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Hepatocytes Undergo Phenotypic Transformation to Biliary Epithelium in Organoid Cultures

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Abstract

Organoid cultures of hepatocytes in the presence of hepatocyte growth factor (HGF) and epidermal growth factor (EGF) display characteristic histologic organization. Biliary epithelium covers the surface of the tissue exposed to the culture medium. Hepatocytes, stellate cells and endothelial cells compose the underlying structures. In order to investigate the origin of the biliary epithelial cells in the organoid cultures, we utilized the retrorsine/DPPIV system of hepatocyte transplantation to create hybrid livers in which clones of DPPIV hepatocytes colonize variable portions of the lobules. We demonstrate that, as others have shown, biliary epithelium in this *in vivo* system remains that of the recipient (DPPIV negative) rat. Hepatocytes are the only cells positive for the DPPIV marker enzyme in the hybrid livers. Organoid cultures were prepared from the hybrid livers. Overall, 46.82% of the hepatocytes placed into culture were positive for DPPIV at time zero (after isolation). At 21 days in culture, 47.54% of the biliary epithelium on the surface of the organoid cultures was positive for DPPIV. Since the only DPPIV cells inoculated in the cultures were hepatocytes, this finding demonstrates that, in the conditions of the organoid cultures, hepatocytes do undergo phenotypic transition to biliary epithelial cells.

Abbreviations

HGF, hepatocyte growth factor; DPPIV, dipeptidyl peptidase IV; EGF, endothelial growth factor; PCNA, proliferating cell nuclear antigen; DMEM, Dulbecco's modified Eagle medium; HGM, hepatocyte growth medium

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Hepatocytes and biliary epithelial cells are the two main epithelial cell types of adult liver. Several studies have shown that when hepatocyte proliferation is impaired, stimulation of hepatic regeneration leads to formation of oval cells, which in turn may become mature hepatocytes. This phenomenon has been documented in rodents (oval cells)[1,2] and in humans (ductular hepatocytes).[3–5] Numerous pieces of evidence from the literature document that oval cells emerge from the biliary epithelium, although it is not entirely clear whether the cellular sources of the oval cells reside in the portal ductules or in the canals of Herring. In the model of suppressed hepatic regeneration by acetylaminofluorene expression of hepatocyte-specific transcription factors appears in the epithelium of the biliary ductules after partial hepatectomy.[6] Oval cells express both biliary and hepatocytic markers.[7] The same hybrid phenotype has also been shown in ductular hepatocytes.[4] Administration of methylene diamiline (DAPM), a toxin specific for the biliary epithelium, immediately before the acetylaminofluorene–partial

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hepatectomy protocol, prevents the appearance of oval cells.[8] The relationship between hepatocytes and small ductular cells is also shown in extrahepatic sites. Cells of small pancreatic ductules are also the cells of origin of hepatocytes in the pancreatic hepatization protocol.[9,10]

Transformation of hepatocytes into biliary epithelium in the whole liver often has been argued on the basis of histologic observations but has not been clearly proven. Hepatocytes often tend to assume acinar configuration during mechanical or chemically induced cholestasis. During embryology, hepatoblasts are assumed to be the origin of the cells forming the ductal plates, which eventually migrate into the portal triads to form biliary ductules.[11,12] Hepatocytes in collagen gels also assume ductular configurations in the presence of hepatocyte growth factor (HGF).[13]

We recently described a system of organoid cultures in which hepatocytes isolated by collagenase perfusion, and the accompanying contaminant nonparenchymal cells, organize into characteristic and reproducible histologic architecture in which the surface exposed to the medium is covered by biliary epithelium.[14] We used the retrorsine/dipeptidyl peptidase IV (DPPIV) hybrid liver system described by Laconi et al.[15] and Shafritz et al.[16] to create hybrid livers in which only hepatocytes, and not the biliary epithelium, carry the specific marker DPPIV. We generated organoid cultures from the hybrid livers to investigate the origin (hepatocytic or biliary) of the biliary epithelium seen in the organoid cultures.

Materials and Methods

Animals

Fischer 344 male rats, DPPIV-positive, were obtained from Charles River Laboratories, Frederick, MD. A colony of German Fischer 344-negative rats was obtained from founders donated by Dr. Bryon Petersen, University of Florida. The colony is maintained in the Animal Facility of the University of Pittsburgh School of Medicine. The animal husbandry and all procedures performed on the rats used for these studies were approved under the Institutional Animal Care and Use Committee protocol 0699068A-1 and conducted according to National Institutes of Health guidelines.

Materials

Epidermal growth factor (EGF) was obtained from Collaborative Biomedical (Waltham, MA). Collagenase for hepatocyte isolation was obtained from Boehringer Mannheim (Mannheim, Germany). Vitrogen (from Celtrix Labs, Palo Alto, CA) was used for collagen coating of roller bottles. General reagents and retrorsine were obtained from Sigma (St. Louis, MO). EGF was purchased from Collaborative Biomedical. HGF used for these studies was the Δ5 variant and was kindly donated by Snow Brand Co. (Toshigi, Japan). Antibodies were obtained from the following sources: proliferating cell nuclear antigen (PCNA) from Signet laboratories (Dedham, MA); HEPPAR from Dako Corp. (Carpinteria, CA).

Retrorsine/DPPIV Protocol and Methodology

The protocol established by Laconi et al.[15] was used for the studies. Male German Fischer rats (DPPIV-negative), weighing 200 g, were given 2 intraperitoneal injections with retrorsine, 30 mg per kg body weight, dissolved in water. The injections were given 15 days apart. A month after the last injection, the rats were subjected to a two-thirds partial hepatectomy, as described by Higgins and Andersen.[17] During the partial hepatectomy operation, the rats were also injected with 3.5 million hepatocytes isolated immediately before the operation from a DPPIV-positive Fisher 344 male rat. The animals were left to recover and were not subjected to any other experimental procedures for the next 3 months.

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Generation and Maintenance of Organoid Cultures

The techniques used to generate the organoid cultures from the livers of the rats subjected to the retrorsine/DPPIV protocol were exactly as described recently.[14] Freshly isolated hepatocytes were added to roller bottles (1,450 cm^2 pleated surface) obtained from Falcon (Franklin Lakes, NJ). Each bottle contained 210 million freshly isolated hepatocytes in 250 mL of hepatocyte growth medium (HGM) supplemented with HGF (40 ng/mL) and EGF (20 ng/m). The bottles were rotated at a rate of 2.5 rotations per minute and kept in an incubator maintained at 37°C, saturated humidity, and 5% CO_2. The medium HGM was used to maintain the cultures. The composition of the medium was also as recently described. Dulbecco's modified Eagle medium (DMEM) liquid medium, HEPES, glutamine, and antibiotics were purchased from GIBCO/BRL (Grand Island, NY). Insulin, transferrin, selenium mixture was purchased from Boehringer Mannheim. All other additives were cell-culture grade (Sigma). Unless otherwise indicated for specific experiments, the basal HGM consisted of DMEM supplemented with purified bovine albumin (2.0 g/L), glucose (2.25 g/L), galactose (2.0 g/L), ornithine (0.1 g/L), proline (0.030 g/L), nicotinamide (0.305 g/L), $ZnCl_2$ (0.544 mg/L), $ZnSO_4{:}7H_2O$ (0.750 mg/L), $CuSO_4{:}5H_2O$ (0.20 mg/L), $MnSO_4$ (0.025 mg/L), glutamine (5.0 mmol/L), and dexamethasone (10^{-7} mol/L). Gentamicin, 50 μg/mL, was added to the basal HGM. The mixed basal HGM was sterilized by filtration through a 0.22-μm low-protein–binding filter system, stored at 4°C, and used within 4 weeks. Insulin, transferrin, selenium mixture 1.0 g/L (rhinsulin 5.0 mg/L, human transferrin 5.0 mg/L [30% diferric iron saturated] selenium 5.0 μg/L), was added after filtration immediately before use. The growth factors, as required, were added to HGM fresh at the specified concentrations every time the medium was changed. Tissue samples were taken at different times during the cultures to assess histology.

DPPIV Histochemistry

Staining for DPPIV was performed as previously described.[15,18] The staining was performed on hepatic tissue, removed before the initiation of the collagenase perfusion, on dried smears of freshly isolated cells immediately after collagenase perfusion, and on tissue removed from the organoid cultures. The tissues were frozen and sectioned by using a cryostat. Sections of 4 to 5 μm in thickness were used for histologic analysis. Smears of freshly isolated cells were fixed by air-drying and were stained on the glass slides.

Immunostains for PCNA and cytokeratin 19 were performed as previously described for organoid cultures.[14]

Results

Histology of the Hybrid Livers

A stain for DPPIV enzyme of a liver fragment (approximately 1 × 2 cm) resected immediately before the initiation of the collagenase perfusion is shown in Fig. 1A and B. Variable proportions of the hepatocytes per lobule express the marker, indicating origin from the donor DPPIV-positive cells injected 3 months before the cell isolation. The percentage of DPPIV-positive cells differed between lobules, averaging 40% overall (Fig. 1A). Biliary ductules were all negative for DPPIV (Fig. 1B). The hepatic fragments were serially sectioned throughout and all sections were stained for DPPIV and counterstained with hematoxylin. All biliary epithelial cells were assessed in all the serial sections. A total of 2,240 biliary epithelial cells were thus counted and assessed for DPPIV. All biliary cells were negative for the DPPIV marker.

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DPPIV Stain in the Freshly Isolated Cells

Staining for DPPIV in a dried smear of freshly isolated hepatocytes from the hybrid livers is shown in Fig. 2. Overall, 46.82% of the hepatocytes were positive for the marker. A suspension from these cell preparations was used to prepare the organoid cultures.

DPPIV Stain of Tissue From the Organoid Cultures

The full histologic maturation of the organoid cultures, as shown in our previous studies, occurs between 15 to 21 days in culture. Tissue was removed from the cultures at 21 days and stained for DPPIV. The results are shown in Fig. 3A and B. Hepatocytes bearing the DPPIV marker as well hepatocytes negative for the marker were seen in the organoid culture tissue. Biliary epithelium cells, arranged linearly on the surface of the tissue section, expressed the DPPIV marker. From 183 cells counted, 47.54% of the biliary cells were positive for DPPIV. Patches of DPPIV-positive and DPPIV-negative biliary epithelial cells were interspersed randomly on the surface of the sections.

The transformation of DPPIV-positive hepatocytes into DPPIV-positive biliary epithelium seems to be a gradual process. Organoid culture tissues removed at day 5 (Fig. 4) show the earliest appearance of DPPIV-positive cells lining up the surface of the sections. By day 11, the histologic picture is comparable to that seen at day 21 (data not shown).

Although the DPPIV-positive cells were interspersed with the DPPIV-negative cells, they did not appear to be distinguishable by any other criterion. A stain for cytokeratin 19 (Fig. 5A) shows that more than 90% of the surface biliary epithelium is positive for cytokeratin 19. A stain for PCNA (Fig. 5B) also shows that more than 90% of the surface biliary epithelium is positive, suggesting that both DPPIV-positive and DPPIV-negative cells are into the cell cycle. A stain for the hepatocyte marker HEPPAR also shows that the surface biliary epithelium is negative for this marker. These results are the same as those we reported previously.[14] Electron microscopic studies of the surface biliary epithelium were also shown in our previous report.[14]

Discussion

The combined evidence presented from our cell culture and whole animal studies shown in Figs. 1 through 5 shows that hepatocytes have the capacity to undergo phenotypic transformation to biliary cells in the environment of the organoid cultures. Because only hepatocytes *in vivo* are positive for the DPPIV marker, the DPPIV-positive biliary epithelium of the organoid cultures must be derived from them. This conclusion is also reinforced by the proximity of the percentage numbers of the inoculated DPPIV-positive hepatocytes (46.82%) and the DPPIV-positive biliary epithelial cells (47.54%). The percentage of the DPPIV-positive hepatocytes was more difficult to assess, varying from 20% to 60% in different organoid tissue fragments.

The biliary epithelium, which is negative for DPPIV, may, theoretically, derive from either the DPPIV-negative hepatocytes of the recipient animals or from contaminant DPPIV-negative biliary epithelium. Such contamination of hepatocytes with a variable small admixture of biliary, stellate, endothelial, and other cell types is always noticed in hepatocyte preparations after collagenase perfusion.[19] It is of interest that, despite the retrorsine treatment, the DPPIV-negative isolated cells of the recipient are capable of participating in the formation of the organoid cultures. Studies with the retrorsine/partial hepatectomy model have shown that not all cells are rendered incapable of proliferation and that a variable percentage of hepatocytes or hepatocyte-like cells escapes the full effect of retrorsine and remains capable of growing into small clones *in vivo*.[20,21] In our studies, the percentage of PCNA-positive hepatocytes

and biliary epithelial cells (90%) exceeded the percentage of DPPIV-positive biliary cells of hepatocytes (<50%), suggesting that at least some of the DPPIV-negative (retrorsine-treated) cells may participate in the cell cycle.

The findings of this work raise the possibility that the phenotypic transformation of hepatocytes to biliary epithelial cells may also occur *in vivo*, as suggested from histologic observation. This cannot be concluded from the current work, which is limited to cell culture. The advantage of the retrorsine model, however, is that it allows for selective tagging of the hepatocytes, and thus it may be applicable to investigate the possibility of phenotypic conversions of hepatocytes to biliary epithelial cells in animal models of chronic or acute biliary damage, such as treatment with DAPM,[8] bile duct ligation,[22] etc. Further studies with these models are required to pursue this issue.

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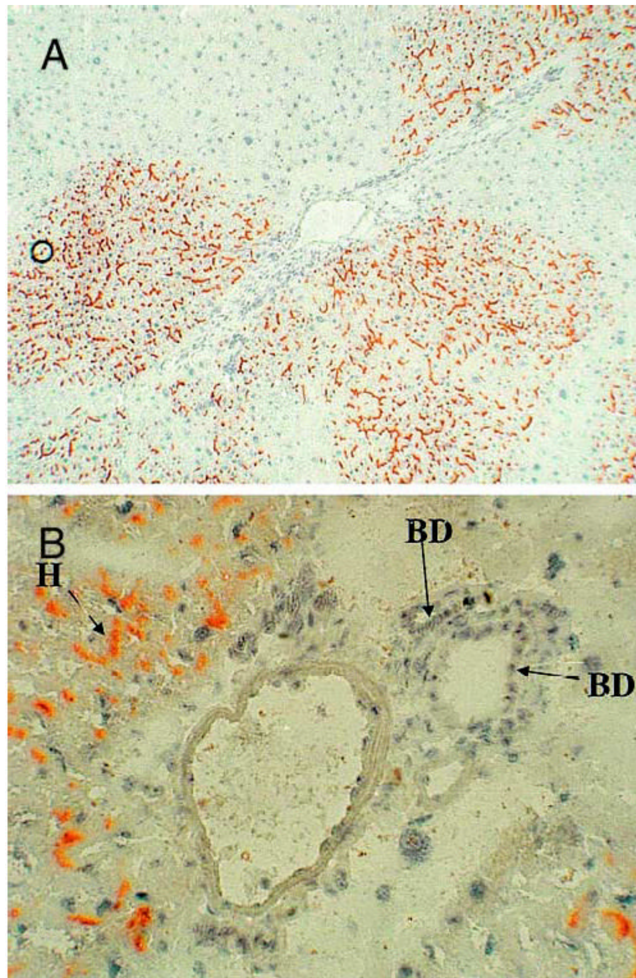

Fig. 1.

(A) Histochemical stain for DPPIV of the hybrid livers at 3 months after infusion of DPPIV-positive hepatocytes into DPPIV-negative rat liver, after treatment with retrorsine and partial hepatectomy. Positive stain result is indicated by the canalicular pattern of **red/orange color**. Variable portions of lobules are occupied by the DPPIV-positive cells. (B) Higher-power photomicrograph of a portal triad from a hybrid liver. Many hepatocytes (H) are positive for DPPIV, whereas biliary epithelium (BD) is uniformly negative.

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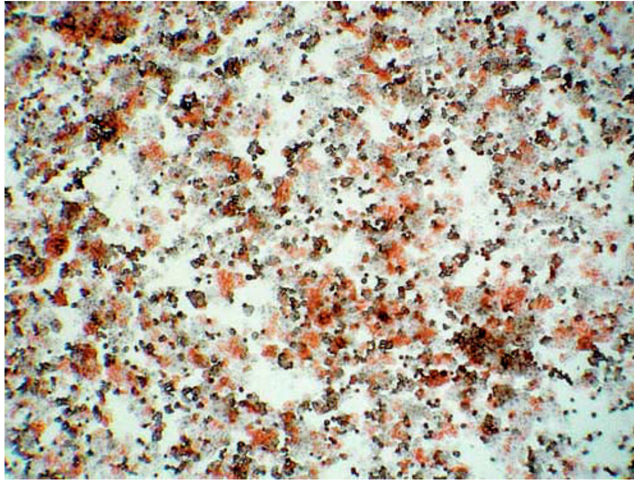

Fig. 2.
DPPIV stain of air-dried smear of freshly isolated hepatocytes from a hybrid liver. Positive cells are stained **red/orange**. Normally the stain is limited to the bile canaliculus, but after collagenase perfusion, the canalicular structures are disrupted and canalicular markers are seen over the entire hepatocyte membrane.[19]

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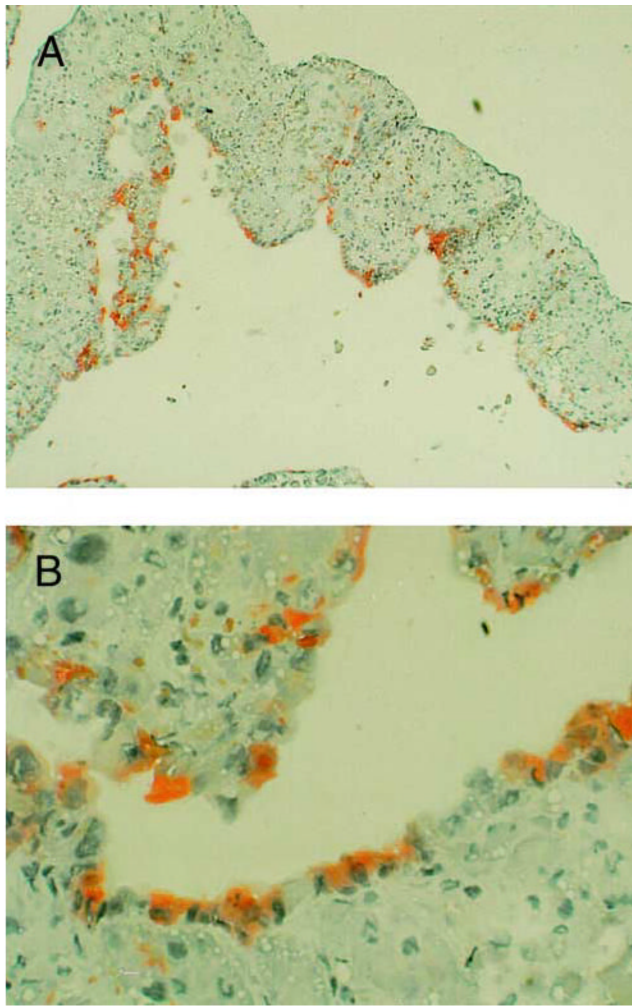

Fig. 3.

(A) Low-power (×20) and (B) high-power (×100) photomicrographs showing patches of DPPIV-positive biliary epithelial cells on the surface of the organoid structures at day 21. DPPIV-negative cells are interspersed between the DPPIV-positive cells, suggesting double origin of the biliary cells from both donor (+) and recipient (−) hepatocytes.

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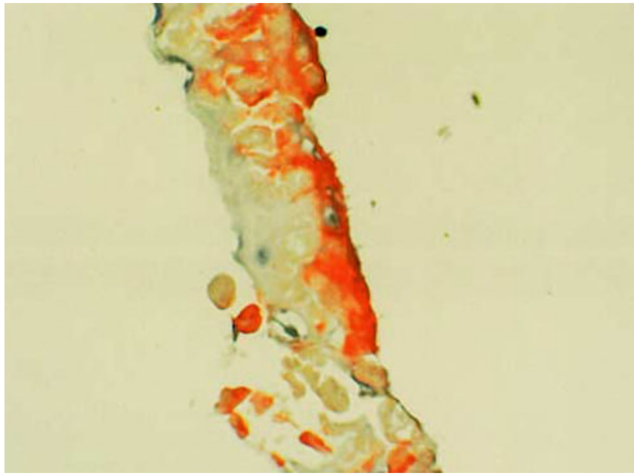

Fig. 4.
DPPIV stain of organoid cultures at day 5. Several DPPIV-positive hepatocyte-like cells are seen near the surface of the cultures, but mature biliary epithelium is not yet present.

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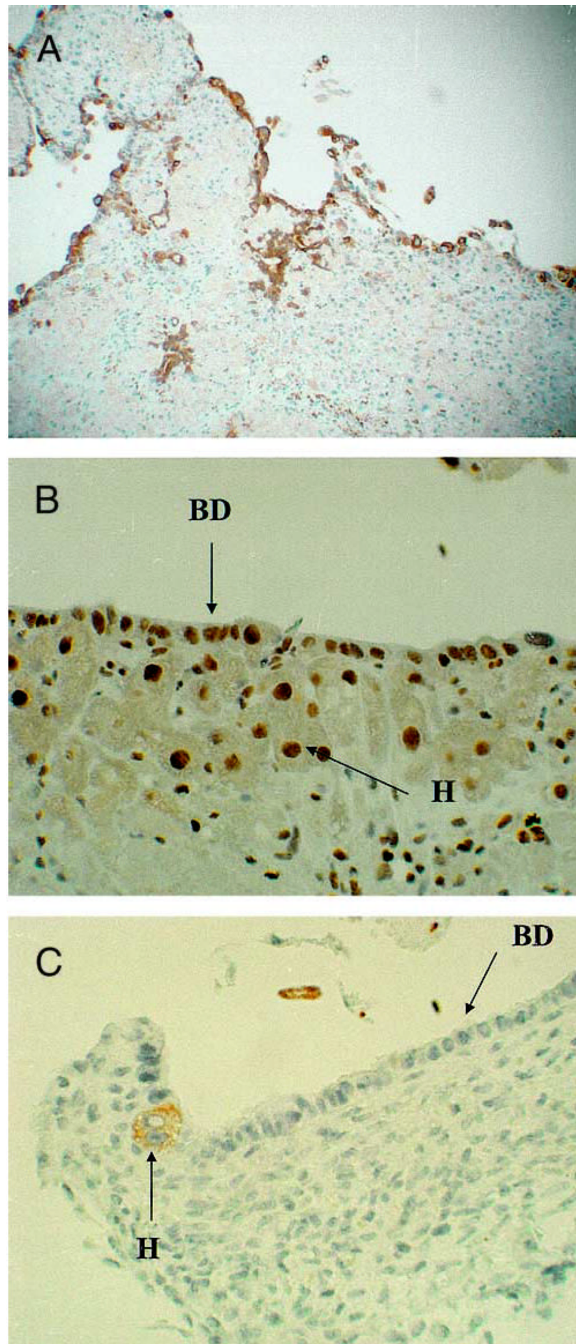

Fig. 5.

Organoid cultures from hybrid livers at day 21. (A) Immuno-histochemical stain for cytokeratin 19, a marker for biliary epithelium. Most of the surface biliary epithelium is positive for cytokeratin 19. (B) Immunohistochemical stain for PCNA. Most of the surface biliary epithelium and the underlying hepatocytes have positive (**brown color**) nuclei, indicating the cells are into the cell cycle. (C) Immunohistochemical stain for the hepatocyte marker HEPPAR shows that the biliary epithelium (BD) is negative for this marker. A single hepatocyte (H) adjacent to the surface biliary epithelium is positive for HEPPAR.