentiation from hES cells and hMAPC. There are several differences in the differentiation to the hepatocyte lineage between the two cell populations. hES cell differentiation was most readily accomplished by formation of three-dimensional EBs prior to culture on an ECM-coated dish, whereas MAPC differentiation was induced by immediate plating directly onto ECM-coated plates. Moreover, the ECM that optimally supported differentiation to hepatic cells differs: for hES cells, type I collagen, and to a lesser extent, Matrigel, but not fibronectin, optimally supported hepatocyte differentiation. In contrast, MAPC differentiation occurred most extensively on Matrigel and fibronectin, but not collagen type I. Despite these differences, the growth factors (FGF4 and HGF) and SF conditions needed to induce hepatocyte differentiation were remarkably similar for both cell types. hES and hMAPC differentiated progeny had similar morphologic, phenotypic, and functional charac-

teristics of hepatocytes. Because the fraction of cells with hepatocyte features after 28 days of differentiation was greater when cultures were initiated with MAPC than after 21 days of differentiation ES cells, differentiation of MAPCs may occur in a less heterogeneous manner, whereas hES cells may undergo stochastic differentiation into different lineages when cultured as EBs. Because we have demonstrated that altering external conditions can increase the degree of differentiation to hepatic cells, we speculate that further optimization of differentiation conditions for hES cells that would circumvent the need for EB formation may yield increased directed differentiation, as has been shown for mES cell differentiation to neuroectoderm (53).

The ability to derive human hepatocytes from ES cells will now enable preclinical testing in animal models of hepatocyte and liver dysfunction. If successful replacement is shown, such ES-derived hepatocytes may be useful in future clinical trials of liver cell transplantation *in vivo* or *in ex vivo* therapy as part of a bioartificial liver (54). This extracorporeal therapy could be particularly advantageous, because immune barriers would be less problematic. Finally, the ability to characterize cell-bound and soluble stimuli that best support development of hepatocytes from early precursor cells will allow better understanding of normal human hepatic development. For example, mesoendoderm, a common precursor for both mesoderm and endoderm in lower organisms, has recently been suggested by studies of murine ES cell development (55), although it is not known whether such a progenitor exists for humans. A more comprehensive appreciation of basic human developmental biology can be applied to further optimize cell replacement resources and methods to regenerate endogenous hepatocyte function.

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FIG. 4. Indocyanine Green stains outgrowth from EBs. Hepatic cells derived from 7-day-old hEBs stained for ICG after attachment to type I collagen. Cells were allowed to grow for an additional 4 days (**A,B**), or 14 days (**C,D**) after attachment to type I collagen.

FIG. 5. PROD assay on hES cell-derived hepatocytes. hES-cell derived hepatocytes were plated on Matrigel and were allowed to aggregate in the presence or absence of 1 mM phenobarbital for 4 days. A PROD assay was done as described in Materials and Methods. (**A** and **D**) Cells with no pentoxifyresorufin. (**B** and **E**) Cells incubated with pentoxifyresorufin. (**C** and **F**) Cells incubated with pentoxifyresorufin and pentobarbital. Representative examples of three studies are shown.

FIG. 4.

Human ES cell derived hepatocytes

Rat Hepatocytes

FIG. 5.

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