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Characterization of Biliary Epithelial Cells Isolated from Needle Biopsies of Human Liver in the Presence of Hepatocyte Growth Factor

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Current methods for the isolation of intrahepatic biliary epithelial cells from human liver rely upon relatively large segments of tissue, thereby limiting studies to cells isolated from patients with end-stage disease. To investigate a greater range of diseases and those at an earlier stage, we have developed a method to isolate biliary epithelial cells from biopsy-sized fragments of human liver. Tissue explants are cultured for >4 weeks, and, in approximately 50% of samples incubated with medium containing hepatocyte growth factor, biliary epithelial cells begin to migrate from the fragments and proliferate. With time they form confluent pavements of cells that express cytokeratin 19 and γ-glutamyl transpeptidase and are negative for markers of non-biliary cell phenotype. After subculturing, cells can be expanded, yielding substantial numbers for subsequent study **in vitro**. ***Cells can be isolated with a similar degree of success from adult normal liver, from a variety of liver diseases, and from post-transplant liver biopsies. Overall, pediatric tissue yielded cells less frequently than adult tissue. This novel technique is likely to have a major impact on the study of biliary pathophysiology, as small fragments of tissue removed from biopsies taken for diagnostic purposes can be used. (Am J Pathol 1995, 146:537–545)***

With the advent of orthotopic liver transplant programs for the treatment of end-stage liver disease, human liver tissue has become more readily accessible for experimental investigations. As a consequence, the study of human liver growth, physiology, and pathology has been greatly facilitated by the ability to isolate different populations of cells with high purity. Human hepatocytes are now used extensively for growth and functional studies.[1–3] Sinusoidal cells from human liver have also been studied *in vitro*[4–6] although to a lesser extent due to the greater technical difficulty in isolating purified preparations and the smaller number of cells obtained.

In recent years, interest in the biology and pathology of the biliary epithelium has received greater attention. Several established methods are now available for the isolation of rat biliary epithelial cells (BECs) from normal and cholestatic tissue.[7–10] With normal or diseased human liver tissue, intrahepatic adult human BECs can also be isolated with high purity. Cells are prepared by collagenase digestion, density gradient centrifugation, and immunomagnetic separation.[11,12] Although this technique does not rely on enzymatic perfusion of the intact biliary tree[13] or microdissection of ducts,[14] one disadvantage is that it requires relatively large amounts of tissue (30 to 50 g) and therefore the small fragments of tissue that become available after hepatic biopsy procedures are insufficient. This effectively limits studies of BEC pathophysiology to cells from the livers of patients with end-stage disease undergoing transplantation.

The ability to use biopsy-derived tissue would offer substantial benefits, for example, in studying early stages of chronic diseases, such as primary biliary cirrhosis (PBC), in which biopsies are taken to monitor disease progression. In addition, it would present the opportunity to investigate other liver diseases that af-

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fect the intrahepatic biliary tree and yet do not progress to end-stage disease. Moreover, it would also enable centers where there are no liver transplant programs and therefore where access to diseased tissue is limited to undertake such studies. Because human BECs proliferate in culture in the presence of hepatocyte growth factor (HGF)[15] and can be maintained for up to 14 weeks and seven passages with retention of biliary epithelial phenotype,[16] even very small initial yields of cells can ultimately be expanded, leading to substantial numbers for subsequent use. Here we describe a novel technique that exploits this property of HGF, to isolate intrahepatic BECs from biopsy-sized fragments of liver.

Materials and Methods

Source of Tissue

Normal liver was obtained from the pediatric segmental liver transplant program. After surgical dissection, tissue was transferred to the laboratory in sterile University of Wisconsin fluid at 4 C. Diseased tissue was from end-stage hepatectomies that were placed on ice immediately after removal at orthotopic liver transplantation. Biopsy fragments were from material taken for routine diagnostic purposes or post-transplant protocol biopsies.

Culture of Explants

Under aseptic conditions, normal and end-stage hepatectomy tissue was biopsied with Menghini needles. Tissue fragments (approximately 10 to 15 mm in length) were washed in phosphate-buffered saline three times at 4 C, divided into 2- to 3-mm fragments, and placed in tissue culture wells in a minimal amount of fluid. They were then allowed to attach in 25-cm^2 flasks or multi-well tissue culture plates for approximately 1 hour in a humidified incubator at 37 C. After this, growth medium[13] (Dulbecco's minimal essential medium/Hams F12 containing 5% fetal calf serum 5 µg/ml insulin, 400 ng/ml hydrocortisone, 10 ng/ml cholera toxin, 10 ng/ml epidermal growth factor, 2 nmol/L triiodothyronine and 10 ng/ml HGF) was carefully added to the wells without disturbing the explants. After 24 hours, medium was renewed and cultures were incubated for 3 to 4 weeks with additional medium changes every 3 to 4 days. The subsequent monolayers were gently trypsinized and were either replated or were subjected to immunomagnetic separation by established techniques.[11,12]

Characterization of Cultures

Cultures were monitored routinely by phase-contrast microscopy. At appropriate intervals, culture wells were washed twice in phosphate-buffered saline and were fixed in ice-cold ethanol for 10 minutes. These cultures were then characterized by a variety of markers for the various liver cell types (specificities are summarized in Table 1). Immunopositivity was visualized by immunoalkaline phosphatase (Dako, Carpenteria, CA) fast red (Sigma Chemical Co., St. Louis, MO) with primary antibodies against cytokeratin-19 (CK-19), desmin, vimentin, factor VIII-related antigen, EBM11 (all from Dako), HEA125 (Bradsure Biologicals, Loughborough, UK), and asialoglycoprotein receptor (Gift from Dr. I. McFarlane, King's College Medical School, London, UK). γ-Glutamyl transpeptidase (GGT) was visualized by histochemistry.[25] For autoradiographic determination of the growth of cells, explant cultures were incubated with [^{3}H]thymidine for 4 hours, fixed, and processed routinely with Ilford K5 photographic emulsion.

Results

Tissue fragments from normal liver attached readily to culture dishes. Specimens from diseased livers, however, required more extensive washes to remove the excess tissue debris and bile often present. After a delay that varied from 3 to 10 days, cells could be seen migrating out from the explants (Figure 1a). These cells, which had an epithelial-like morphology, were seen to expand substantially with time in culture, yielding confluent colonies (Figure 1b). The process was relatively slow, requiring as long as 3 to 4 weeks. Omission of HGF from the culture medium resulted in little outgrowth of cells, indicating that the response was dependent upon the presence of the growth factor. Autoradiography after [^{3}H]thymidine addition showed numerous labeled nuclei, demonstrating that cells were clearly both migrating out from the tissue

Table 1. *Phenotypic Markers Used to Characterize Liver Cell Isolates*

Cell type	Marker	Reference
BECs	HEA125	17
	CK-19	18
	GGT	19
Hepatocytes	Asialoglycoprotein receptor	20
Fat-Storing cells	Desmin	21
Kupffer cells	Endogenous peroxidase	22
	EBM-11	23
Endothelial cells	Factor VIII-related antigen	24

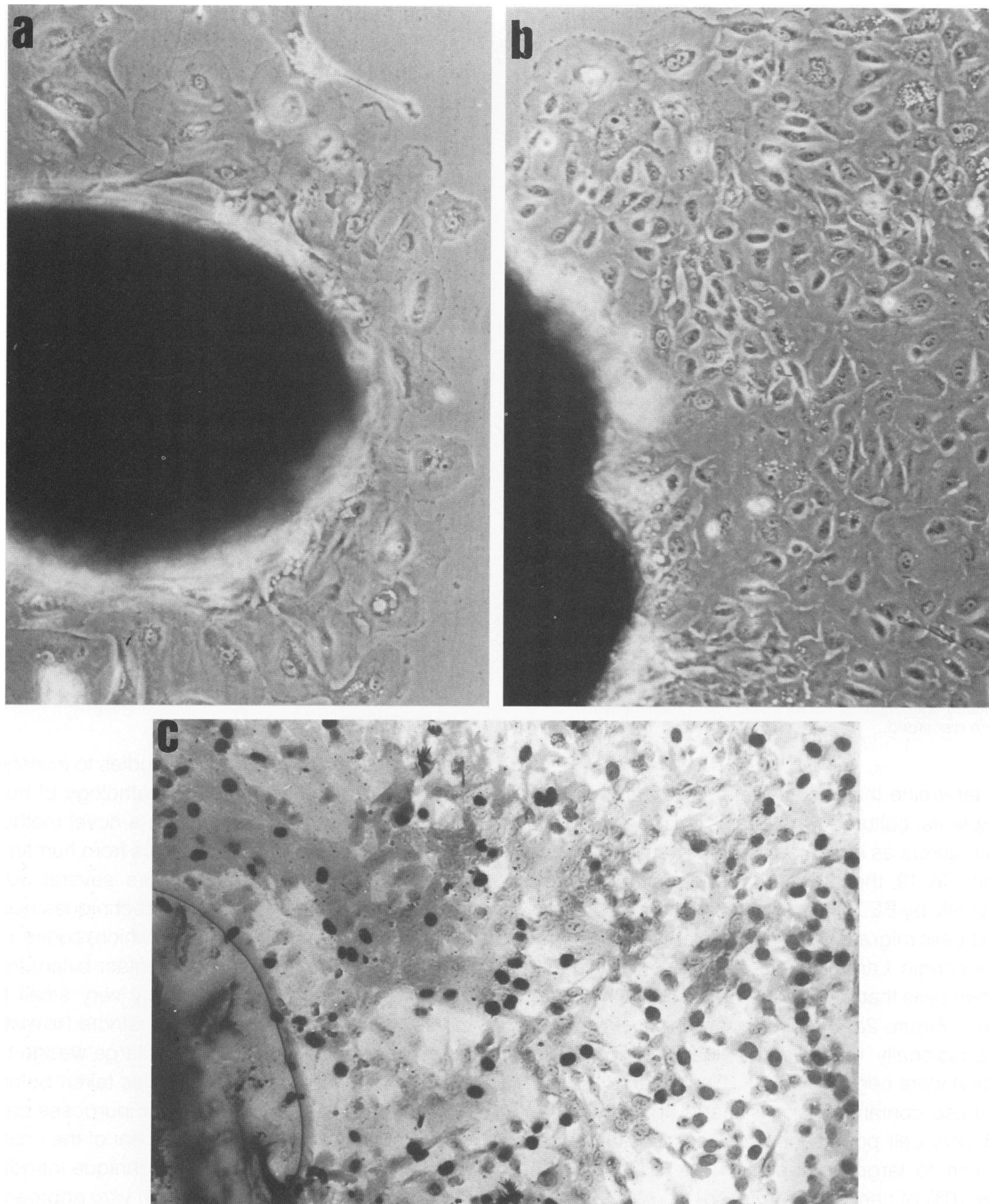


Figure 1. a *and* b: *Phase contrast photomicrographs of liver biopsy fragments. Biopsies were cultured for 3* (a) *or 14* (b) *days.* c: *Autoradiograph of 21-day culture after [^{3}H]thymidine exposure. Normal liver was from 52-year-old* (a *and* b) *and 57-year-old* (c) *donors. Magnification, ×250.*

and proliferating (Figure 1c). When colonies had expanded sufficiently after trypsin treatment and growth in HGF-containing medium,[15] large numbers of cells for subsequent studies were obtained in approximately 50% of cases. Over the course of 4 to 6 weeks with this approach, the final yield of BECs was in the range 0.5×10^7 to 5×10^7 cells per biopsy specimen.

Cells grew out of tissue fragments from a variety of normal donors and liver diseases. Details are summarized in Table 2. Overall, in approximately 50% of samples, cells were successfully grown from explants and cell numbers subsequently increased. However, there did appear to be variation that was dependent upon the age of the donor. Fragments from individuals below the age of 11 years yielded cells with much lower frequency (2 of 8; Table 2). Additionally, in tissue from adults, the success rate was higher in normal (4 of 5) compared with diseased samples (6 of 13).

Table 2. *Culture of Biopsy-Sized Fragments from Normal and Diseased Livers*

Diagnosis	Age*	Sex	BEC	Non-BEC
Normal	11	M	–	–
	9	M	–	–
	52	F	–	–
	52	M	+	+
	57	F	+	–
	10	F	+	+
	6	F	–	–
	38	M	+	–
	7	M	+	–
	35	F	+	+
Alcoholic hepatitis	27	M	+	+
	56	F	+	+†
Biliary atresia	5	M	–	–
Cystic fibrosis	8	M	+	+
	2	M	–	–
PBC	68	F	+	+
	57	F	–	+
	61	F	+	+
	49	F	–	–
	53	M	–	–
	62	F	+	+
	38	F	+	+†
	42	F	–	–
	50	F	–	–
	42	M	–	–
Neonatal hepatitis	2 m	M	–	–
Primary sclerosing cholangitis	26	F	–	+

*Age is in years except for neonatal hepatitis in months (m).
†Cultures were heavily contaminated with mesenchymal cells and were discarded.

To determine the phenotype of cells derived from the explants, cultures were characterized by a number of markers as indicated in Table 1. Immunostaining with CK-19, the intermediate filament expressed specifically by BECs in adult liver sections, indicated that the cells migrating out from explants were clearly of biliary origin. Large colonies of confluent polygonal epithelial cells that were 100% positive for CK-19 are shown in Figure 2a, b. Careful screening of cultures did occasionally reveal contaminant fibroblast-like cells that were negative for CK-19 (Figure 2c). However, these contaminant cells made up <2% of the total biliary cell population, and those cultures that gave rise to large numbers of non-BEC (approximately 10% of cases) were discarded (see Table 2). Cells uniformly retained GGT activity as determined by histochemical visualization (Figure 2d) throughout the primary culture period and after trypsinization and growth in the presence of HGF.

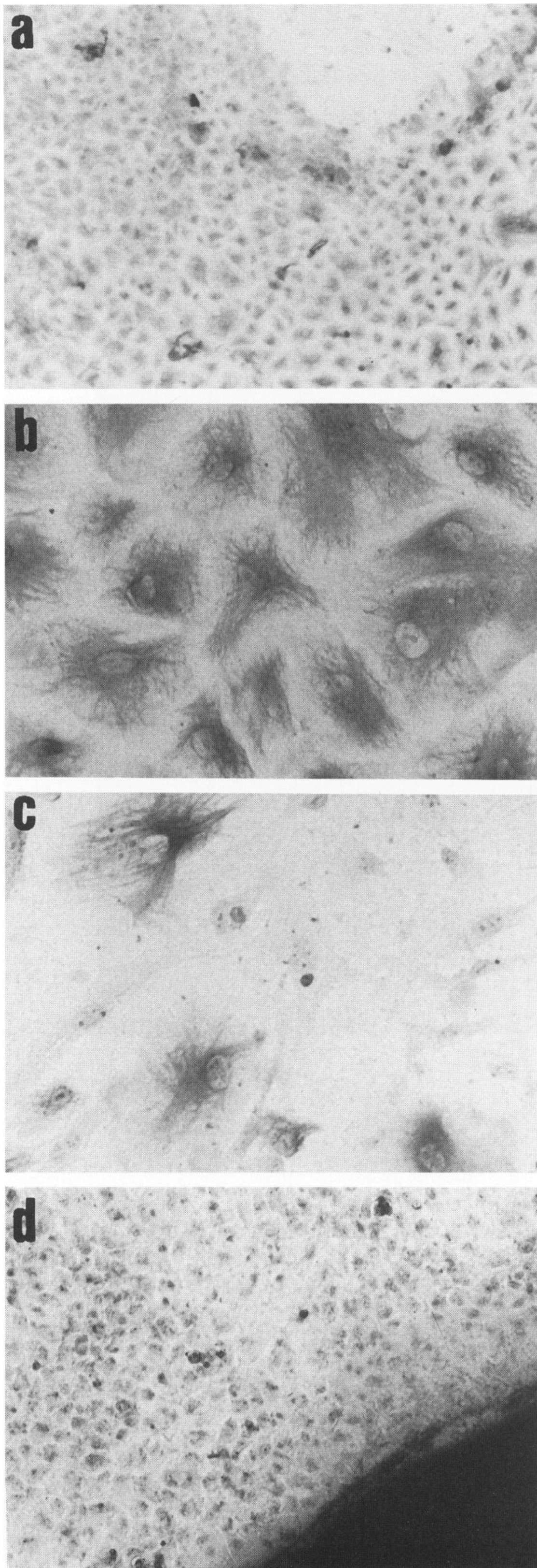
Cultures immunostained with the vimentin antibody showed that cells adjacent to the explants were largely negative, the percentage of vimentin-positive cells then rising with increasing distance from the tissue (Figure 3a) resulting in >95% positivity. At the edge of the outgrowths, where 100% of the cells were vimentin positive, they demonstrated a more fibroblast-like morphology with a ragged scatter-like edge to the colonies (Figure 3b). Conversely, HEA125 staining showed the opposite pattern. Only a relatively small number of cells in close proximity to the biopsy were HEA125 positive (Figure 3c) and beyond this expression was lost. Cultures stained with antibodies against the markers factor VIII-related antigen (Figure 3d), desmin, and asialoglycoprotein (not shown) were negative.

An additional series of experiments was undertaken with tissue obtained at the time of protocol biopsy sampling after liver transplantation. Results are summarized in Table 3. As indicated, cells were successfully grown in 7 of 16 samples (45%). This is similar to that found with fragments from normal or end-stage tissues (Table 2) and indicates that the technique can be applied equally well to samples obtained by percutaneous biopsy. Detailed phenotypic analyses similarly confirmed these cells to be of biliary epithelial phenotype (not shown). Although many of the samples were from biopsies subsequently histologically confirmed to be undergoing an episode of acute rejection (Table 3), the relative ability to isolate cells did not appear to correlate with the diagnosis.

Discussion

As part of an ongoing series of studies to investigate the growth, differentiation, and pathology of human biliary epithelium, we report here a novel methodology to obtain preparations of BECs from human liver biopsies. This development offers several advantages over the other established techniques used to isolate intrahepatic human BECs, which require >10 g of tissue[11,12] or perfusion of the intact biliary tree.[13] The explant method requires only very small fragments of liver and is therefore not restricted to material from transplant hepatectomies or large wedge biopsies. Percutaneous needle biopsies taken before or after transplantation for diagnostic purposes can be used because only a minor fragment of the core obtained is necessary. Thus, this technique introduces the exciting possibility that, with *in vitro* approaches, investigations of the pathophysiology of the biliary epithelium during progressive chronic liver disease can be now undertaken. Additionally, it will allow centers where there are no liver transplantation programs (and therefore where tissue availability is much more limited) to initiate studies of this nature.

The observation that BECs migrate and proliferate from adherent tissue explants was unexpected, as even in normal liver tissue, they represent a minor cell population (<5% by cell number[26]). Typically, fibroblasts that proliferate relatively rapidly might be anticipated to grow out from such explants. Although



contaminating cells were present (Figure 2c), in most cases they were found in very small numbers (<2% of cells that were positive for both CK-19 and GGT) and did not compromise the quality of the subsequent biliary cell cultures. Although in the initial stages of the procedure, relatively small cell numbers are derived from the biopsies directly, with subculture and proliferation in HGF-supplemented medium as previously reported for intrahepatic human BECs,[15] substantial expansion of cell numbers is attainable. Contaminating mesenchymal cells, which do not respond to HGF,[27] therefore are lost from the cultures. Thus, generation of $>10^7$ BECs from very small tissue fragments can be obtained.

Clearly, the success of the technique is dependent upon the presence of HGF because few cells are obtained when HGF is omitted from the culture medium. In addition to its potent mitogenicity for epithelial cells, HGF, also known as scatter factor,[27,28] induces cell motility and/or three-dimensional morphogenetic responses in isolated cells, including tubule formation by kidney epithelium[29] and capillary formation by endothelial cells.[30] Therefore, it seems possible that the ability to generate BEC cultures from explants as described here is at least partly attributable to this property of HGF. Furthermore, as seen in Figure 3b, under certain conditions the cells appear to alter morphology, at times becoming more fibroblast-like and forming less compact colonies with a much less well defined border than seen in previous cultures.[15] These morphological changes are analogous to the scattering effect of HGF on Manine Darby canine kidney cells,[31] suggesting that human BECs may respond to HGF in more than one way, namely, mitogenically and motogenically. Indeed, recent evidence that HGF induces either mitogenesis or morphogenetic changes in rat liver epithelial cell lines supports this possibility.[32] Other cells, including microvascular endothelials cells, can grow or form tubular capillary-like structures in response to HGF.[30,33] Although there is evidence that a truncated HGF variant (NK2) binds to the HGF (c-*met*) receptor and induces motogenicity but not mitogenicity on cells,[34,35] the mechanisms whereby cells respond to full length mature HGF with one response and not the other remains to be elucidated.

Figure 2. *Phenotypic characterization of BEC monolayers derived from liver biopsy fragments. Confluent colonies of cells, derived from a 57-year-old donor, were grown for 4 weeks and were immunostained with an antibody against CK-19. Cells show cytoplasmic filamentous staining* (a *and* b)*. Note detachment of the tissue fragment before staining, leaving a vacant area* (a) *and the presence of immunonegative non-BEC at high power* (c)*. Cells stained for GGT activity* (d)*. Magnification,* ×*250* (a *and* d) *and* ×*400* (b *and* c).

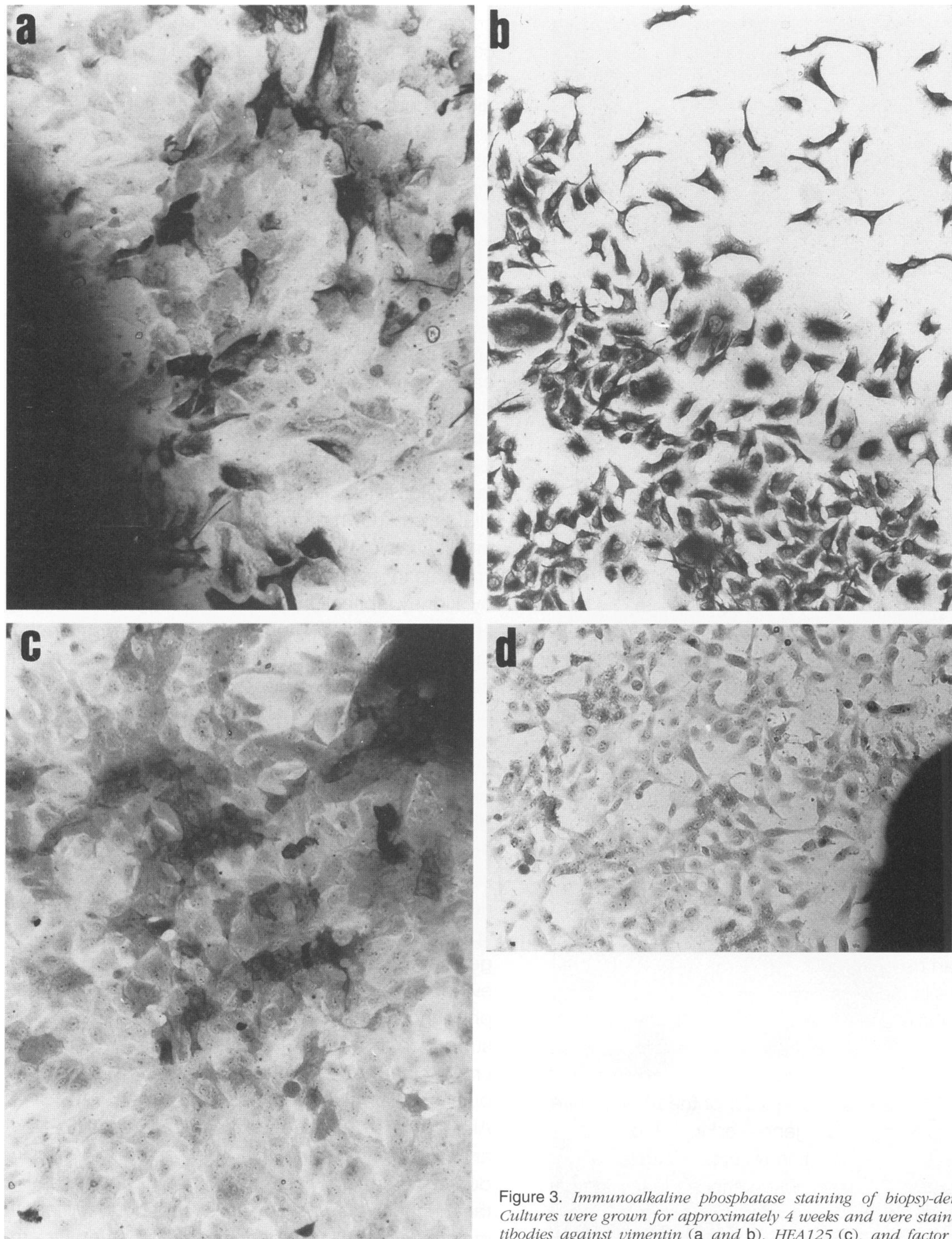


Figure 3. *Immunoalkaline phosphatase staining of biopsy-derived BECs. Cultures were grown for approximately 4 weeks and were stained with antibodies against vimentin* (a *and* b), *HEA125* (c), *and factor VIII-related antigen* (d). *Magnification, ×250.*

The percutaneous post-liver transplant samples yielded BEC preparations with a rate of success similar to that seen with normal or end-stage liver tissue. Many of the biopsies were from patients subsequently histologically confirmed to be undergoing an episode of acute rejection (Table 3). Because inflammatory damage to bile ducts is a characteristic feature of acute rejection,[36] a paucity of bile ducts might be expected to lead to a lower rate of success in their isolation. However, the relative ability to isolate cells

Table 3. *Culture of Post-Transplant Hepatic Biopsies*

Patient		Donor		Biopsy	Original	Histological		Non-
Age	Sex	Age	Sex	time*	disease†	diagnosis‡	BEC	BEC
56	M	44	F	27 m	HBV	No rej	−	−
37	F	20	M	24 m	PSC	No rej	+	+
4	F	16	F	8 m	Hepbl	No rej	−	−
23	F	34	M	20 m	CAH	No rej	−	−
1	F	11	F	10 d	BAT	No rej	−	−
55	M	43	M	6 d	AC	Acute rej −	+	+
33	F	38	F	58 d	NANB	Acute rej +	−	−
37	F	34	F	7 d	PBC	Acute rej +++	−	−
33	F	22	M	67 d	NANB	Acute rej −	−	−
66	M	20	F	7 d	PBC	Acute rej ++	+	+
32	F	19	F	2 m	CAH	Acute rej −	+	−
33	F	38	F	5 m	NANB	Acute rej +	−	−
66	M	20	M	8 d	PBC	Acute rej −	+	+
21	M	28	M	3 m	CAH	Acute rej −−	+	−
41	M	29	M	21 m	PSC	Mild hepatitis	−	−
50	F	13	M	35 m	PBC	Mild hepatitis	+	+

*Biopsy time in days (d) or months (m) after liver transplantation.
†NANB, Non-A, non-B hepatitis; CAH, chronic active hepatitis; PSC, primary sclerosing cholangitis; AC, alcoholic hepatitis; Hepbl, hepatoblastoma; BAT, biliary atresia.
‡Degree of histological rejection (rej) scored − or +.

from the tissue did not appear to correlate with the histological diagnosis. This may relate to the presence of proliferative bile ductular cells that are known to arise during acute rejection.[37] Current phenotypic markers do not distinguish between cells derived from patent bile ducts and ductular proliferative cells, and this is currently under investigation.

BECs from biopsies demonstrated expression of GGT and CK-19 throughout, indicating stability of these markers of adult biliary epithelial phenotype. However, HEA125 positivity was limited to cells adjacent to the explant (Figure 3a). These observations are consistent with our recent findings with long-term cultures of BEC from collagenase digests of liver, which can be cultured for up to 14 weeks and seven passages in the presence of HGF.[16] Although during this time the expression of GGT and CK-19 is similarly maintained, HEA125 is expressed only in early cultures and is gradually lost from approximately 4 weeks and beyond.[15,16] In view of the time required for sufficient cells to be generated from biopsies (approximately 4 weeks), this reflects a similar phenotypic response. These observations clearly indicate that conditions for the growth and maintenance of adult human BECs remain suboptimal, and current efforts are in progress to improve the phenotypic stability of cultures.

We have also previously noted that in BEC cultures expression of vimentin, the intermediate filament originally regarded as a mesenchymal cell marker *in vivo*, increases with time in culture[15] and, from approximately 4 to 5 weeks (or two to three passages), all CK-19- and GGT-positive cells coexpress vimentin (manuscript in preparation). As seen in Figure 3, the pattern of vimentin expression by BECs in the present study is consistent with these previous observations. Coexpression of vimentin and cytokeratins by cells has been widely documented[38–41] and reflects a general response of many epithelial cells when isolated and maintained in culture. Interestingly, in certain situations vimentin/cytokeratin intermediate filament coexpression also occurs in epithelia *in vivo*, for example, in cells of newly formed bile ducts after bile duct ligation in the rat[42] and in damaged and regenerating renal tubular epithelium.[43] Indeed, the phenomenon has been described in a wide variety of tissues (reviewed in reference 44). The functional significance of intermediate filament coexpression has been suggested to correlate with the proliferation/differentiation status of cells, but this has yet to be clarified.

This means of preparing human BECs is a novel and therefore potentially extremely valuable technique and is likely to have a major impact on the study of biliary cell pathophysiology in the future. For example, in previous studies, we have found that BECs isolated from end-stage PBC but not from normal liver express aberrantly the E2 component of pyruvate dehydrogenase complex on the plasma membrane.[45] E2 is the antigen recognized by the anti-mitochondrial antibody that is present in the plasma of >95% of PBC patients,[46] and this observation may possibly have a bearing on the pathogenicity of the disease. Thus, the ability to isolate and study BECs from biopsies of patients with, for example, chronic liver diseases such

as PBC may provide novel insights into such progressive disorders.

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