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Hepatocytic Differentiation of Cultured Rat Pancreatic Ductal Epithelial Cells after *in Vivo* Implantation

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We have investigated the differentiation potential of propagable cultured rat pancreatic duct epithelial cells after* in vivo *implantation in isogeneic Fischer-344 rats. Cells genetically labeled with* Escherichia coli *β-galactosidase (*lacZ*) reporter gene were embedded in a mixture of collagen and Matrigel (basement membrane matrix) and implanted either subcutaneously or intraperitoneally. Tissues from the two locations were harvested 4 to 8 weeks later. The great majority of the* lacZ*-labeled epithelial cells colonizing both sites phenotypically resembled hepatocytes, although they demonstrated different degrees of hepatocytic differentiation. Less than 5% of* lacZ*-labeled cells formed ductular structures. The hepatocyte-like cells from the subcutaneous implantation site expressed mixed phenotypes of both hepatocyte and ductal cell, including the expression of α-fetoprotein, tyrosine aminotransferase, γ-glutamyl transpeptidase, carbonic anhydrase II, and cytokeratin 19. In contrast, the hepatocyte-like cells colonizing the mesentery showed the phenotype of mature hepatocytes, including an abundant glycogen storage and a lack of α-fetoprotein and carbonic anhydrase II expressions. Neither acinar cell nor endocrine differentiation was seen. These findings demostrate that pancreatic ductal cells can be the progenitor cell for transdifferentiated hepatocytes. (Am J Pathol 1995, 147:707–717)

The induction of hepatocytes in the pancreas of rats and hamsters after treatment with chemical carcinogens or copper-depleting diets[1,2] and the development of pancreatic acinar cells in rat liver after treatment with polychlorinated biphenyls[3] are examples of metaplasia or transdifferentiation. Initially it was postulated that the pancreatic hepatocytes were derived from the acinar cells,[2,4] hence representing transdifferentiation.[5] However, subsequent studies suggested that they originated from the duct epithelial and/or periductal cells.[6,7,8] An elucidation of the histogenetic origin of these pancreatic hepatocytes is complicated by the multiplicity of epithelial cell types present in this organ and the dynamic nature of *in vivo* experiments. A more direct approach is to establish an *in vitro* model with cell lines derived from specific pancreatic epithelial cell types and to investigate subsequently whether they are capable of differentiating into other cell types. Although pancreatic acinar and islet cells in primary cultures can be maintained functionally for an extended period of time, they have not been shown capable of continuous proliferation *in vitro* to form cell lines.[9,10] In contrast, we and others have reported the establishment of propagable cell lines from normal pancreatic duct epithelium of adult rats and hamsters[11–13] and of a periductal (oval) cell line from copper-deficient rats.[14,15] In this study, we have genetically labeled cultured normal rat pancreatic duct epithelial cells with a bacterial enzyme whose activity can be detected by histochemistry and have examined the differentiation capacity of these ductal cells in two *in vivo* microenvironments.

Materials and Methods

Cell Line and Culture

The RP-2 pancreatic duct epithelial cell line was established from the pancreas of an adult male Fi-

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scher-344 rat by a previously described method.[13] Cells were cultured in Richter's improved minimal essential medium with zinc option (Irvine Scientific, Santa Ana, CA) containing 10% fetal bovine serum (ICN Canada, St. Laurent, Quebec, Canada), 4 μg/ml insulin, and 40 μg/ml gentamycin. The cell line was routinely passaged before reaching confluence.

With the lipofectin reagent (GIBCO BRL, Grand Island, NY), the RP-2 cells at passage 10 were genetically labeled by transfection with the pRSV*lac*Z expression vector, which contained a 3.7-kb *Hin*dIII-*Xba*I fragment of the bacterial *lac*Z gene.[16] Stably transfected cells were selected by culturing in a medium containing 800 μg/ml G-418 (GIBCO BRL). After 14 days, the surviving transfected cells were pooled and then reseeded at a plating density of 500 cells per 100-mm tissue culture plate (Beckon Dickinson, Mississauga, Ontario, Canada) and grown in medium containing G-418. With cloning cylinders, several cell strains were isolated from individual colonies. Cells forming these colonies were designated as passage 1 and they were expanded by additional subcultures in 100-mm tissue culture plates. Cells of strain 2 and 5 at passage 2 were randomly chosen and used for implantation.

Cell Implantation

Male Fischer-344 rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 120 to 150 g were used in all implantation experiments. All animals were fed normal chow diet.

Male rats were used for subcutaneous implantation because of their lack of mammary glands. Under ether anesthesia, a skin incision was made along the midline of the upper abdomen. The skin and underlying fascia were bluntly dissected from the abdominal muscle, and cells suspended in an extracellular matrix gel were placed in the space between the skin and the rectus muscle. The extracellular matrix gel was reconstituted at 4°C from 6.4 parts of rat tail collagen type I[17] solution (approximately 3.33 mg/ml), 1.8 parts of 5X concentrated Richter's improved minimal essential medium with zinc option, and 1 part of an aqueous solution containing 200 mmol/L HEPES, 200 mmol/L $NaHCO_3$, and 50 mmol/L NaOH,[18] and 1 part Matrigel solution (Collaborative Research, Bedford, MA). A total of 2×10^6 cells were used in each implantation. Cell-free gel was implanted in control animals. The skin was closed with 2–0 nylon suture and the animal allowed to recover.

Intraperitoneal implantation was carried out in a similar fashion except that the gels were placed between the mesenteric fat and omentum within the peritoneal cavity.

Four to eight weeks after implantation, the animals were sacrificed by cervical dislocation and tissue from the implantation site was harvested. Part of the tissue was fixed immediately in 10% buffered formaldehyde for routine paraffin embedding, and a small portion was fixed in 1% glutaraldehyde-4% paraformaldehyde solution for electron microscopic studies. When available, tissue was also snap-frozen in liquid nitrogen and stored at −80°C.

Fresh implantation tissue was also subjected to an enzymatic reaction for bacterial *Lac*Z. Rat liver, pancreas, kidney, and skeletal muscle were also subjected to this reaction as controls for mammalian β-galactosidase activity. After thorough rinsing with phosphate-buffered saline (PBS), the 2- to 3-mm tissue fragments were fixed at 4°C in 2% (v/v) formaldehyde/0.2% (v/v) glutaraldehyde in PBS for 40 to 60 minutes. The reaction consisted of an overnight incubation at 4°C in a solution containing 20 μg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactoside, 2 mmol/L $MgCl_2$, 20 mmol/L potassium ferricyanide, 20 mmol/L potassium ferrocyanide, 0.02% Nonidet P-40, and 0.01% sodium deoxycholate. A positive reaction was indicated by the formation of a bluish precipitate. After the tissue was rinsed sequentially in PBS containing 3% dimethyl sulfoxide and PBS alone, it was processed for paraffin embedding.[19]

Histochemistry and Enzyme Histochemical Studies

Paraffin sections were routinely stained with hematoxylin and eosin (H&E), periodic acid-Schiff with or without previous digestion with diastase, and reticulin stains. Frozen sections of unfixed tissue were stained with Sudan IV and were used in various procedures of enzymatic histochemistry.

Histochemistry on tissue sections for bacterial β-galactosidase was performed by using the same reaction as described above. Alkaline phosphatase histochemistry was performed on tissue sections fixed in PBS containing 2% (v/v) formaldehyde/0.2% (v/v) glutaraldehyde by incubation at room temperature in a Tris-HCl buffer, pH 9.5, containing 1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 1 mg/ml nitroblue tetrazolium.[19] γ-Glutamyl transpeptidase and carbonic anhydrase (CA) histochemistry were performed by using published methods.[20,21] For CA, a positive reaction was revealed by the formation of black cobalt sulfide precipitates, and the specificity of this staining was confirmed by performing a par-

allel reaction in the reagent solution containing acetazolamide, which strongly inhibits the CA activity.

Immunocytochemistry

Immunocytochemistry was performed by the streptavidin-biotin-peroxidase system (Zymed Laboratories, San Francisco, CA) coupled with microwave antigen retrieval treatment.[22] Endogenous peroxidase and nonspecific binding were blocked, respectively, with 3% hydrogen peroxide and 10% normal goat serum. Antigen-antibody complex formation was visualized with either aminoethyl carbazole or 3,3′-diaminobenzidine. Polyclonal antibodies against rat albumin (ALB), α-fetoprotein (AFP), and transferrin were obtained from Nordic Immunological Laboratories (Capistrano Beach, CA) and were used at 1:6000, 1:2000, and 1:6000 dilutions, respectively. The rabbit polyclonal antibody against rat tyrosine aminotransferase (TAT) was used at 1:600 dilution.[23] A mouse monoclonal antibody against *Eschicheria coli* β-galactosidase was obtained from Oncogene Science (Uniondale, NY) and used at 1:10 dilution. The mouse monoclonal antibodies against human cytokeratin 19 (Amersham Canada, Oakville, Ontario, Canada) and rat cytokeratin 8[24] were used at 1:50 dilution. The mouse monoclonal antibodies HES6 and BDS7, respectively, recognize the surface antigens of hepatocytes and biliary epithelial cells in adult rat liver.[24] HES6 was used at 1:200 dilution whereas BDS7 was used undiluted. The rabbit polyclonal antibody against porcine α-amylase[25] was used at 1:200 dilution. Polyclonal antibodies against insulin, glucagon, and chromogranin were purchased from Dako Corp. (Carpinteria, CA) and used at 1:150 to 1:250 dilutions, as suggested by the manufacturer. Negative controls included replacement of the primary antibodies with nonimmune sera or their omission.

Most antibodies were used on formalin-fixed, paraffin-embedded sections. Tissues for positive control included rat liver and pancreas. We found that HES6 antibody, which was previously reported to be reactive only with frozen tissue sections,[24] worked well on paraffin sections treated by antigen retrieval procedure. In contrast, immunostaining with BDS7 or antibodies to cytokeratin 8 and 19 were only possible on cold acetone-fixed frozen sections.

Southern Blot Analysis

Genomic DNA was isolated according to the standard method.[26] Fifteen micrograms of DNA were digested overnight with 5 U/μg *Eco*RI (Pharmacia, Piscataway, NJ) and resolved by electrophoresis in a 0.8% agarose gel in 40 mmol/L Tris-HCl buffer, pH 7.9, containing 50 mmol/L sodium acetate and 1 mmol/L EDTA. After partial hydrolysis in base solution, DNA was transferred to a Hybond-N nylon membrane (Amersham) in 20X standard saline citrate (SSC) solution (1 X SSC: 150 mmol/L sodium chloride, 15 mmol/L sodium citrate, pH 7.0). The air-dried membrane was cross-linked by exposure to the ultraviolet light and then hybridized to a 3.7-kb *Hin*dIII-*Xba*I fragment of the *E. coli* β-galactosidase cDNA labeled with [^{32}P]dCTP to a high specific activity with the oligolabeling kit (Pharmacia). Hybridization and washing were performed according to Church and Gilbert,[27] and membranes were exposed to Kodak XAR-5 film at −80°C with an intensifying screen.

Northern Blot Analysis with cRNA Probes

Total cellular RNA was isolated from confluent cultured cells and frozen tissue by lysis in 4 mol/L guanidine isothiocyanate as described previously.[28] Thirty micrograms of RNA were electrophoretically separated in 1% formaldehyde-agarose gel in an aqueous buffer containing 5 mmol/L sodium acetate, 1 mmol/L EDTA, and 20 mmol/L 3-[*N*-morpholino] propanesulfonic acid, pH 7.0. Northern transfer was carried out in 10X SSC solution, and the membrane was cross-linked as described.

The cDNAs of rat ALB (n19–638), AFP (n147–408), CA II (n35–619), and aldolase B (n140–945) were cloned by the reverse transcription-polymerase chain reaction (RT-PCR),[29] and subcloned at the *Sma*I site of the pGEM-4Z plasmid (Promega, Madison, WI). The sequences of these genes were obtained from the Genebank file contained in Entrez (National Center for Biotechnology Information, Bethesda, MD), and the PCR primers were selected by using the Oligo 4.0 (Plymouth, MN) software. The identity of these cloned cDNA fragments was confirmed by sequencing with the Sequenase version 2.0 DNA kit (Amersham). Recombinant plasmids were linearized with a suitable restriction endonuclease to avoid the generation of 3′-overhang.[30] [^{32}P]UTP-labeled sense and anti-sense cRNAs were generated from the linearized cDNA templates with the SP6/T7 riboprobe Gemini II core system (Promega). These probes were used for Northern or *in situ* hybridization.

Membranes were prehybridized at 60°C for 2 hours in a solution containing 50 mmol/L Na_2HPO_4, pH 6.5, 0.8 mol/L NaCl, 1% sodium dodecyl sulfate, 1 mmol/L EDTA, 50% deionized formamide, 100

μg/ml salmon sperm DNA, and 0.5 ng/ml yeast tRNA. After adding the labeled cRNA probes, hybridization was carried out overnight at 60°C in the same solution minus the salmon sperm DNA and yeast tRNA. Washing was performed at 70 to 75°C in a solution containing 0.0125 mol/L NaCl and 0.125% sodium dodecyl sulfate for three to five times of 10 minutes each. Membranes were exposed to Kodak XAR-5 film for 5 to 10 days.

In Situ *Hybridization*

Formaldehyde-fixed paraffin sections or frozen sections of tissue previously fixed in PBS containing 4% paraformaldehyde were used. Sections were first treated with 20 μg/ml proteinase K (Boehringer Mannheim Canada, Laval, Quebec, Canada) at 37°C for 20 minutes, and the reaction was stopped by immersing in 4% paraformaldehyde solution for 5 minutes. After three washes in PBS, sections were immersed in a solution of 0.25% acetic anhydride in 0.1 mol/L triethanolamine to reduce nonspecific noise,[32] dehydrated in ethanol and then air dried. ^{32}P- or ^{35}S-labeled anti-sense or sense cRNA probes were prepared as described above, and the length of the probes was reduced to approximately 200 bp by partial alkali hydrolysis.[33] Labeled probe (1×10^6 to 5×10^6 cpm) was added onto each section and incubated for 16 hours at 50°C in a humidified chamber. Unhybridized probes were removed by treatment with 20 μg/ml RNAse A (ICN Biochemical) in 2X SSC at 37°C, followed by washes in SSC solution at decreasing concentrations (from 2X to 0.1X). After dehydration in increasing concentrations of ethanol, sections were air dried, coated with autoradiographic emulsion (Amersham), and incubated at 4°C in a light-tight box for 5 to 14 days. Step sections were stained with H&E for histological evaluation. Sections hybridized with a sense probe or sections treated with RNAse solution before hybridization served as negative controls.

Results

Genetic Labeling of Normal Pancreatic Duct Epithelial Cells

We found that the liposome-mediated transfection method was very efficient in labeling these propagable cultured rat pancreatic duct epithelial cells with the *E. coli* β-galactosidase reporter gene. From more than 50 colonies present in a 100-mm tissue culture plate, five cell strains (RP2-*Lac*Z C1–5) were isolated. The integration of the bacterial *lac*Z gene in these cell strains was confirmed by Southern analysis showing a random integration of the *lac*Z gene into the genomic DNA (Figure 1), by the expression of the *lac*Z mRNA, and by a positive histochemical staining for β-galactosidase enzyme activity (data not shown). The parental untransfected cells did not show any of these features.

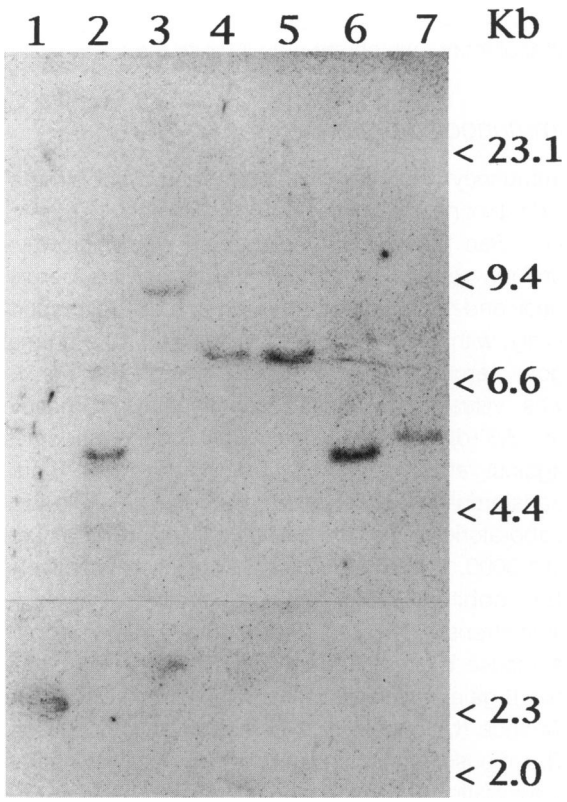

Figure 1. *The Southern blot analyses of DNA from the parental cell line* (**lane 1**), Lac*Z gene-transfected stock cell line* (**lane 2**), *and five clonal cell strains* (**lanes 3–7** *correspond to strains 1–5) showing the random integration of* Lac*Z gene in the genome of the transfected cells.*

Phenotypic Properties of Labeled Duct Epithelial Cells

Table 1 summarizes the *in vitro* phenotypic properties of these *lac*Z-labeled duct epithelial cell strains, as studied by immunocytochemistry, enzyme histochemistry, and mRNA expression. All five duct epithelial cell strains showed identical phenotype. These cells expressed CA II[13] and alkaline phosphatase activities but were negative for γ-glutamyl transpeptidase activity and glycogen storage. RT-PCR technique also demonstrated the presence of low levels of mRNA transcripts for CA II, ALB, and AFP, but they were not detectable by Northern hybridization (Figure 2). Aldolase B mRNA was not detected by either technique. Immunohistochemistry

Table 1. *Phenotypic Profiles of Adult Rat Hepatocytes, Cultured* lacZ*-Labeled Cells, and* lacZ*-Positive Hepatocyte-Like Cells Located Subcutaneously and Intraperitoneally*

Phenotypes	Adult parenchymal hepatocytes	Cultured *lac*Z-labeled RP-2 cells	Hepatocyte-like cells located subcutaneously	Hepatocyte-like cells located intraperitoneally
ALB*	+++	−	+	+++
AFP	−	−	+	−
Transferrin	+++	−	+	+++
TAT	+++	−	+	+++
Aldolase B†	+	−	+	+
CA II	−	+	+	−
Cytokeratin 8	+	+	+	ND
Cytokeratin 19	−	−	−	−
Alkaline phosphatase	−	+	−	−
γ-glutamyl transpeptidase	−	+	+	ND
HES6	+	−	−	+
BDS7	−	−	−	−
Glycogen storage	+	−	−‡	+

ND, not done; +, positive; −, negative.
* The relative levels of ALB, AFP, transferrin, and TAT were determined by the intensity of immunostaining. For other phenotypic markers, only − or + was marked.
† Determined by mRNA expression.
‡ Only a few cells (<2%) contained a scant cytoplasmic glycogen deposit.

failed to detect the presence of ALB, AFP, transferrin, TAT, chromogranin, insulin, glucagon, and amylase. The great majority of the cells stained positively for cytokeratin 8 but negatively for cytokeratin 19. Immunohistochemistry with the monoclonal antibodies HES6 and BDS7 were negative.

Subcutaneous Implantation

Two RP-2 cell strains (RP2-*Lac*ZC2 and RP2-*Lac*ZC5) were randomly chosen for *in vivo* implantation study. The abdominal subcutaneous tissue was selected because of its easy accessibility and the absence of host epithelial cells. Extracellular matrix as cell embedding medium was essential for the localization, improved colonizing efficiency, and survival of these implanted cells. Four to eight weeks after implantation, a piece of pinkish-white fat-like tissue localized over the rectus abdominis muscle was found. No similar tissue was noted in the control animals transplanted with only extracellular matrix gel. After incubation in the reagent solution for bacterial β-galactosidase enzyme activity, numerous blue spots appeared within the tissue (Figure 3A). These blue foci represented aggregates of cells that had cytoplasm containing numerous blue granules (Figure 3C). The same enzymatic reaction performed on fresh tissues of rat kidney, liver, pancreas, and muscle did not produce blue foci or cells containing cytoplasmic blue granules (data not shown). H&E staining of paraffin sections of formalin-fixed tissue showed nests of epithelioid cells with abundant eosinophilic but finely vacuolated cytoplasm scattered among adipocytes (Figure 3B). Sudan IV stain dem-

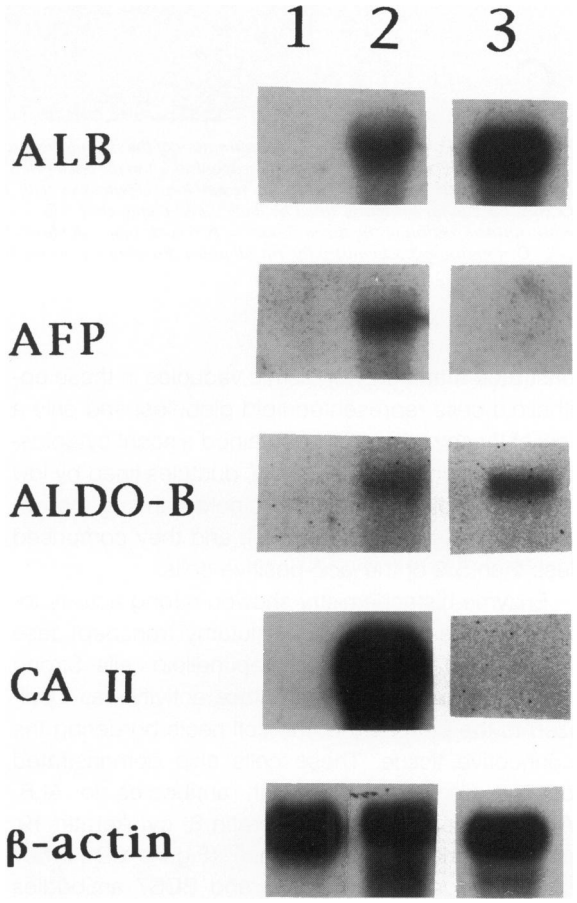

Figure 2. *Northern blot analyses showing the relative expression of ALB, AFP, aldolase B (ALDO-B), and CA II in the transfected cell strain used in implantation* (lane 1)*, in the subcutaneous tissue containing the implanted cells* (lane 2)*, and in adult rat liver* (lane 3)*. RNA was hybridized with* ^{32}*P-labeled cRNA (ALB and AFP) or cDNA (CA II and aldolase B) probes. The mRNA sizes are 2.3 kb for ALB, 2 kb for AFP, 1.6 kb for aldolase B, and 1.5 kb for CA II.*

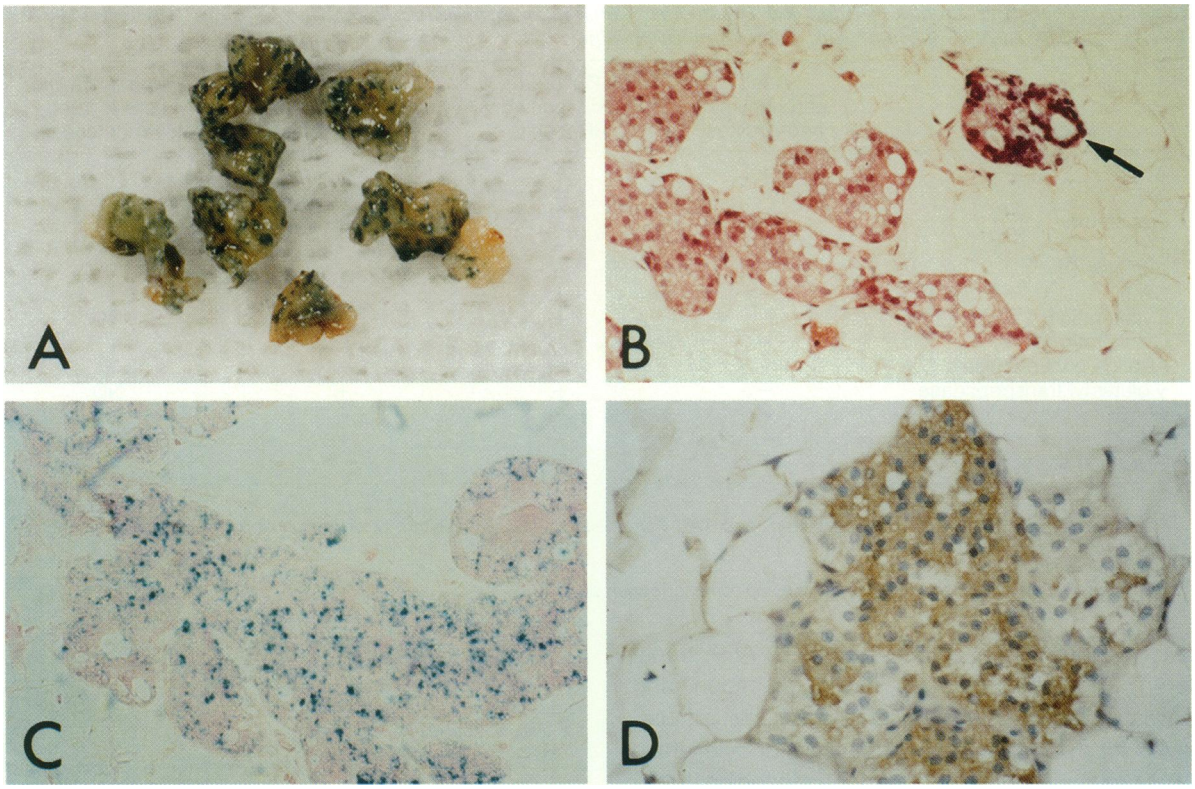

Figure 3. *Morphological/histological examination of the subcutaneously placed cells after 8 weeks of implantation.* **A:** *Incubation of the subcutaneous tissue containing implanted cells in the reagent solution for bacterial β-galactosidase activity reveals the presence of large* LacZ*-transfected epithelioid cells.* **B:** *Nests of large epithelioid cells resembling hepatocytes with finely granular and vacuolated cytoplasm are scattered among adipocytes. Occasional ductal structures lined by low cuboidal epithelial cells are also noted adjacent to these nests of hepatocyte-like cells (arrowhead).* **C:** *An eosin-stained section of the tissue shown in* **A** *reveals blue cytoplasmic granules in these hepatocyte-like cells, confirming the* in vitro *origin of these cells.* **D:** *Immunohistochemistry for rat albumin also shows a strong positive staining in these cells. Magnification, ×30 (***A***); ×180 (***B***); ×300 (***C***); ×300 (***D***).*

onstrated that the cytoplasmic vacuoles in these epithelioid cells represented lipid globules, and only a few of these cells (<2%) contained a scant cytoplasmic glycogen deposit. Focally, ductules lined by low columnar epithelial cells were noted among the epithelioid cell nests (Figure 3B), and they comprised less than 5% of the *lac*Z-positive cells.

Enzyme histochemistry showed strong activity for CA II and weak activity for γ-glutamyl transpeptidase in the cytoplasm of the large epithelioid cells. Strong staining for alkaline phosphatase activity was localized to the periphery of the cell nests bordering the connective tissue. These cells also demonstrated positive immunostaining with antibodies to ALB, AFP, transferrin, TAT, cytokeratin 8, cytokeratin 19, and bacterial β-galactosidase (Figure 3D). They stained negatively for HES6 and BDS7 antibodies and antibodies to several pancreatic enzymes/hormones, including amylase, insulin, glucagon, and chromogranin. In contrast to the nests of epithelioid cells, the ductular epithelium showed strong cytoplasmic alkaline phosphatase activity.

Transmission electron microscopic examination revealed the presence of bile canalicular-like structures between adjoining epithelioid cells (Figure 4). Other organelles were sparse, but lipid globules were evident.

Northern blot analysis of RNA isolated from the subcutaneous tissue confirmed the presence of mRNA transcripts for albumin (2.3 kb), α-fetoprotein (2 kb), aldolase B (1.6 kb), and CA II (1.5 kb) (Figure 2). These mRNA transcripts were also detected by the RT-PCR technique. *In situ* hybridization studies confirmed that the CA II, albumin, aldolase B, and AFP mRNA were expressed in the large vacuolated hepatocyte-like cells.

Intraperitoneal Implantation

Six weeks after implantation with cell-containing gel matrix, a whitish mass was found wrapped around the mesentery of a segment of small bowel. Sections stained with H&E showed trabeculae and nests of large polygonal epithelioid cells scattered in a des-

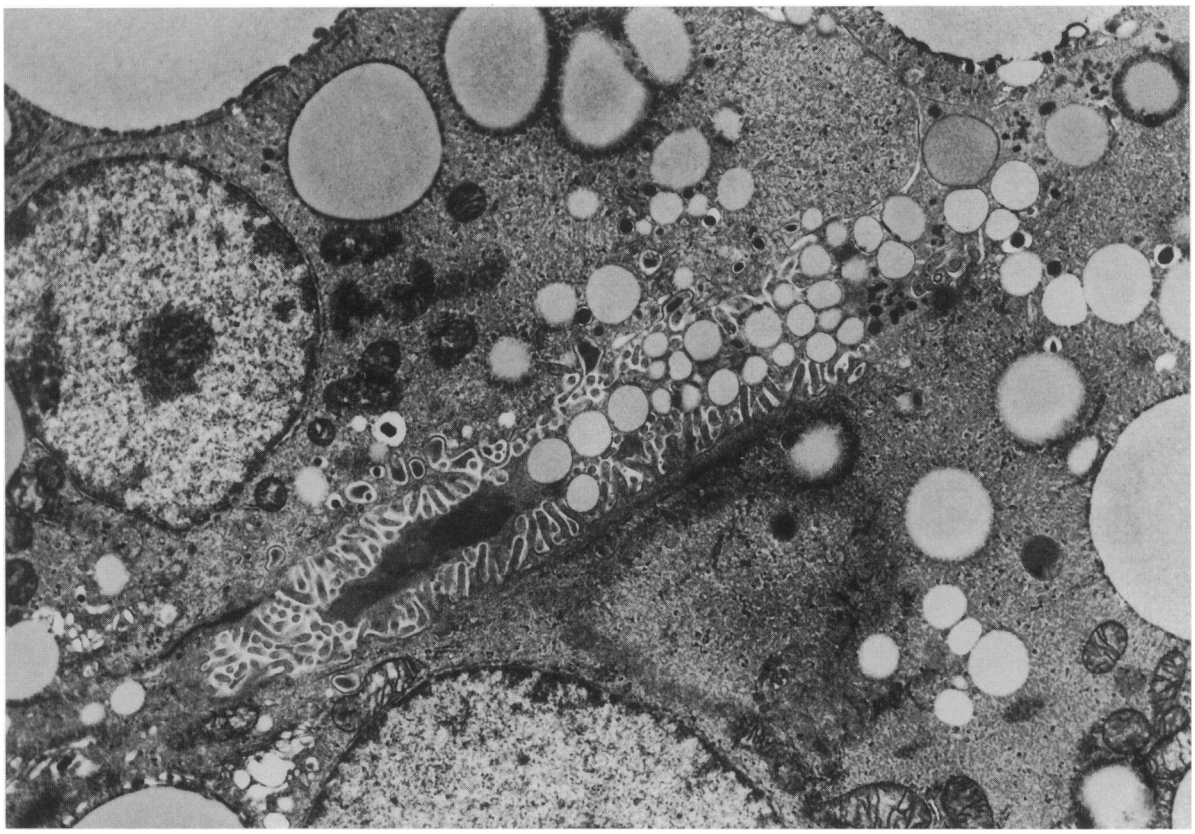

Figure 4. *Transmission electron microscopy reveals the presence of bile canalicular-like structures between adjoining hepatocyte-like cells present in the subcutaneously implanted tissue. Magnification, ×9280.*

moplastic mesentery. The cells histologically resembled the mature hepatocyte of adult rat liver, demonstrating central nuclei and granular eosinophilic cytoplasm (Figure 5A). They contained abundant cytoplasmic glycogen (Figure 5B) but were devoid of lipid globules. Immunohistochemistry with monoclonal antibody to the *E. coli* β-galactosidase yielded a strong positive reaction, confirming the *in vitro* origin of these cells (Figure 5C). As a negative control, hepatocytes of normal adult rat stained negatively with the same antibody. Similar to the subcutaneous site, the hepatocyte-like cells were predominant, representing approximately 95% of the *lac*Z-positive cells, although *lac*Z-positive ductules were also noted among these hepatocyte-like cells (Figure 5C). With the same titer of primary antibodies, the hepatocyte-like cells showed stronger immunoreactivities for TAT (Figure 5D), ALB, and transferrin than the similar cells localized subcutanously. These cells stained positively for HES6 but negatively for BDS7 antibodies. Staining with antibodies against various pancreatic enzymes/hormones also proved negative. *In situ* hybridization studies on these tissue sections confirmed the presence and localization of aldolase B and ALB (Figure 6, A and B) mRNA transcripts in these hepatocyte-like cells, whereas mRNA of AFP and CA II were not detected.

Discussion

We have demonstrated that, after *in vivo* implantation, propagable cultured cells derived from the duct epithelium of adult rat pancreas developed the phenotypic features of a hepatocyte and that the extent of this phenotypic expression is influenced by the microenvironment in which these cells are localized. The propagable normal pancreatic epithelial cell line used in this experiment was established from a duct fragment selectively picked with a Pasteur pipette under a phase contrast microscope.[13] This was performed to confirm specifically that the cells we used were of ductal origin. These cultured cells show low level expression of *in vivo* phenotypes of ductal cells such as CA II, alkaline phosphatase, and γ-glutamyl transpeptidase as well as low level expression of ALB and AFP mRNAs. The expression of ALB and AFP has been reported in the oval cells of rat liver *in vivo* and *in vitro* and in cultured normal rat liver epithelial cells.[33,34] Periductal oval cells from copper-

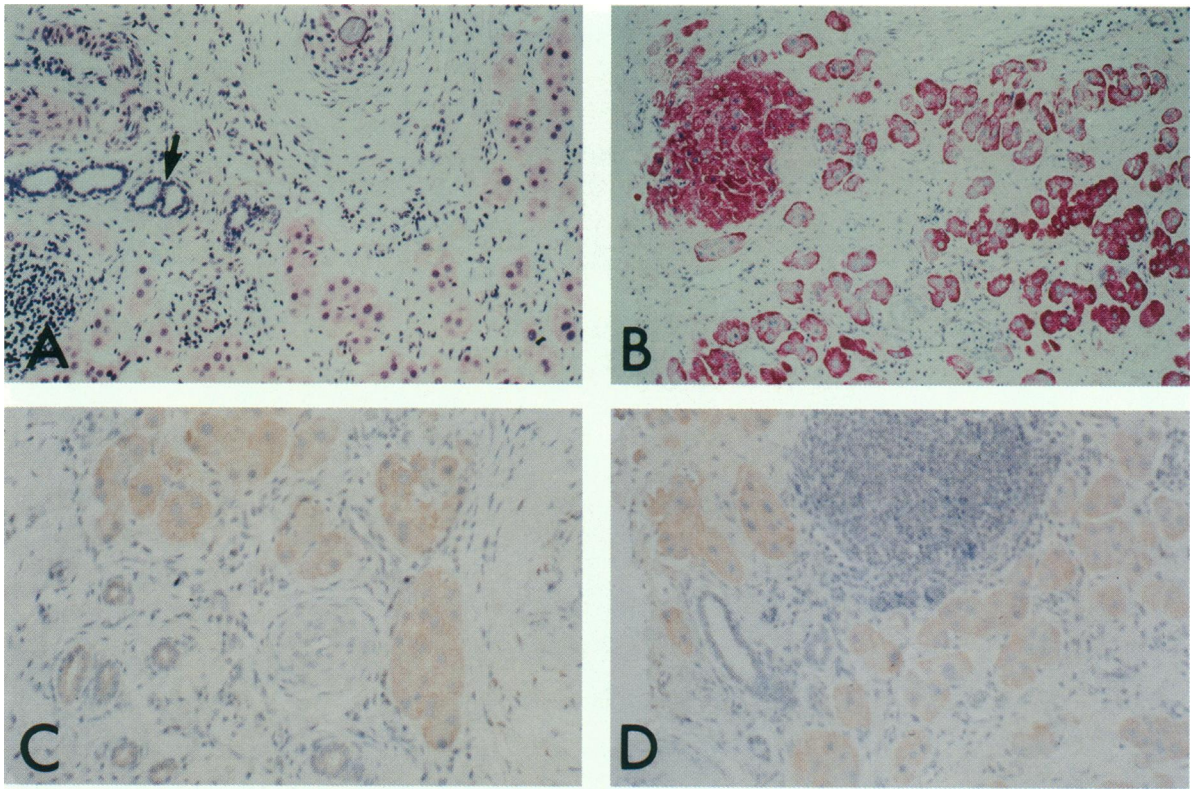

Figure 5. *Histology of the intraperitoneally implanted tissue.* A: *An H&E section shows islands/cords of hepatocyte-like cells with eosinophilic granular cytoplasm embedded in a desmoplastic stroma. Ductal structures were also noted focally (arrowhead).* B: *These hepatocyte-like cells contain abundant diastase-sensitive periodic acid-Schiff (PAS)-positive material consistent with glycogen.* C: *Both hepatocyte-like and ductal cells show strong immunostaining with antibody against the bacterial β-galactosidase.* D: *These hepatocyte-like cells demonstrate strong immunostaining for tyrosine aminotransferase. Magnification, ×180 (A); ×180 (B); ×300 (C); ×300 (D).*

deficient rats also express albumin *in vivo* and *in vitro*.[6,15] These data support the hypothesis that the cells of duct epithelia of adult rat pancreas and hepatobiliary tree share common phenotypic and histogenetic properties.[12,35]

The acquisition and maintenance of cell differentiation result from synergistic interplays between intrinsic and environmental factors; the latter include growth factors, cell surface molecules, and extracellular matrix proteins.[36] The environment can regulate

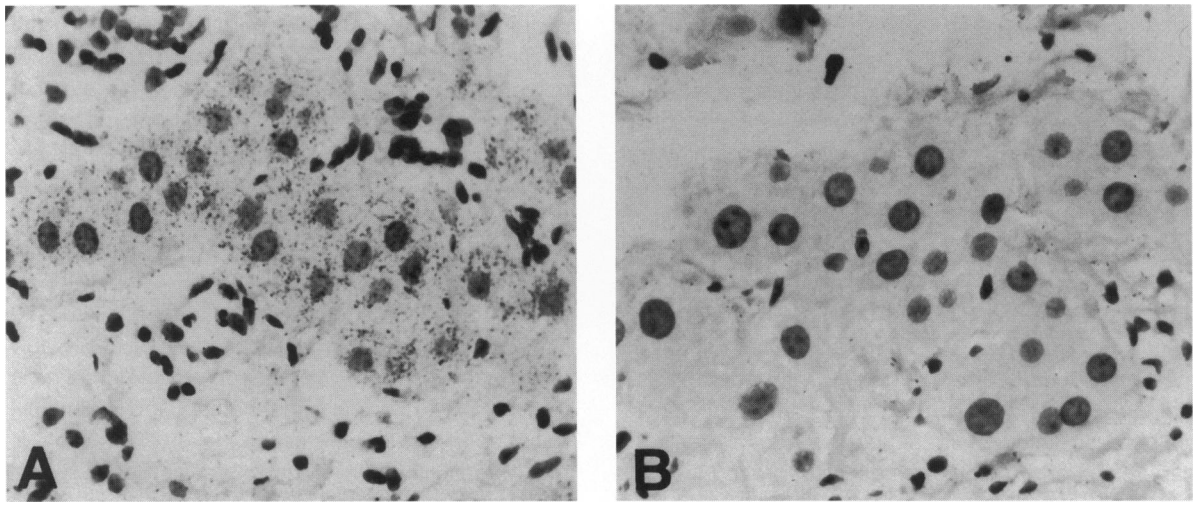

Figure 6. *An* in situ *hybridization with ^{32}P-labeled albumin cRNA on a section from the intraperitoneal tissue shows the presence of abundant albumin mRNA in the hepatocyte-like cells* (A) *and an absence of silver granules in the negative control with the sense cRNA probe. Magnification, ×700.*

not only the decision to differentiate but also the range of genes expressed during the differentiation process. However, the responses to such environmental stimuli depend on the intrinsic programming mechanism of the cell.[37] Thus, implantation of cells in different cellular milieu will not only reveal the nature of these cells but also reflect the effects of the microenvironment on their phenotypic expression. The results of our study represent the most direct evidence yet to support the ductal cell origin of transdifferentiated pancreatic hepatocytes. When localized in the systemic circulatory compartment, the cells displayed partial differentiation toward hepatocytes but retained some of their ductal phenotype. In contrast, when the same cells were implanted intraperitoneally, they expressed the full phenotypic properties of mature hepatocytes, including their morphological appearance and the loss of AFP and CA II expression. Additional studies are needed to determine whether the modulating effect of the microenvironment on the extent of differentiation of these cells is related to an exposure to the portal circulation. Coleman et al[38] reported that, when cultured rat liver epithelial cells were transplanted into the liver parenchyma, they fully differentiated into mature hepatocytes and became integrated into the hepatic cell plates, thus becoming morphologically indistinguishable from native parenchymal cells. We have demonstrated further that direct contact with host hepatocytes is not a prerequisite for inducing hepatocytic differentiation, suggesting that factors other than cell surface molecules are important in the induction of this differentiation pathway. Wu et al[39] have reported that, in some hepatocyte cell lines derived from mice transgenic for transforming growth factor-α, the expression of liver-specific proteins such as ALB, TAT, and transferrin gradually diminished when these cells were continuously cultured and passaged in serum containing medium. However, a short-term culture in serum-free medium can restore the high levels of expression of these genes. These results confirm that the expression of hepatocyte-specific genes is modulated by extracellular factors.

Although hepatocytes can be identified in the pancreas of aged rats,[40] the incidence is exceptionally low, indicating that the normal pancreatic microenvironment is not conducive for hepatocytic differentiation. Although pancreatic acinar cells demonstrate the ability to proliferate after partial resection or subtotal acinar cell necrosis,[41,42] complete regeneration is not seen after the severe injury used in the experimental protocol used for the induction of pancreatic hepatocytes in adult rats and hamsters.[1,2,4,6,43,44] The extensive destruction/atrophy of the acinar cell population may alter the reticulin framework of the pancreatic acini. Instead, proliferation of ductular and periductular cells occurs, and these cells are committed to hepatocyte rather than acinar cell differentiation. Our findings that pancreatic duct cells can differentiate toward hepatocytes after *in vivo* implantation, in the absence of stringent extracellular matrix requirements, suggests a propensity for hepatocyte differentiation for the hepatobiliary-pancreatic duct epithelial stem cells of adult animals. In contrast, acinar or endocrine cell differentiation did not occur. It is possible that the differentiation into these cells requires a more precisely defined microenvironment such as the composition and organization of the extracellular matrix and growth factors. These may be present in *in vivo* situations in which nesidioblastosis and regeneration of islets from duct cells can occur.[45,46]

The definition of differentiation potential and lineage of the duct epithelial cells in the adult pancreas may have important implications for the treatment of various pancreatic diseases. There is ample evidence that pancreatic ductal cells from various animal species can be propagated in culture to yield a large number of these multipotential stem cells. Hubchak et al[11] have demonstrated that, when propagable hamster pancreatic duct epithelial cells are grown on basement membrane matrix, they can recapitulate the morphology of differentiated ductal cells. Our results have demonstrated the possible use of these cells as stem cells of hepatocytes. We are currently investigating the capacity of these cells to differentiate along acinar or endocrine cell lineages. An understanding of the precise conditions and mechanisms undertaken by these cells to pursue a specific differentiation pathway would have a significant implication in pancreatic and liver bioengineering technologies. Moreover, these stem cells could also be used to introduce new genes into the pancreas for gene therapy purposes.

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