American Journal of Pathology, Vol. 150, No. 2, February 1997
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Selective Bipotential Differentiation of Mouse Embryonic Hepatoblasts *In Vitro*

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A line of hepatic endoderm cells, hepatoblast cell line 3 (HBC-3), was derived from the liver diverticulum of the mouse on day 9.5 of gestation by culture on a mitomycin C treated STON$^+$ feeder layer in a hepatoblast culture medium consisting of Dulbecco's modified Eagle's medium, nonessential amino acids, fetal calf serum, and β-mercaptoethanol. This line, HBC-3, stains positively for α-fetoprotein, albumin, and cytokeratin 14 (CK-14), protein markers expressed by the embryonic liver diverticulum, indicating that HBC-3 cells retain an undifferentiated hepatoblast phenotype. HBC-3 cells acquire hepatocyte-like ultrastructural characteristics, including bile canaliculi, peroxisomes, and glycogen granules, when maintained in culture for 3 weeks without passage. Treatment with dimethylsulfoxide or sodium butyrate induces a rapid hepatocytic differentiation. The cells cease to express α-fetoprotein and CK-14, maintain albumin expression, and become positive for glucose-6-phosphatase activity (a profile consistent with differentiation along the hepatocyte lineage). On Matrigel, HBC-3 cells form elaborate ductular structures, which are positive for γ-glutamyl transpeptidase and CK-14 and CK-19 and do not express detectable amounts of albumin, a phenotypic change consistent with differentiation along the bile ductular lineage. Thus, HBC-3 cells behave in culture as bipotential hepatoblasts and provide a model system to identify factors that regulate bipotential differentiation in the liver. (Am J Pathol 1997, 150:591–602)

The cells that give rise to the mammalian liver are first recognizable as a thickening of the ventral foregut in the region that is in contact with the precardiac mesoderm, at approximately 8.5 to 9 days of development in the mouse.[1] The cells of this region of the gut epithelium proliferate to form the liver diverticulum. At approximately 9.5 days of gestation, cells of the liver diverticulum begin to migrate from the gut epithelium into the surrounding mesoderm, the septum transversum. Classically, the cells that have migrated from the diverticulum into the septum transversum are called hepatoblasts, to indicate that these cells have been determined along the hepatic epithelial cell lineage. That is, this population of cells represents the progenitor cells from which mature hepatocytes and bile ductular epithelial cells are derived through a series of as yet undefined differentiative events. Hepatoblasts express α-fetoprotein (AFP) and albumin,[2,3] whereas the remaining cells of the gut epithelium do not acquire albumin expression at this early point of development.

Recent work with explant cultures of foregut endoderm has suggested that specification of the hepatic lineage may occur as a result of the interaction of the foregut endoderm and adjacent cardiac mesenchyme prior to migration of endodermal cells into the septum transversum, because a low level of albumin expression can be detected in foregut endoderm explants that are accompanied by adherent cardiac mesoderm.[4] Experiments using explants and primary cultures of hepatoblasts isolated from the liver at later stages of development have indicated that this committed precursor population is capable of at least bipotential differentiation.[5] However, the molecular and cellular events that lead to differentiation of hepatoblasts into mature hepatocytes or bile ductular cells are not yet clearly defined because of the lack of clonal cell lines that possess the characteristics of hepatoblasts.

Supported by an American Liver Foundation Liver Scholar Award (to LER) and by grant 5P30DK41296 from the National Institutes of Health.

Accepted for publication October 19 1996.

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The adult liver is capable of significant regeneration, suggesting that there is a population of cells residing in the adult liver that has the capacity to behave as progenitor cells. The identity of this population within the liver has been a subject of considerable debate. In regeneration after partial hepatectomy, quiescent, apparently terminally differentiated, hepatocytes retain the capacity to reenter the cell cycle and to proliferate to restore liver mass.[6,7] The extensive proliferative capacity of hepatocytes has been demonstrated by transplanting genetically marked hepatocytes into regenerating livers.[8] Thus, in these experimental paradigms, as well as in unmanipulated normal liver, hepatocytes exhibit a unipotential capacity to proliferate *in vivo*. Recent work, however, has indicated that hepatocytes that have been stimulated to proliferate in culture can acquire the capacity to give rise to both hepatocytes and ductular structures *in vitro*.[9]

After viral or chemical injury, in which hepatocyte proliferation is inhibited, a population of facultative stem cells is found in the liver.[10–15] These cells were originally described as "oval cells" because of their large oval nuclei and scant cytoplasm.[16] These cells exhibit multipotent differentiation capacity, because they can give rise to pancreatic acini and intestinal epithelium as well as hepatocytes and bile ducts[17–19] and can differentiate into hepatocytes after transplant to the liver.[20]

Although liver epithelial cells and, in some circumstances, hepatocytes isolated from the adult liver have characteristics consistent with a hepatoblast phenotype, there has been no point of reference with which to compare these cells, because until now embryonic hepatoblast cell lines have not been available. In this report, we present the isolation and characterization of a cell line established from mouse liver diverticulum microdissected on day 9.5 of gestation. This line, hepatoblast cell line 3 (HBC-3), can be maintained as an undifferentiated hepatoblast cell line, which can be induced to undergo bipotential differentiation into hepatocyte- or bile ductular epithelia-like cells, when treated with two chemical inducers of differentiation or when provided with an altered culture substratum. Thus, HBC-3 cells represent the first candidate hepatoblast cell system in which to study molecular and cellular aspects of hepatic epithelial cell differentiation.

Materials and Methods

The following procedure was used to isolate hepatic endodermal cells (hepatoblasts) from mouse embryos on day 9.5 of gestation. Pregnant female C57Bl6/DBA F_1 female mice were sacrificed on day 9.5 of gestation by cervical dislocation. Gravid uteri were dissected into 1 × phosphate-buffered saline (PBS; 130 mmol/L NaCl, 7 mmol/L Na_2HPO_4, and 3 mmol/L $NaH_2PO_4 \cdot H_2O$, pH 7.2). Individual 9.5-day-old embryos were dissected free of decidua and extra embryonic membranes in 1 × PBS. Liver diverticula (Figure 1A) from approximately 40 embryos were microdissected. Microdissected diverticula were transferred to a dish of hepatoblast culture medium (HBM; Dulbecco's modified Eagle's medium (high glucose, no pyruvate) plus 1 × nonessential amino acids, 1 × antibiotics and antimycotics (GIBCO, Grand Island, NY), 2.86×10^{-5} mol/L β-mercaptoethanol, and 10% fetal bovine serum). Individual diverticula were washed through 50-μl drops of 1 × PBS and placed in a second 50-μl drop of 0.25% trypsin-EDTA solution and incubated at 37°C for 5 minutes. The microdrops were triturated to dissociate the diverticula. The cell suspension was plated in 35-mm tissue culture dishes containing a mitomycin C-treated $STON^+$ feeder layer and 5 ml of HBM. Cultures were established using cells from 1 to 10 diverticula.

The cultures were maintained at 37°C in a humidified 5% CO_2 in air atmosphere. The cultures were observed daily for the outgrowth of colonies bearing an endodermal morphology, ie, polyhedral cells with phase bright boarders (Figure 1B). After approximately 7 to 10 days in culture, individual colonies of cells were scraped from the feeder layer using a micropipette. Cell clumps were washed in a 50-μl drop of 1 × PBS and transferred into a 50-μl drop of 0.25% trypsin-EDTA to dissociate cells. The cell suspension was replated on a $STON^+$ mitomycin C-treated feeder layer in a 2-cm well of a 24-well tissue culture plate in 1.5 ml of HBM. The cultures were placed at 37°C in a humidified 5% CO_2 in air atmosphere. Cultures were observed for the outgrowth of cells that maintained an endodermal morphology. These cultures were passaged 1:3 every 7 to 10 days. Once confluent plates of cells were obtained, the cells were passaged 1:5 every 3 to 5 days. Frozen stocks of early passage cells were prepared and stored at −140°C.

Preparation of Mitomycin C-Treated $STON^+$ Feeder Layers

The line of STO cells used as a feeder layer in these studies ($STON^+$) has been transformed with a plasmid containing the neomycin resistance gene, mak-

ing these cells useful as a feeder layer when selecting genetically altered cell lines. Mitomycin C-treated $STON^{+}$ cells were prepared by standard methods as previously described.[21]

In Vitro *Differentiation*

Spontaneous differentiation was obtained by prolonged culture on a mitomycin C-treated $STON^{+}$ feeder layer without passage. Cultures were maintained for 3 to 4 weeks without passage. Cells were fed with fresh medium every 3 to 5 days. Chemically induced differentiation was accomplished by plating on tissue culture plastic in HBM containing either 5 to 10 mmol/L sodium butyrate or 2 to 4% dimethylsulfoxide (DMSO). Cultures were assayed for differentiation 4 to 7 days after plating.

Substrate-induced differentiation was obtained by plating HBC-3 cells on a layer of Matrigel in the absence of a feeder layer (Collaborative Research Inc., Bedford, MA). Fibronectin, laminin, collagen I, and collagen IV substrates were used as supplied by Collaborative Research.

Electron Microscopic (EM) Analysis

The ultrastructure of undifferentiated and spontaneously differentiated cultures of HBC-3 cells was determined by EM analysis. Two-day- and 4-week-old cultures of HBC-3 cells were washed with ice-cold PBS and subsequently fixed using 2.5% glutaraldehyde and 0.1 mol/L cacodylate for 1 hour. The samples were then processed using standard techniques for EM analysis.

Immunofluoresence and Immunohistochemistry

Immunodetection of AFP, cytokeratin 14 (CK-14), and CK-19 were performed as follows. Undifferentiated HBC-3 cells were grown on mitomycin C-treated STO feeder layers in Nunc (Naperville, IL) chamber well tissue culture plastic slides for 1 to 7 days prior to fixation with ice-cold acetone. Differentiated HBC-3 cells were treated as described above prior to acetone fixation. Fixed slides were stored at −20°C before staining. Culture-bearing slides were washed five times for 5 minutes in 1 × PBS. The slides were then air dried at room temperature. A rabbit anti-mouse AFP (ICN Immunochemicals, Costa Mesa, CA) was used to detect AFP. This antiserum was diluted 1:50 in PBS containing 5% normal sheep serum (The Binding Site, Inc., San Diego, CA). A rabbit anti-mouse CK-14 antiserum (kindly provided by Dr S. Yuspa, National Institutes of Health, Bethesda, MD) was used to detect CK-14. This antiserum was diluted 1:1000 in PBS containing normal sheep serum. A guinea pig anti-mouse CK-19 antiserum (kindly provided by Dr L. Germain, Laval University, Quebec, Canada) was used to detect CK-19. This antiserum was diluted 1:1000 in PBS containing 5% normal sheep serum. Slides were incubated in diluted antiserum overnight at room temperature in a humidor. After incubation in the primary antisera, slides were washed seven times for 5 minutes in 1 × PBS at room temperature. Fluorescein isothiocyanate-labeled sheep anti-rabbit IgG A and M were diluted 1:50 in PBS plus 5% normal sheep serum. The slides were incubated with this secondary antiserum for 45 minutes at room temperature in a humidor and washed seven times for 5 minutes in 1 × PBS, followed by a 2-minute wash in distilled water. Coverslips were mounted using Gelmount (Biomedia Corp., Foster City, CA) before observation.

HBC-3 cells for albumin detection were grown for 1 to 7 days on Nunc glass slides coated with gelatin. Gelatin coating was used to facilitate the adherence of cells to the glass slides. The slides bearing HBC-3 cells were fixed with ice-cold 95% ethanol for 5 minutes before staining. Albumin protein was detected using a sheep anti-mouse albumin antiserum (IgG fraction; The Binding Site) Positive staining was visualized using the Vectastain Elite staining kit and affinity-purified, biotinylated anti-sheep IgG (H and L) (Vector Laboratories, Burlingame, CA). The cells were counterstained with Gill's hematoxylin to visualize nuclei.

Histochemistry

Histochemical analysis of γ-glutamyl transpeptidase (GGT) was performed as previously described.[22] Histochemical analysis of glucose-6-phosphatase activity was performed as previously described.[13]

Results

A cell line was isolated from the 9.5-day-liver diverticulum by placing the microdissected liver diverticulum (Figure 1A) in culture on a mitomycin C-treated STO fibroblast feeder layer. The rationale for this approach was based on previously developed methods for the isolation of embryonic stem cells.[23]

Using this approach, the dissociated liver diverticulum gave rise to many small colonies of small

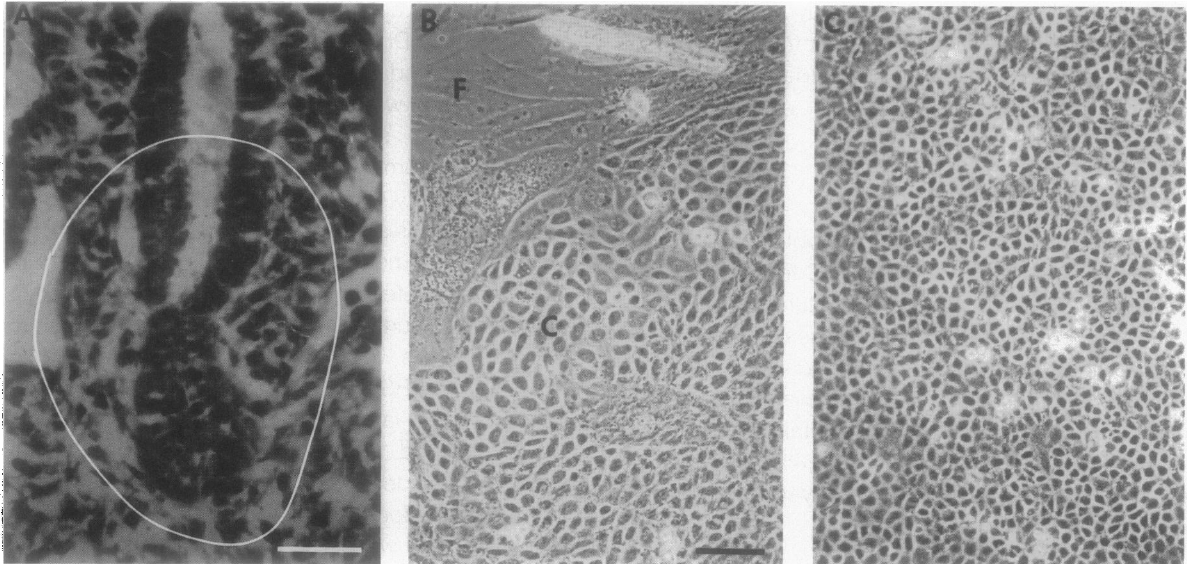

Figure 1. *Isolation of hepatic diverticula on embryonic day 9.5 and establishment of these cells in tissue culture.* A: *Transverse section through a mouse embryo isolated on embryonic day 9.5 at the level of the hepatic diverticulum. The* circled *region represents the approximate region that was microdissected and introduced into culture.* B: *Photomicrograph of the edge of a colony of cells with an endodermal morphology, which grew out of the initial diverticulum culture.* C: *Photomicrograph of a monolayer of HBC-3 cells at passage 5.* Bar: *0.1 mm.*

polygonal cells with phase bright boarders (Figure 1B); muscle cells and fibroblasts also arose. When cells from dissociated diverticula were plated on tissue culture plastic rather than a feeder layer, no colonies with phase bright boarders were observed.

Several of the polygonal cell colonies, which were candidate hepatoblast colonies, were selected for further growth into cell lines, as described in Materials and Methods. Initially, eight of these colonies showed a limited capacity to expand after trypsinization and replating on feeder layers. Seven of these early cultures maintained their unique phenotypes; however, they were incapable of sustaining growth at clonal cell densities. In contrast, one culture maintained its original phenotype and grew well at low cell density. This colony was expanded into a cell line, which has been designated HBC-3 (Figure 1C). HBC-3 cells have continued to retain their original hepatoblast morhpology when grown on a feeder layer of mitomycin C-treated STON$^+$ fibroblasts after more than 60 passages, which represent at least 150 cell generations. Karyotype analysis of HBC-3 cells at passage 40 revealed a euploid chromosome number. In addition, these cells can be cryogenically preserved and thawed, and the revived cells produce cultures that maintain their hepatoblast morphology.

Because our goal was to isolate a hepatoblast cell line, we analyzed HBC-3 cells for evidence of liver-specific gene expression. We hypothesized that HBC-3 cells should express biochemical markers in common with the cells of the liver diverticulum at 9.5 days of gestation in the mouse if they were indeed hepatoblasts. Early in liver ontogeny, a relatively small number of cellular markers are available to describe the embryonic hepatoblast phenotype, among these are AFP and albumin.[2] CK-14 expression has been detected in cells isolated from fetal rat liver as early as day 12 of gestation.[24] GGT is expressed transiently by all hepatic epithelial cells during liver ontogeny, with expression being detectable as early as day 12.5 of gestation in the rat. This enzyme activity is retained by cells of the biliary epithelial lineage but is lost from the hepatocyte lineage during the early postnatal period.[2]

To determine whether HBC-3 cells express these early liver cell markers, HBC-3 cells were stained for albumin, AFP, and CK-14 expression immunochemically and for GGT activity histochemically. The results of this analysis are shown in Figure 2. No staining was obtained using normal rabbit serum as a control (Figure 2A). Specific albumin staining was detected in HBC-3 cells 1 day after plating (Figure 2B). This staining increased in cultures that had been maintained for 1 week or longer without passage (Figure 2C). HBC-3 cells were stained histochemically for GGT activity (Figure 2, D–F).[22] A few cells in 1-week-old cultures exhibited GGT activity (Figure 2D). In cultures younger than 1 week old, no cells exhibited GGT activity. After 2 weeks without passage many small patches of cells stained positively for GGT activity (Figure 2E). After 3 weeks of culture without passage, many large areas that stain

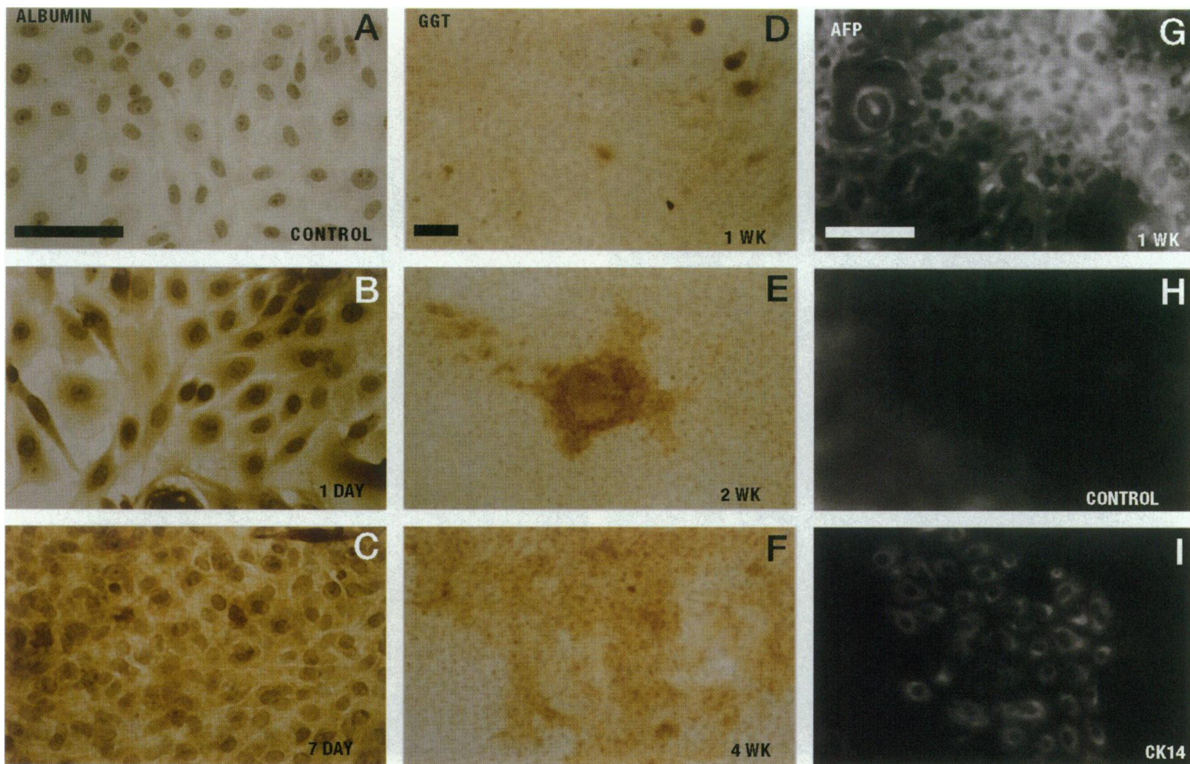

Figure 2. *Expression of liver-related markers by cultures of HBC-3 cells.* **A** *to* **C**: *Immunoperoxidase staining of HBC-3 cultures for albumin. The cells were counterstained with Gill's hematoxylin to permit visualization of nuclei.* **A**: *No staining was obtained using normal rabbit serum as a control.* **B**: *Specific albumin staining was detected in HBC-3 cells 1 day after plating.* **C**: *This staining increased in cultures that had been maintained for 1 week or longer.* **D** *to* **F**: *HBC-3 cells were stained histochemically for GGT activity.* **D**: *A few cells in 1-week-old cultures exhibited GGT activity, measured here by the deposition of a rust-colored product. In cultures younger than 1 week old, no cells exhibited GGT activity.* **E**: *After 2 weeks without passage, many small patches of cells stained positively for GGT activity.* **F**: *After 3 weeks of culture, many large areas that stained positively for GGT activity were observed.* **G** *and* **H**: *HBC-3 cells were stained for AFP by indirect immunofluoresence.* **G**: *In 1-week-old cultures, all HBC-3 cells stained positively with the AFP antisera.* **H**: *No staining was observed using normal rabbit serum as a control.* **I**: *One-week-old cultures were stained using anti CK-14 antiserum. Patches of cells were observed that stained strongly with anti CK-14 antibody. These patches were surrounded by HBC-3 cells, which were not decorated with anti-CK-14 staining.* **Bar**: *0.1 mm.*

positively for GGT activity were observed (Figure 2F). Thus, HBC-3 cells are initially albumin positive and GGT negative. However, when maintained without passage the cells become more strongly positive for albumin, and some cells also become GGT positive.

AFP expression in HBC-3 cells was investigated by indirect immunofluoresence using a rabbit anti-mouse AFP antisera. In 1-week-old cultures all HBC-3 cells stained positively with the AFP antisera (Figure 2G). The large cells in Figure 2G, which are negative for AFP staining, are the STO feeder cells. No staining was observed using normal rabbit serum as a control (Figure 2H). CK-14 expression was examined using a rabbit anti-mouse CK-14 antibody. When 1-week-old cultures were stained using this antibody, the cultures stained heterogeneously (Figure 2I). Patches of cells were observed that stained strongly with anti CK-14 antibody. These patches were surrounded by HBC-3 cells, which were not decorated with anti CK-14 staining. Thus, HBC-3 cells are AFP positive and are heterogeneous with respect to CK-14 expression.

In the adult liver, differentiated hepatocytes display characteristic morphological features; among these are a brush boarder of microvilli on their apical and sinusoidal surfaces, bile canaliculi, peroxisomes, and glycogen granules. These ultrastructural features are acquired during liver ontogeny; therefore, it might be expected that these features would be lacking in undifferentiated HBC-3 cells. However, if HBC-3 cells are capable of differentiating *in vitro* along the hepatocyte lineage cells, they may acquire these hepatocyte-like features.

EM examination of 1-day-old cultures of HBC-3 cells at passage 10 showed that the cells have a relatively undifferentiated morphology with a few surface microvilli and also that the cells exhibit some apical tight junctions and a few fat droplets (Figure 3A).

It has been shown that some pluripotential cell types (teratocarcinoma cells and mouse embryonic

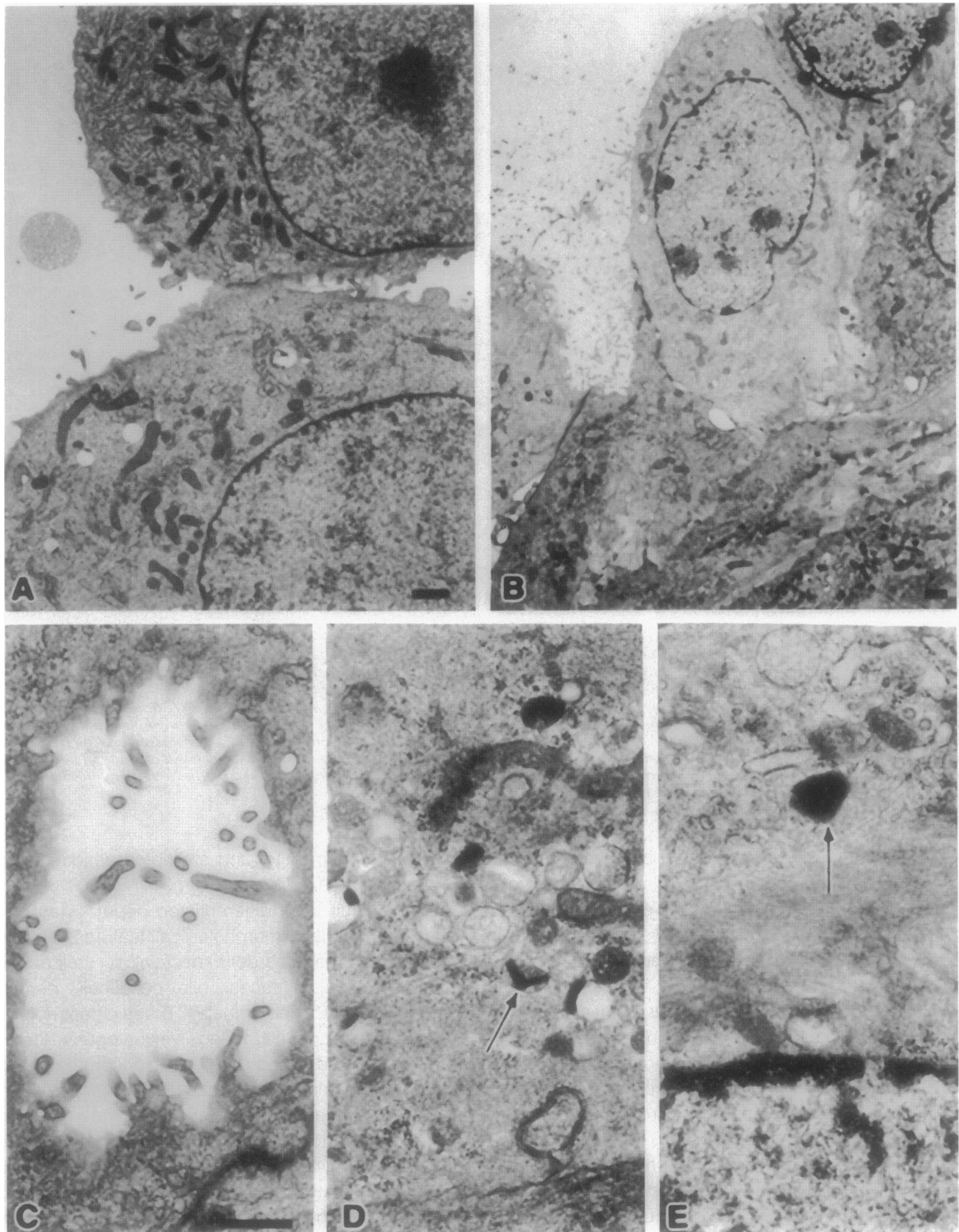

Figure 3. *Ultrastructural analysis of HBC-3 cells.* **A**: *HBC-3 cells at passage 10, 2 days after seeding on a STON⁺ feeder layer.* **B** *to* **E**: *HBC-3 cells at passage 10, 4 weeks after seeding on a STON⁺ feeder layer.* **B**: *The cells exhibited hepatocyte-like microvilli on their apices as well as along their basolateral surfaces.* **C**: *Further examination revealed that these cells formed structures that resembled bile canaliculi.* **D**: *The cells also contained vesicles that morphologically resembled mouse peroxisomes* (**arrow**), *as well as* (**E**) *a few sparse glycogen granules* (**arrow**). **Bars**: *1 μm* (**A** *and* **B**) *and 0.5 μm* (**C** *to* **E**).

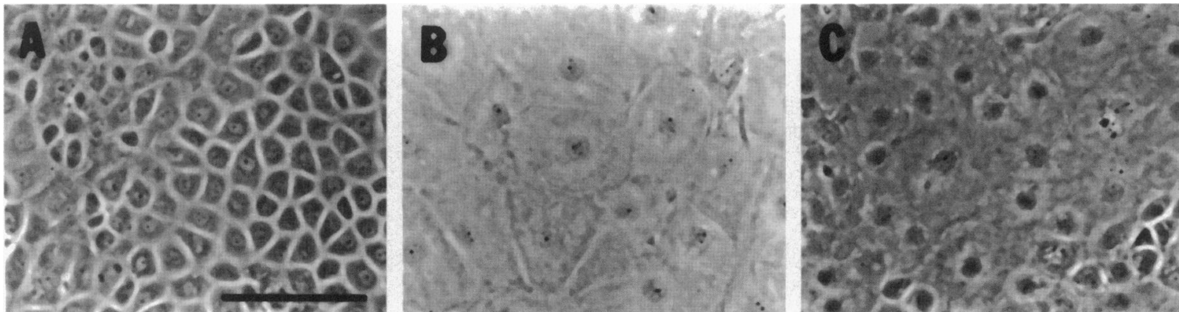

Figure 4. *Chemically induced differentiation of HBC-3 cells.* **A:** *Undifferentiated HBC-3 cells, passage 35.* **B:** *HBC-3 cells, passage 35, treated with 5 mm of sodium butyrate for 4 days.* **C:** *HBC-3 cells, passage 35, treated with 2% DMSO for 4 days.* **Bar:** *0.1 mm.*

stem cells) exhibit density-dependent growth in culture and are capable of undergoing differentiation *in vitro* when they are maintained in culture at high density without passage.[21] To determine whether long-term high-density cultures of HBC-3 cells also exhibit a similar density-dependent alteration of phenotype, HBC-3 cells were plated on STON$^+$ feeder layers and cultured without passage for approximately 3 weeks. The long-term cultures were examined every 3 to 4 days for changes in morphology. Surprisingly, the cells in these cultures did not exhibit contact-inhibited growth; rather, the cells continued to form successive layers of cells, until a layer of cells three to four cells thick was present in the plate. When cells in the layered formation were examined electron microscopically, we observed hepatocyte-like microvilli on their apices as well as along their basolateral surfaces, and these structures were similar to that seen along the sinusoidal surface of hepatocytes in intact liver (Figure 3B). Further examination revealed the presence of bile canaliculi between adjacent cells (Figure 3C). Finally, we observed vesicles that were morphologically identical to mouse peroxisomes in the cells (Figure 3D, arrow), as well as a few sparse glycogen granules (Figure 3E, arrow). These observations led us to conclude that HBC-3 cells had the capacity to spontaneously differentiate along the hepatocyte lineage in culture.

The occurrence of spontaneous differentiation of HBC-3 cells in long-term culture raised the possibility that the differentiation process in HBC-3 cells might be amenable to regulation using chemical inducers of differentiation. Sodium butyrate and DMSO have been shown to be capable of inducing differentiation in a variety of different normal and transformed cells, including those of hepatic origin. Furthermore, these compounds have been shown to stabilize the differentiated phenotype of primary cultures of hepatocytes in culture[25–28] and to promote the functional differentiation of rat ductular oval cells[29] and rat liver epithelial cells.[30]

To determine the differentiation potential of HBC-3 cells, we treated HBC-3 cells with increasing concentrations of sodium butyrate or DMSO. We found that both sodium butyrate and DMSO were capable of inducing phenotypic differentiation of HBC-3 cells along the hepatocytic lineage in a dose-dependent manner. The optimal concentrations for induction of the hepatocytic phenotype were 5 mmol/L sodium butyrate and 4% DMSO (Figure 4). In both cases the phenotypic alteration was accompanied by a cessation of cell growth. Analysis of liver-specific gene expression showed that sodium butyrate or DMSO caused HBC-3 cells to cease to express AFP, CK-14, and GGT activity but to maintain albumin expression and become uniformly positive for glucose-6-phosphatase activity, which was not expressed in undifferentiated cultures (Table 1).

It became apparent during the initial isolation of HBC-3 cells that feeder layers were essential for the growth and maintenance of hepatoblasts in culture. In addition to mimicking the endodermal-mesodermal tissue interaction that is present in the developing liver, the feeder layers may contribute both growth factors and extracellular matrix to the culture milieu. Because cell-matrix interactions play a major role in establishing and maintaining differentiated cell phenotypes, we tested the effect of various substrates, which have been shown to effect hepatocyte differentiation in culture, on the differentiation of HBC-3 cells. The culture substrates we tested were collagen I, collagen IV, fibronectin, laminin, poly-L-lysine, Matrigel, gelatin, and tissue culture plastic. HBC-3 cells plated on collagen I, collagen IV, fibronectin, laminin, poly-L-lysine, gelatin, or tissue culture plastic were similar in morphology to those plated on a feeder layer, indicating that these different substrates alone were not sufficient to cause differentiation of HBC-3 cells. In addition, when HBC-3 cells were plated on these matrices in the presence of 5 mmol/L sodium butyrate, the cells acquired a hepatocyte-like phenotype identical to

Table 1. *Marker Analysis of HBC-3 Cells after Treatment with 5 mmol/L Sodium Butyrate, 2% DMSO, or Matrigel*

Marker	Untreated	5 mmol/L sodium butyrate	2% DMSO	Matrigel	Spontaneously differentiated cultures (3–4 weeks)
AFP	++	−	−	+	−
Albumin	+	+	++	−	++
GGT	−	−	−	+	+
G6Pase	−	+	+	ND	ND
CK-14	+	−	−	+	−
CK-19	−	−	−	+	−

HBC-3 cells were treated with either 5 mmol/L sodium butyrate, 2% DMSO, Matrigel, or HBM for 4 days. Spontaneously differentiated cultures were held without passage for 3 to 4 weeks in HBM. Cultures were assayed for marker proteins as described in Materials and Methods. G6Pase, glucose-6-phosphatase; ND, not determined.

that obtained when the cells were plated on tissue culture plastic in the presence of 5 mmol/L sodium butyrate (data not shown).

A different and unexpected response was obtained when HBC-3 cells were plated on Matrigel. When HBC-3 cells are plated on a layer of Matrigel, the cells form elaborate ductular structures (Figure 5A). This morhpological change was observed in the presence or absence of sodium butyrate in the cell culture medium. The induction of ductular structures was accompanied by biochemical alterations within the cells. Cells that have formed these structures do not express albumin and are weakly positive for CK-14 and -19 (Table 1). GGT activity was detected as early as 1 day after plating on Matrigel (Figure 5B).

Discussion

The broad functional definition of a stem cell is that the stem cell is capable of self-renewal and giving rise to one or more cell types different from itself. Included in this definition are undifferentiated totipotent stem cells, such as the totipotent cells of the mammalian inner cell mass, as well as the committed stem cell that has become more restricted in its potential. As a result of the commitment process, committed stem cells or progenitor cells may express lineage-specific markers but retain an otherwise undifferentiated phenotype, eg, hematopoetic stem cells. Although it has been hypothesized that a liver stem cell must exist in the mammalian embryo, the phenotype of the true embryonic liver stem cell has remained illusive because of to its relative inaccessibility and to difficulties in establishing culture conditions that would allow such cells to be maintained for more than a few passages in culture. We have for the first time described a procedure that uses a combination of microdissection, growth on a mitomycin C-treated STON$^+$ feeder layer, and a modification of embryonic stem cell culture medium,[21] which has enabled us to isolate cells that have the characteristics of embryonic liver stem cells (hepatoblasts).

The cell line we developed (HBC-3) was derived from the liver diverticulum of the mouse at 9.5 days of gestation. Selection of embryos on day 9.5 of gestation as a starting point for isolation of our cell line was an important aspect of our procedure, because hepatic diverticula are relatively free of contaminating cell types that have been problematic in previous attempts of this type of cell isolation. Using microdissected diverticula, three cell types are introduced into the primary cell cultures: 1) hepatic endoderm, 2) adherent mesodermal cells of the cardiac region and septum transversum, and 3) gut endoderm. Several of our initial diverticulum cultures generated foci of beating cardiac cells and fibroblasts. It is possible that these cells contributed to the culture environment that led to the initial establishment of HBC-3 cells. Contamination of the initial diverticulum explant cultures with cells of the hematopoetic lineage was minimal, because these cells had not yet completed their migration from the yolk sac blood islands to the liver at this stage of development.[1] In addition, contamination from other cell types was reduced, because many of the anlage for other organs have not been established at 9.5 days of gestation in the mouse.[31]

At 9.5 days of development, the hepatic endoderm is entering a proliferative phase, which accompanies the migration of endodermal cells into the surrounding mesoderm of the septum transversum. This may aid in the establishment and maintenance of endodermally derived hepatoblasts in culture. Finally, because endodermal-mesodermal tissue interactions seem to be important in liver development, STO feeder layers were used in an attempt to substitute for the septum transversum in the cell culture system. A combination of these factors

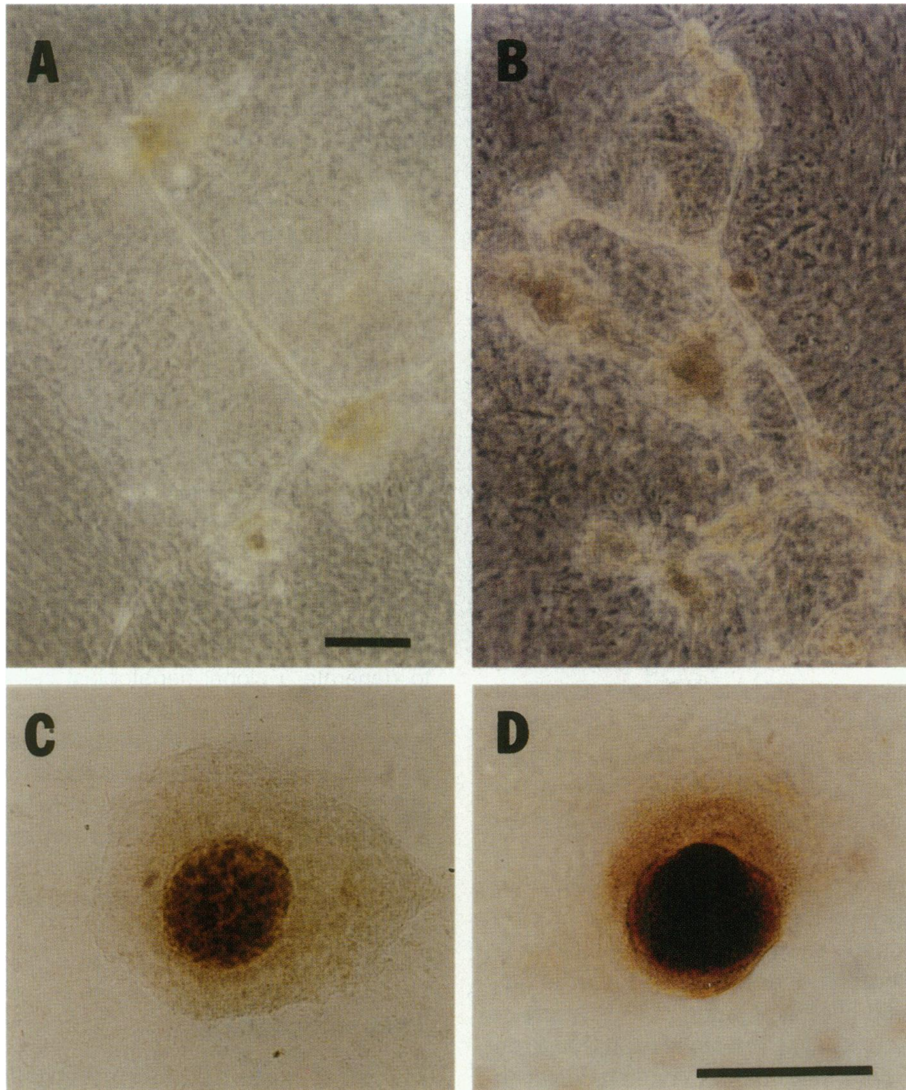

Figure 5. *Culture of HBC-3 cells on Matrigel.* **A** *and* **B**: *HBC-3 cells plated on a thick layer of Matrigel, 3-week old culture.* **C**: *HBC-3 cells plated on a layer of Matrigel, 1-day-old culture stained with hematoxylin to visualize cluster of cells.* **D**: *HBC-3 cells plated on a layer of Matrigel, 1-day-old culture stained for GGT activity by the method of Rutenberg et al.*[22] **Bar**: *0.1 mm.*

may have contributed to culture conditions that were favorable for the propagation of hepatic endoderm cells into a cell line.

Although the array of markers available to describe the cellular phenotype of the embryonic hepatoblast of the mouse is limited, HBC-3 cells show a pattern of expression of liver-related markers that is similar to that which has been reported for 9.5-day mouse liver diverticulum.[32,3] When HBC-3 cells are maintained on a $STON^+$ feeder layer with regular passage, these cells are positive for AFP, albumin, and CK-14 expression, as are the cells of the 9.5-day liver diverticulum. In addition, HBC-3 cells did not express detectable GGT activity, a liver-related enzymatic activity that is first detected later in liver ontogeny.[2] Thus, in terms of these markers, undifferentiated HBC-3 cells seem to retain the properties of the embryonic hepatoblast.

Our data demonstrate that HBC-3 cells retain another critical feature of the embryonic hepatoblast, that is, the capacity to differentiate into at least two different cell types. This characteristic has been demonstrated for explants of rat fetal liver tissue.[2] In the above experiments, it is not possible to determine whether the starting tissue already consists of two well-defined precursor cell populations. In contrast, our HBC-3 cells are a clonally derived cell line that can differentiate along the hepatocyte or bile ductular lineage in response to altered culture conditions.

HBC-3 cells spontaneously acquire ultrastructural features characteristic of differentiated hepatocytes when they are maintained without passage for 3 to 4 weeks. Concurrently, some of the cells in these cultures are positive for GGT activity, an enzyme marker that is transiently expressed by cells of the hepatic epithelial lineage during fetal development but is lost in hepatocytes during the early postnatal period. These data, taken together with the results of our ultrastructural analysis, suggests that HBC-3 cells are capable of differentiating into cells that resemble late fetal or early postnatal hepatocytes spontaneously when maintained without passage. In addition, when undifferentiated HBC-3 cells are treated with chemical inducers of differentiation, eg sodium butyrate and dimethyl sulfoxide, these cells undergo a relatively rapid change of phenotype, which includes cessation of growth, change in morphology, and change in marker expression consistent with a hepatocyte-like phenotype. A similar hepatocyte-like morphology as has been previously reported for oval cells and rat liver epithelial cells[29,30] after treatment with these chemical inducers of differentiation. These data suggest that liver epithelial cell lines derived from adult liver have some properties in common with the embryonic hepatoblasts we have isolated.

The mechanism of action by which sodium butyrate and DMSO induce differentiation is at present unclear. It has been shown that sodium butyrate is an inhibitor of histone deacetlyase and causes histones to become hyperacetylated.[33–35] It is postulated that hyperacetylation of histones can lead to an alteration in gene expression by precipitating changes in chromatin structure.[36–38] DMSO has been shown to affect the intracellular calcium ion concentration by causing a release of intracellular calcium ion stores.[39] It is proposed that this change in intracellular calcium may play a role in the induction of differentiation by DMSO.

HBC-3 cells undergo a second type of differentiation when grown on Matrigel. Under these conditions, HBC-3 cells exhibit a striking morphological change, eg, the cells form a network of tubules. Concurrently, the cells lose albumin expression and become positive for GGT expression, suggesting that these cells have differentiated along the bile ductular lineage. Thus, HBC-3 cells, originally isolated from the liver diverticulum of the 9.5-day-old mouse embryo, have been determined to become hepatoblasts and seem to retain the capacity to function as a bipotential stem cell population when cultured *in vitro*.

Matrigel has been used by others in the culture of primary hepatocytes to maintain the differentiated hepatocyte cell functions.[40] It is interesting that HBC-3 cells undergo differentiation along the bile ductular lineage under similar culture conditions. Between 13.5 and 15.5 days of gestation in the mouse, bipotential hepatoblasts that are located adjacent to portal veins within the developing liver parenchyma begin to express CK markers that are commonly associated with cells of the biliary tract. The expression of the CKs is first observed around larger vessels that were surrounded by a layer of connective tissue.[41] It has been postulated that the interaction of these hepatoblasts with the extracellular matrix surrounding these vessels is important in the induction of a bile ductular phenotype. Therefore, it is not surprising that HBC-3 cells, which have the phenotype of an undifferentiated hepatoblast, differentiate along the bile ductular lineage when presented with the complex extracellular matrix of Matrigel.

In summary, it has been possible for the first time to generate a clonal hepatoblast cell line from the liver diverticulum of the 9.5-day-old mouse embryo, to propagate these cells in the undifferentiated state, and to induce these cells to selectively differentiate *in vitro* along the hepatocyte or bile ductular lineages. Therefore, HBC-3 cells represent a unique and useful model system in which to begin to explore the cellular and molecular events that are important for liver development.

Acknowledgments

The author thanks Dr. Charles Rogler for his encouragement, helpful discussions, and critical reading of the manuscript, Dr. David Shafritz for critical reading of the manuscript, Mr. Frank Macaluso and the Analytical Ultrastucture Core of the Albert Einstein Cancer Center for their expert assistance with EM analysis, Drs. Sanjeev Gupta and Pankaj Rajvanshi for advice on sodium butyrate treatment and help with the initial glucose-6-phosphatase assays, and Drs. S. Yuspa and L. Germain for graciously providing anti-mouse CK antibodies.

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