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Cell Behavior in the Acetylaminofluorene-Treated Regenerating Rat Liver

Light and Electron Microscopic Observations

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When hepatocyte regeneration is impaired, facultative stem cells and their descendants, also called oval cells, become activated and produce cell progeny that eventually differentiate. We have observed these cells in the rat liver after partial hepatectomy when the animals have been fed 2-acetylaminofluorene. Oval cells emerge from the portal areas and stain strongly with monoclonal antibodies raised against cytokeratins 8 and 19 and vimentin, the intermediate filament traditionally associated with mesenchymal cells. The majority of oval cells appeared to be part of a bile ductular reaction, manifest by their cytokeratin expression, and the bile duct injection of pigmented gelatin confirmed that these oval cells were essentially tortuous, arborizing duct-like structures (cholangioles) branched from and continuous with preexisting bile ducts.* In situ *hybridization studies showed that hepatocyte growth factor mRNA-expressing sinusoid lining cells were most numerous in the periportal areas during the period of ductular proliferation. At 1 week after partial hepatectomy, we observed morphological evidence of areas of* in situ *focal differentiation in the ductular structures, either to a columnar intestinal-type epithelia or to a hepatocyte phenotype, with abundant large mitochondria and membranous cytokeratin 8 immunoreactivity contrasting with the diffuse staining of the ductular cells. By following the fate of oval cells the authors conclude that in this model proliferated bile ductules represent the oval cell compartment capable of producing pluripotential progenitor cells. (Am J Pathol 1994, 145:1114–1126)

The ability of the liver to regenerate in response to the loss of hepatocytes is widely recognized and this is usually accomplished by the triggering of normally proliferatively quiescent hepatocytes into the cell cycle.[1,2] Liver regeneration is commonly studied in the rat after a two-thirds partial (PH) hepatectomy. However, when hepatocyte regeneration is impaired, relatively undifferentiated cells emerge from the portal space and take over the burden of regenerative growth. These facultative or potential stem cells are called oval cells[3] and in humans they are seen in chronically damaged liver,[4] in association with hepatocellular carcinoma[5] and necrotic lesions,[6] whereas in rats they appear in a variety of circumstances including galactosamine poisoning[7,8] and when hepatocyte regeneration is prevented by the presence of cytotoxic carcinogens such as 2-acetylaminofluorene (2-AAF).[9–12] The antiproliferative effects of chemical carcinogens were recognized by Haddow[13] in 1935 and this property has been exploited in the development of the so-called resistant hepatocyte (RH) model of carcinogenesis.[14] By simply omitting the initial exposure to diethylnitrosamine, the RH model has been adapted to study the differentiation of oval cell progeny into both hepatocytes and intestinal-type epithelia.[9,10,15]

Interest in liver stem cells has reached new heights[16–19] but the exact origin of these cells is not resolved. Periductular cells, transitional duct cells, terminal bile ductules (also called canals of Hering or cholangioles), and bile ducts themselves have been variously proposed as the normal sites for these progenitor cells, however, current thought is tending to move away from a single location for these cells, adopting the more flexible view that any component of the biliary tree can give rise to oval cells.[20] With the

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latter view in mind, bile ductular proliferation is commonly seen in a variety of liver diseases,[21–24] raising the possibility that the ductular cells are in fact the stem cell progeny capable of hepatocyte differentiation.

In this study we describe a prominent ductular reaction emanating from preexisting bile ducts occurring in the AAF-treated rat after PH, with morphological evidence that these cells can focally differentiate into hepatocytes. Moreover, these cellular changes were accompanied by an increased expression of hepatocyte growth factor (HGF) mRNA in the liver, and we show that sinusoid lining cells expressing HGF mRNA become most abundant in the periportal regions immediately adjacent to the emerging ductular cells.

Materials and Methods

Treatment of Animals

Male Fischer rats (B & K Universal Ltd., Hull, UK) weighing 200 g were used and maintained on standard pelleted chow and had access to water ad libitum. To inhibit hepatocyte cell proliferation, all rats received daily oral gavage of 0.2 ml of 1% 2-AAF for a period of up to 14 days. After 7 days of this regime all rats underwent a two-thirds PH under diethyl ether anesthesia; animals were killed under diethyl ether anesthesia by exsanguination via the hepatic vein in groups of four at daily intervals up to 8 days after PH. For routine light microscopy (hematoxylin and eosin staining) and *in situ* hybridization studies, thin (2- to 3-mm) liver slices were immersion fixed in neutral-buffered formalin for 24 hours before processing and embedding in paraffin wax. For the immunocytochemical detection of intermediate filament proteins, liver slices were fixed in methacarn (60% methanol: 30% chloroform: 10% glacial acetic acid) for 24 hours before processing.

Bile duct injection of pigmented medium: a stock solution of pigmented gelatin was made up to a final volume of 25 ml and consisted of 14.5 ml of distilled water, 6.5 g of potassium iodide, 2.2 ml of phosphate-buffered saline (PBS), pH 7.2, 0.15 ml of an octanol/phenol mixture (40%/60%, respectively), 6.5 g of gelatin (BDH) and 8 ml of Monastral blue pigment (Sigma, St. Louis, MO). The two rats injected at 8 days after PH were anesthetized with ether and a microcannula filled with PBS was inserted into the common bile duct and secured with a suture. Immediately before injection, one part 10% formalin was added to nine parts of the gelatin stock solution, mixed thoroughly with a plastic pipette, and aspirated into a 1-ml syringe. The syringe was connected to the cannula and the mass injected slowly into the bile duct using light finger pressure until the subcapsular ducts were filled. The gelatin was allowed to solidify for 10 minutes after which the animal was killed and the whole liver removed and immediately placed in formalin. The tissue was then routinely processed, blocked in paraffin wax, and sectioned.

Electron Microscopy

Tissue samples not exceeding 1 mm^3 in volume were fixed in 2% glutaraldehyde for 2 hours. After washing in phosphate buffer (pH 7.2), tissues were osmicated and dehydrated in acidified 2,2-dimethoxypropane (DMP) before routine embedding in TAAB resin. One-micron sections were cut and stained with toluidine blue for observation at light microscope level and selection of relevant blocks, followed by ultrathin sections of approximately 100 nm, collected on nickel grids and stained with uranyl acetate and lead citrate for observation on a Philips CM-10 electron microscope.

In Situ *Hybridization for Detection of HGF mRNA*

Blocks of tissue were stored at room temperature without special precautions.

Probes

Plasmids containing cDNA for regions of rat HGF (*Eco*RI subclone from RBC1[25] in pBluescript SK vector; kindly provided by Dr. T. Nakamura, Kyushu University, Fukuoka, Japan) and rat β-actin (produced by Dr. R. Chinery, ICRF/RCS Histopathology Unit) were used to produce antisense riboprobes labeled internally with ^{35}S (~800 Ci/mmol; Amersham, Arlington Heights, IL). The orientation of the clones was verified by sequencing. A rat HGF probe of approximately 1.4 kb was generated from *Eco*RV linearized plasmid using T3 RNA polymerase and was used without alkali hydrolysis. Rat β-actin probes of approximately 240 kb were generated from *Eco*RI linearized plasmid also with T3 RNA polymerase.

Hybridization

The method used was essentially that of Senior et al[25] with minor modifications. Sections were hybridized to 1×10^6 cpm of either probe in 20 μl of hybridization mixture and incubated overnight at 55 C. Subsequently, any unhybridized probe was destroyed by digestion with RNAse A, and high stringency washes were conducted to reduce nonspecific

binding. Slides were dipped in photographic emulsion and exposed at 4 C for approximately 40 days before development. Sections were counterstained weakly by Giemsa's method. Patterns of hybridization were studied under dark field, reflected light conditions.

Immunocytochemistry

For the detection of intermediate filament proteins, tissues were fixed for 24 hours in methacarn then processed routinely and embedded in paraffin wax. After blocking for endogenous peroxidase, 3-μ sections were incubated for 10 minutes with normal serum from the donor species of the secondary antibody followed by a 1-hour incubation with the primary antibody (Table 1). After rinsing with PBS, biotinylated rabbit anti-mouse immunoglobulin (Dako, Carpinteria, CA) diluted 1:200 in PBS was applied for 45 minutes then sections were rinsed with PBS and incubated with horseradish peroxidase-conjugated streptavidin/biotin complex (Dako) for 30 minutes. The sections were rinsed with PBS and peroxidase developed for 2 to 7 minutes with 0.05% diaminobenzidine and 0.03% H_2O_2 in PBS, counterstained with Harris' hematoxylin, dehydrated, cleared, and mounted in Pertex. Negative controls in each batch of slides involved replacing the primary antibodies with PBS, and the possibility that endogenous biotin was responsible for staining was excluded by the use of an avidin-biotin blocking kit (Vector, Burlingame, CA) before the application of the primary antibodies, performed according to the manufacturer's instructions. Substitution of the primary antibodies with PBS resulted in no staining and staining due to endogenous biotin was excluded.

Results

Cell Proliferation after Hepatectomy in the AAF-Treated Rat is in Sprouted Bile Ductules

At 1 day after PH small basophilic cells with a high nuclear:cytoplasmic ratio (oval cells) were evident in and around the portal tracts. These cells became more numerous with time and by day 8 long strings of these cells could be seen fanning out into the periportal and midzonal parenchyma (Figure 1, A and B) with most of the cells apparently organized into ductular structures. A bile duct injection of an animal at day 8 with a blue pigmented gelatin medium resulted in the filling of the lumens of these new structures strongly suggesting they were sprouted from preexisting bile ducts (Figure 1C). At 24 hours after PH bromodeoxyuridine (BrdUrd) labeling (data not shown) was largely confined to small bile ductules within the portal tracts, but at later times labeling occurred primarily in the cell cords in the region just distal to the leading cells.

Table 1. *Details of Antibodies Used to Detect Intermediate Filaments*

Antibody	Specificity	Source	Dilution in PBS
LE 41	Cytokeratin 8	ICRF	1:10
LP2K	Cytokeratin 19	ICRF	1:5
Clone V9 (V-6630)	Vimentin	Sigma	1:2000

Oval Cells Express Abundant Intermediate Filaments

Liver parenchymal cells have a simple cytokeratin composition expressing only one cytokeratin pair, cytokeratins 8 and 18.[26] On the other hand, intrahepatic bile ducts also express cytokeratins 7 and 19 in addition to 8 and 18. In this study the expanding population of oval cells stained strongly with antibodies raised against each of these four cytokeratins. In the normal control liver, cytokeratin 19 immunoreactivity was restricted to bile ductular epithelial cells but all oval cells stained intensely and the pattern of staining at later times after PH clearly demonstrated that most oval cells were in fact contiguous cells organized into long, branching ducts that were continuous with preexisting ducts in the portal spaces (Figure 2A). These ductular cells also expressed cytokeratin 8 immunoreactivity. In the normal liver cytokeratin 8 immunoreactivity was present not only in biliary epithelium but also in periportal hepatocytes, albeit at a low level. In biliary epithelia there was intense diffuse cytoplasmic staining, whereas hepatocyte staining was distinctly membranous. After PH all oval cells showed strong staining typical of biliary epithelia (Figure 2B), but staining diminished as cells appeared to undergo differentiation toward a hepatocyte phenotype with the acquisition of the hepatocyte pattern of staining, with a gradual change from cytoplasmic to membranous staining. This differentiation did not occur simultaneously in all ductular cells but rather occurred focally and often into columnar epithelia (Figure 2C). In the normal liver staining with the antivimentin antibody was restricted to some of the sinusoid lining cells, fibroblasts in the portal space, and the smooth muscle of blood vessel walls. However, oval cells were very strongly stained, though when these cells

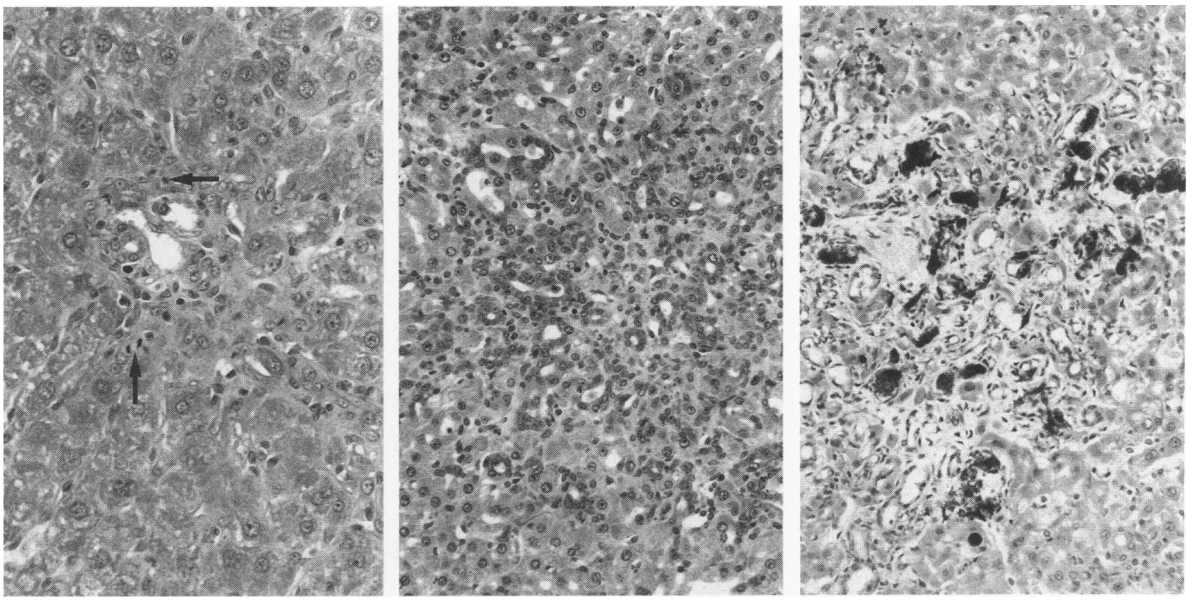

Figure 1. *Morphological changes in the liver after PH in the AAF-treated rat. At 5 days after PH* (A) *the portal areas are noticeably hypercellular with mitotic activity apparent* (*arrows*) *and by 8 days* (B) *long cords of cells are seen with numerous ductular profiles. Injection of common bile duct with pigmented gelatin resulted in the filling of most lumena* (C), *indicating these ducts were continuous with the preexisting bile ducts. Hematoxylin and eosin staining. Original magnifications:* A ×400, B ×200, C ×200.

appeared to differentiate they also lost vimentin immunoreactivity (Figure 2D).

These events at 7 to 8 days after PH were accompanied by substantial cell death in the ductular population, with dead and dying cells being shed into the duct lumen seemingly undergoing apoptosis from the appearance of chromatin margination in the affected cells (Figure 2D), confirmed by electron microscopy (Figure 6C). Cells that budded to the outside of the ductular structures appeared to survive and differentiate (Figure 2C).

Ultrastructural Analysis of the Morphological Changes after PH in the AAF-Treated Rat

After PH, large numbers of small cells with a high nuclear:cytoplasmic ratio, a round or cigar-shaped euchromatic nucleus, and relatively few cytoplasmic organelles can be found in the portal spaces and beginning to migrate between hepatocytes (Figure 3A), and their similarities to neighboring bile duct epithelial cells are readily apparent. Cells budding from preexisting portally situated bile ducts are common (Figure 3B). Typically, undifferentiated cells could be found in the space of Disse (Figure 4A), sometimes occurring singularly with features more akin to hepatocytes than biliary epithelia (Figure 4B) and cells resembling small hepatocytes appeared to be able to insinuate themselves between hepatocytes in an existing plate (Figure 4C). More usual was the occurrence of focal differentiation in the cords of ductular cells with the altered cells resembling small hepatocytes (Figures 2B and 5, A–D). Apoptosis was common during this remodeling process, sometimes affecting single cells (Figure 6A), but was more common in the ductular structures where the apoptotic cells were shed into the duct lumena (Figures 2D and 6, B and C).

HGF Expression after PH in the AAF-Treated Rat

HGF mRNA expression was only seen in sinusoid lining cells. Sinusoid lining cells expressing HGF transcripts became more numerous with time after PH and by 7 days were far more numerous in the periportal regions (Figure 7) immediately adjacent to the cords of oval cells. Neither oval cells nor hepatocytes expressed HGF mRNA, however, there was abundant intact mRNA in oval cells as judged by β-actin mRNA expression in these cells.

Discussion

This report describes the response of the rat liver to PH when the proliferative response of differentiated hepatocytes is prevented by the chronic oral administration of 2-AAF. An absence of proliferative activity in normal hepatocytes was confirmed. However,

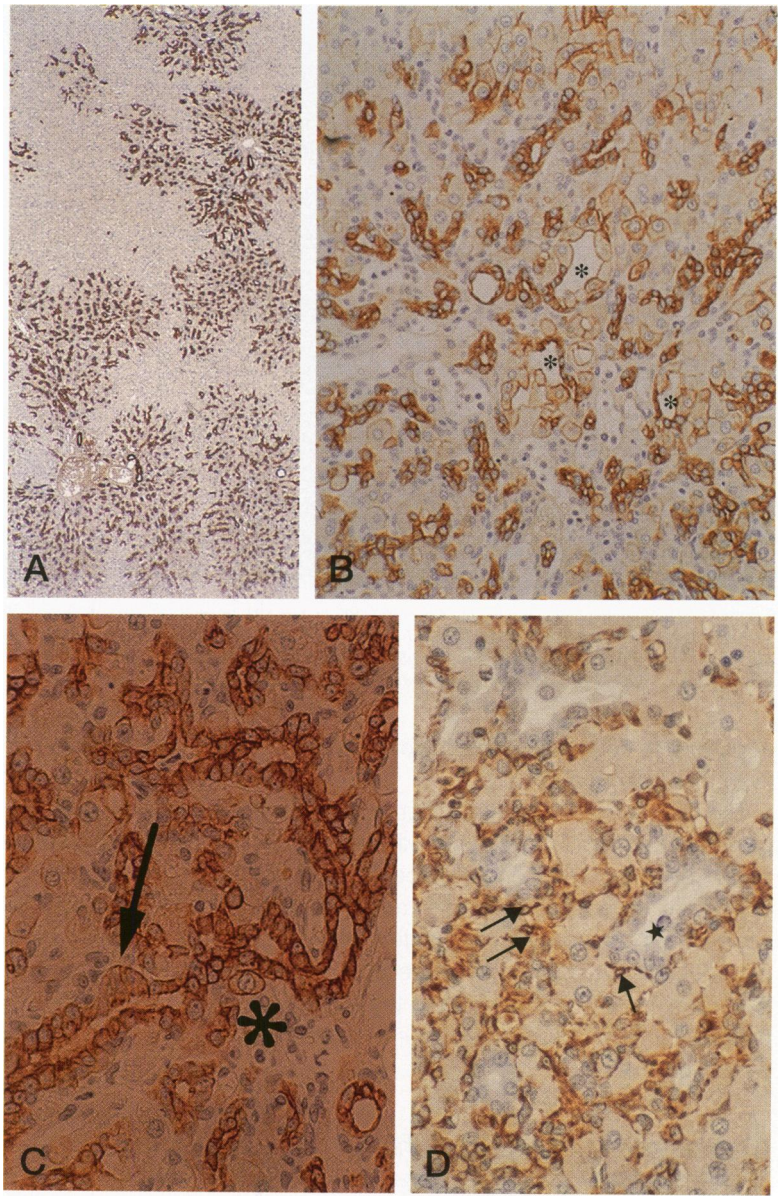

Figure 2. *Intermediate filament expression after PH in the AAF-treated rat. Cytokeratin 19 immunoreactivity at 8 days after PH* (**A**) *illustrates the branching ductular nature of the oval cells, whereas small cells lining ducts at this time also strongly express cytokeratin 8* (**B**). *Note change to the membranous pattern of staining in those ducts* (*) *with hypertrophied cells. The tortuous nature of the ducts can be appreciated by cytokeratin 8 staining of a duct cut in longitudinal section* (**C**), *again showing focal differentiation* (*arrow*) *and possibly budding of a cell to the outside* (*). *These strings of cells also showed intense vimentin immunoreactivity* (**D**), *particularly along the lateral plasma membranes, but again loss of staining occurred where differentiation into cuboidal epithelium was taking place. Note apoptotic cell* (*) *in lumen. Not all vimentin-expressing cells will be ductular cells, and as expected small cells with stellate cytoplasmic processes* (*Ito cells*) *were also positive* (*arrows*). *Original magnifications:* **A** ×*40*, **B** ×*200*, **C** ×*400*, **D** ×*400*.

small cells with a high nuclear:cytoplasmic ratio spread out from the portal tracts and by 8 days after PH there was morphological evidence of differentiation of these cells into either hepatocytes or columnar epithelium. These small cells are commonly referred to as oval cells,[16] facultative liver stem cells that appear to not participate in normal regeneration to any noticeable extent.

Intrahepatic bile ducts express cytokeratins 7, 8, 18, and 19, and we can demonstrate that the expanding population of oval cells stain strongly with antibodies raised against each of these four cytokeratins. Because cytokeratins 7 and 19 are normally only expressed by biliary epithelia in the liver, these observations imply an ancestry of oval cells from biliary epithelium, though phenotypic traits are not necessarily markers of histogenesis because biliary cytokeratins can be expressed by hepatocytes undergoing ductular metaplasia[27] or neoplastic transformation.[28]

Routine hematoxylin and eosin staining revealed relatively little about the spatial organization of the emerging oval cells, though many oval cells formed ductular profiles by 8 days after PH (Figure 1B) and such structures have been commonly referred to as either disorganized bile ductular cells, pseudoducts, or neocholangioles. In liver disease at least three types of bile ductular reaction can be distinguished:[23]

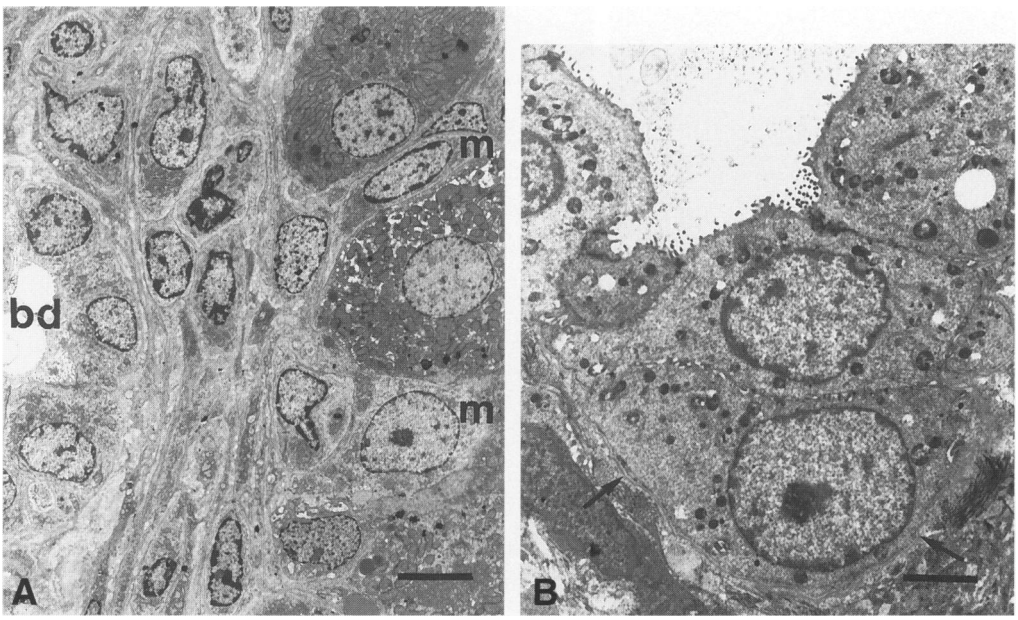

Figure 3. *Electron micrographs of early oval cell behavior after PH in the AAF-treated rat. Many small cells resembling the cells lining preexisting portally situated bile ducts (bd) can be seen at 6 days after PH* (**A**) *and some are clearly migrating (m) between hepatocytes.* **B:** *The budding of a cell from a preexisting portally situated duct is apparent from the continuity of the surrounding basal lamina (arrows) at day 5. Scale bars:* **A** *5.9 μ,* **B** *2.3 μ.*

1) a proliferation of ductules at the parenchymal border due to mechanical obstruction of large bile ducts, 2) a ductular metaplasia of hepatocytes due to chronic damage and inflammation, and 3) a sprouting of bile ductules that invade the hepatic parenchyma.

There has been considerable debate about the origin and function of proliferating bile ductules in human and rodent livers, particularly regarding whether they represent the ductular metaplasia of hepatic cords or the lengthening and/or sprouting from preexisting ducts. In alcoholic liver disease, the presence of alcoholic hyaline (Mallory bodies) in some ductular cells was taken as evidence of an origin from hepatocytes,[29] and in guinea pigs fed α-naphthyl isothiocyanate, bile canalicular ectasia was deemed responsible for the tubularization of hepatic plates leading to ductular metaplasia.[30] On the other hand, the demonstration of both α_1-antitrypsin (a hepatocellular export protein) and carcinoembryonic antigen (a marker of biliary epithelia) in some ductular cells in partially necrotic human liver has suggested that sprouted bile ducts can differentiate into liver cells,[6] and similar conclusions were drawn from observing hepatitis B surface antigen in ductular hepatocytes expressing biliary cytokeratin from human cirrhotic liver.[31] In addition, in rodents many observations support the proposition that hepatocytes can be produced from duct cells; in rats after galactosamine injury ducts lined by an admixture of small (bile duct-like) and large (hepatocyte-like) cells can be seen,[7] whereas in mouse liver injured by nutritional deficiency, Wilson and Leduc[32] were firmly of the opinion that the newly forming cholangioles were the source of new hepatocytes.

With a combination of anticytokeratin antibodies and the bile duct injection of pigmented medium it was quite clear that the oval cells observed in this study were largely a population of long, branching ductular structures sprouted from preexisting bile ducts and continuous with them. However, the emergence of some oval cells from the portal areas either singly or in small groups does occur (Figure 4, B and C), and indeed others[33] have noted that periductular cells proliferate in rat liver in the early stages of chemical hepatocarcinogenesis, which could presumably give rise to migrating single cells. The direct connection of ductular oval cells to portal bile ducts is in itself not a novel finding and has been observed under a variety of conditions. For example, it has been shown in rats fed AAF in a choline-deficient diet,[34] and likewise Lenzi et al[20] feeding rats ethionine in a choline-devoid diet observed long strings of cytokeratin 19-positive cells apparently connected to portal bile ducts, and physiological measurements confirmed a massive increase in biliary tree volume coincident with the oval cell proliferation. Rats treated chronically with carbon tetrachloride to induce cirrhosis develop a similar ductular reaction in the parenchyma, and casts of the biliary tree confirmed a connection to preexisting bile ducts.[35] However, these experiments ex-

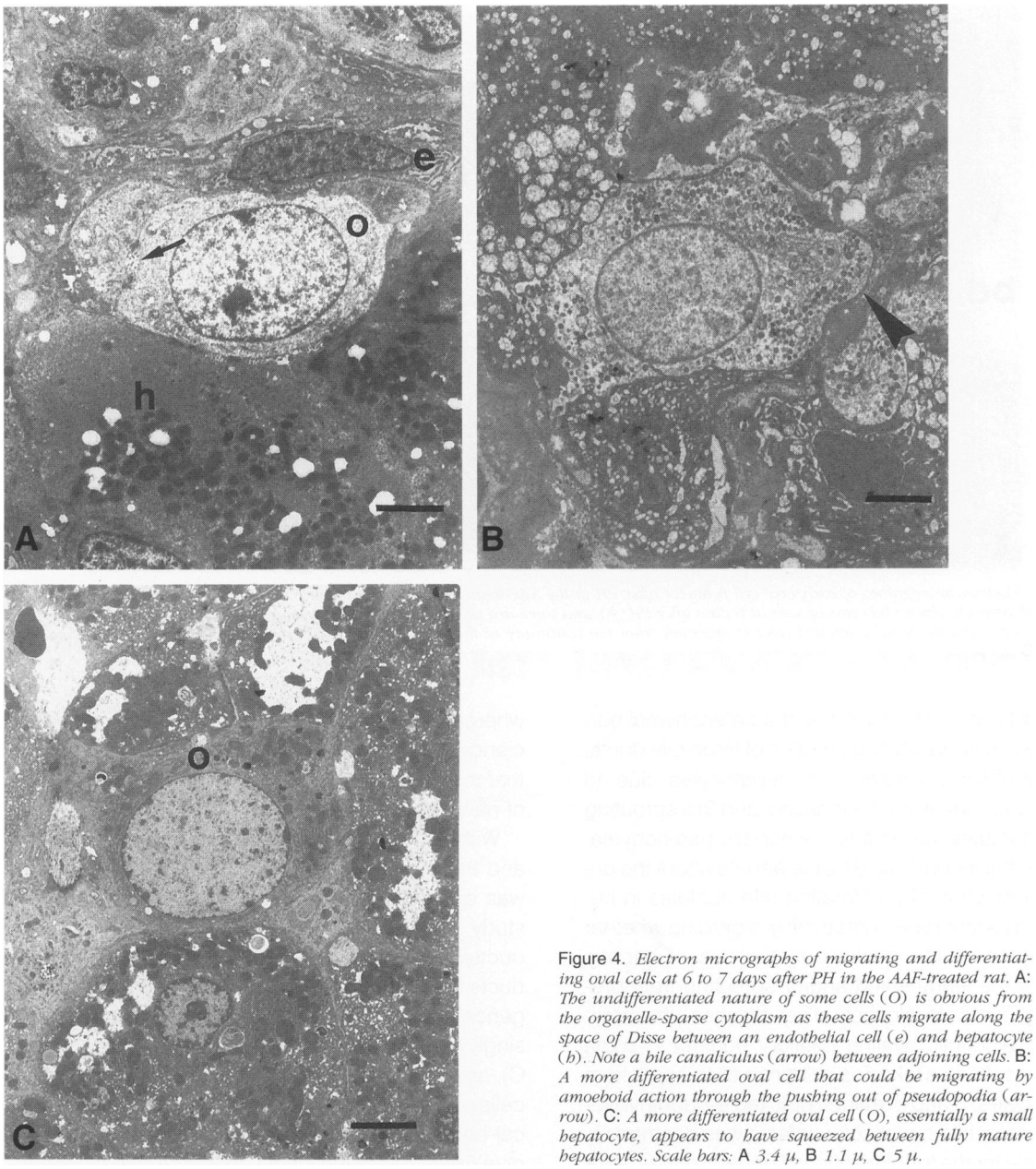

Figure 4. *Electron micrographs of migrating and differentiating oval cells at 6 to 7 days after PH in the AAF-treated rat.* A: *The undifferentiated nature of some cells (O) is obvious from the organelle-sparse cytoplasm as these cells migrate along the space of Disse between an endothelial cell (e) and hepatocyte (h). Note a bile canaliculus (arrow) between adjoining cells.* B: *A more differentiated oval cell that could be migrating by amoeboid action through the pushing out of pseudopodia (arrow).* C: *A more differentiated oval cell (O), essentially a small hepatocyte, appears to have squeezed between fully mature hepatocytes. Scale bars:* A *3.4 μ,* B *1.1 μ,* C *5 μ.*

tend these observations to suggest that the epithelial cells forming these sprouted ducts can differentiate *in situ*. Transformation of these small reactive ductular cells to a tall columnar epithelium with occasional interspersed goblet cells was a common occurrence and has already been noted[15] but we also observed that some lining cells progressively enlarged and accumulated increased numbers of large mitochondria typical of hepatocytes (Figure 5). This focal differentiation was accompanied by a high incidence of apoptotic cell death,[36] with the affected cells budding off into the duct lumen. Of course, we should add the caveat that morphology alone is not evidence for functional hepatocytic differentiation,[18] nevertheless, these ductular cells synthesized α-fetoprotein (data not shown), an event that signals a hepatic commitment in endodermal cells of the ventral foregut during development.[37]

High levels of serum α-fetoprotein in severe forms of acute hepatitis are a favorable prognostic sign, perhaps indicative of regeneration from the stem cell compartment. Interestingly, we observed that this apparent hepatic differentiation of ductular cells occurred focally and inspection of published micro-

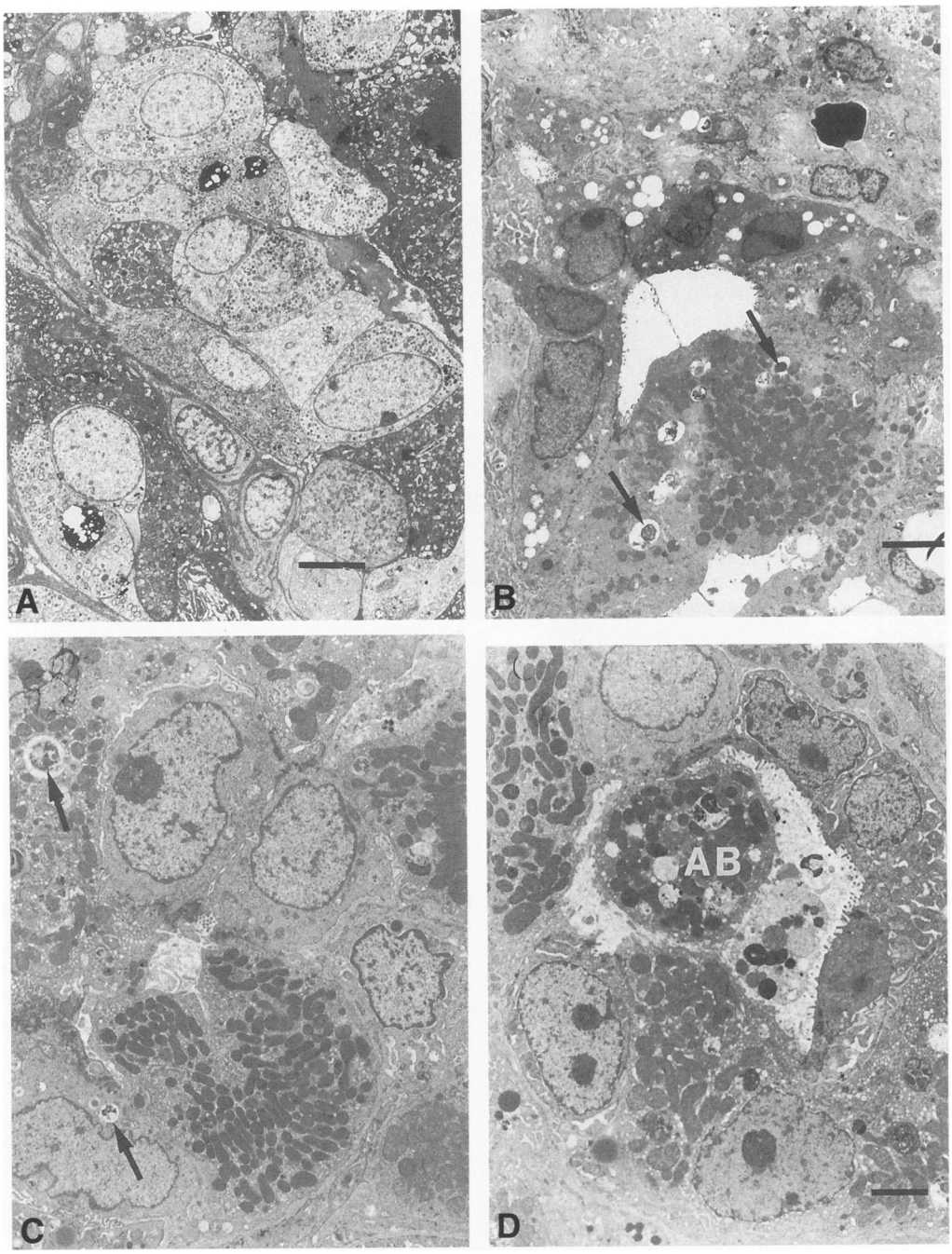

Figure 5. *Electron micrographs of oval (ductular) cell differentiation at 7 to 8 days after PH in the AAF-treated rat.* **A:** *Apparent differentiation of a column (presumably a duct cut in longitudinal section) of oval cells into small hepatocytes, whereas in transverse sections of ducts* (**B** *to* **D**) *the often erratic nature of this process is apparent. Note the numerous phagocytic vacuoles (arrows) in the hypertrophied ductular cells probably indicating enhanced organelle turnover in these cells, and also the frequent occurrence of apoptotic bodies (AB). Scale bars:* **A** *5.5 μ,* **B** *4.2 μ,* **C** *3.1 μ,* **D** *3.1 μ.*

graphs show the erratic nature of this process in ductular cells.[7,38] Labeling proliferating oval cells with tritiated thymidine in the model used here has shown that the label soon appears in small basophilic hepatocytes suggesting a direct lineage relationship,[9] and oval cells induced by feeding rats the choline-deficient diet can be seen to be undergoing hepatocyte differentiation through the acquisition of glucose-6-phosphatase activity.[39] Likewise *in vitro* studies have suggested that hepatocytes are derived

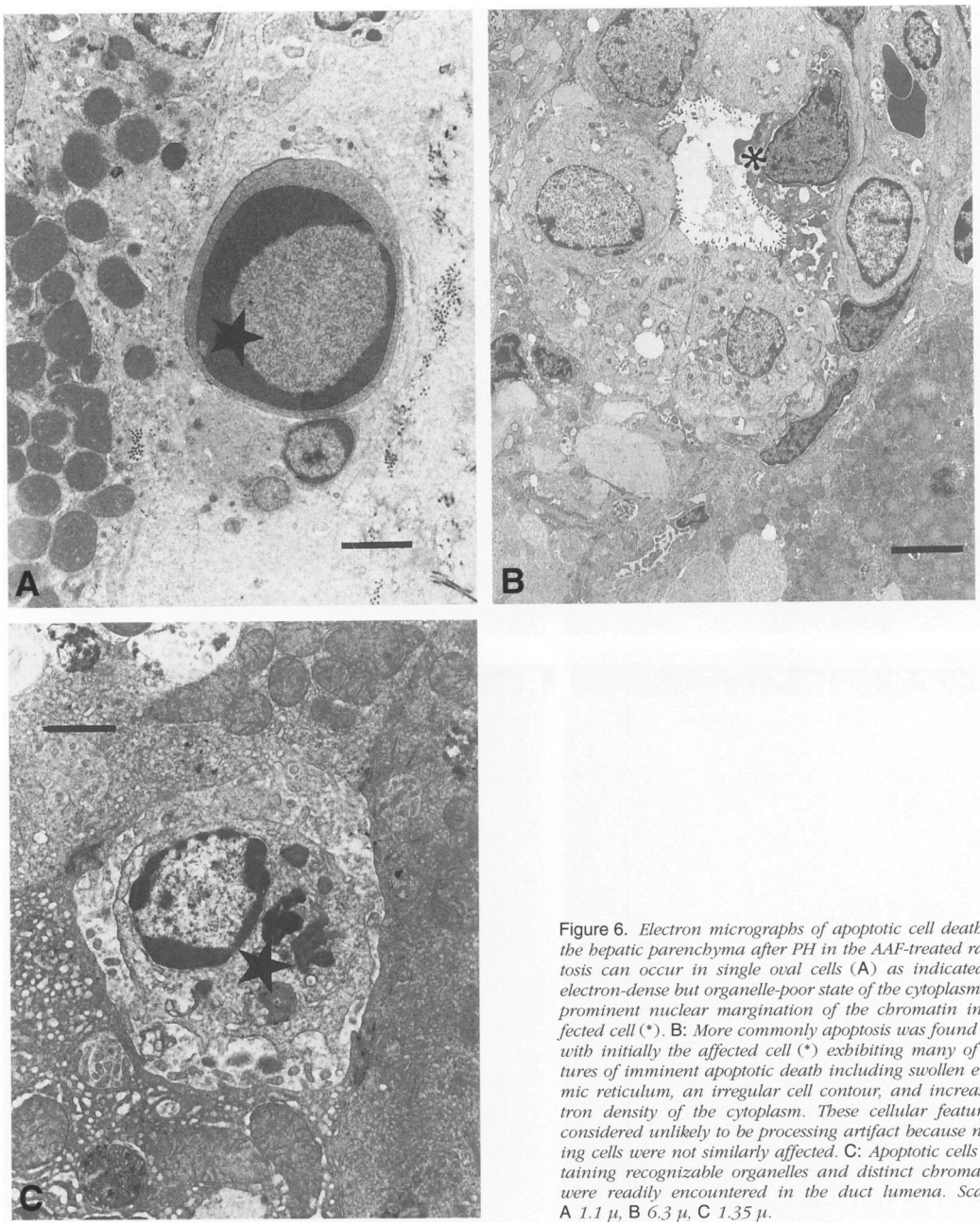

Figure 6. *Electron micrographs of apoptotic cell death among the hepatic parenchyma after PH in the AAF-treated rat. Apoptosis can occur in single oval cells* (A) *as indicated by the electron-dense but organelle-poor state of the cytoplasm and the prominent nuclear margination of the chromatin in the affected cell* (*). B: *More commonly apoptosis was found in ducts with initially the affected cell* (*) *exhibiting many of the features of imminent apoptotic death including swollen endoplasmic reticulum, an irregular cell contour, and increased electron density of the cytoplasm. These cellular features were considered unlikely to be processing artifact because neighboring cells were not similarly affected.* C: *Apoptotic cells* (*) *containing recognizable organelles and distinct chromatin caps were readily encountered in the duct lumena. Scale bars:* A *1.1 μ,* B *6.3 μ,* C *1.35 μ.*

from biliary ductular cells,[40] whereas intrahepatic transplantation of a diploid oval cell-like cell line into Fischer rats caused their differentiation into hepatocytes, at least when judged by morphological criteria.[41]

It has not been possible to identify the exact location of the first cells that respond to PH in the AAF-treated rat. BrdUrd-labeled bile ductular and periductular cells can be found as early as 6 hours after PH, though at 24 hours small bile ducts appeared to be the most proliferatively active cells. At later times after PH the advancing sprouts are the sites of cell proliferation and the parent ducts are relatively quiescent. Largely similar findings have been reported before,[42] suggesting small bile ducts are the prime movers in the initiation of the oval cell response in this model. Cells with the features of oval cells are seen proliferating in human liver after submassive liver necrosis,[43] whereas proliferating portal bile ducts are seen in acute viral hepatitis in humans but in chronic

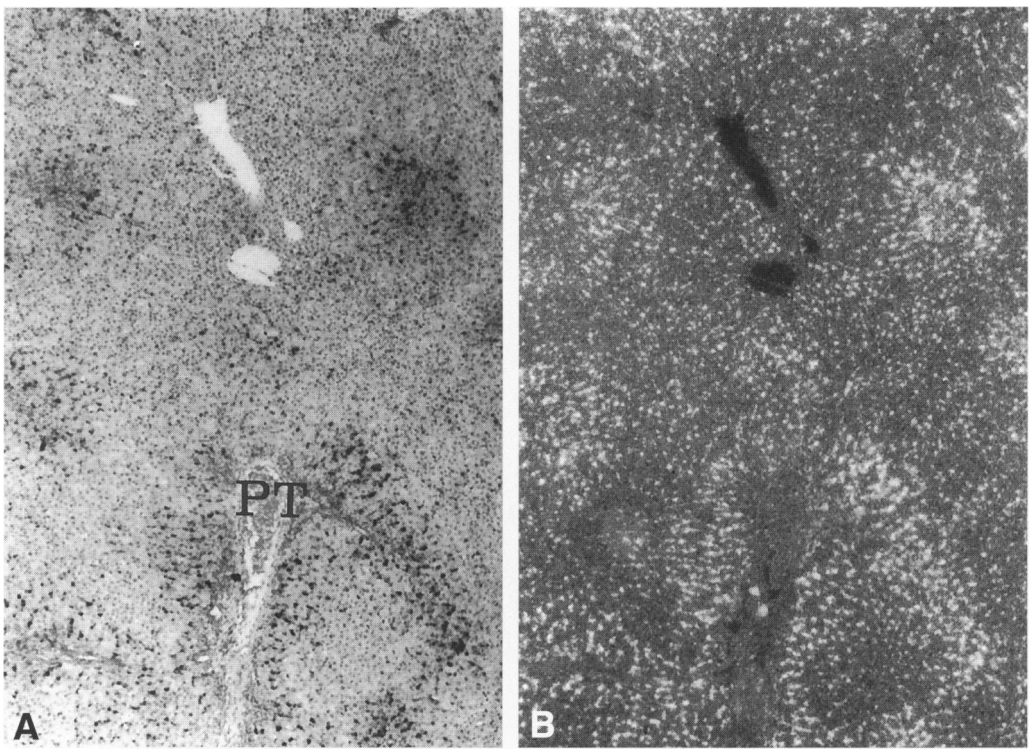

Figure 7. *HGF mRNA expression at 7 days after PH in the AAF-treated rat as viewed by bright field* (**A**) *and dark field reflected light* (**B**) *illumination. At the time of PH there were 46 ± 16* (n = 4 ± *SD*) *labeled cells per mm² in the periportal areas but at 7 days there was a preferential accumulation of labeled cells around the portal tracts* (*PT*) *with 429 ± 78 labeled cells per mm².*

hepatitis cell proliferation occurs in cytokeratin 19-positive strings of ductular cells in areas of confluent necrosis,[44] findings mirrored in this experimental study.

Cytokeratin 8 immunoreactivity was common to both ductular cells and hepatocytes, though the intracellular patterns of staining were distinctly different. In ductular cells there was intense diffuse cytoplasmic staining, whereas in periportal hepatocytes the immunostaining was distinctly membranous as previously found.[45] Cultured rat hepatocytes under a differentiating environment also have dense cytokeratin filament networks at the cell periphery[46] but mitogenic stimuli induce a filament rearrangement from the cell periphery to the cytoplasm.[47] Similarly, pure cultures of rat hepatocytes have a uniformally even distribution of cytokeratin immunoreactivity in the cytoplasm, whereas hepatocytes co-cultured with rat liver epithelial cells (presumed to originate from primitive biliary epithelia) promotes expression of liver-specific genes and the organization of dense cytokeratin fibrils at the cell periphery.[48] In this study it was quite clear that ductular cells showing morphological evidence of hepatocyte differentiation had a distinct change in staining from the ductular pattern (cytoplasmic) to the hepatocyte pattern (membranous), lending further support to the idea that hepatic differentiation is occurring in these cells.

Of further interest was the emergence of vimentin immunoreactivity in the proliferating ductular cells and its subsequent loss as cells differentiated. Vimentin is the intermediate filament characteristic of mesenchymal cells but is known to be present in some carcinomas. Co-expression of intermediate filament classes is rare, though cultured mesothelial cells switch from cytokeratin to vimentin synthesis during the rapid growth phase.[49] Vimentin expression has been noted in hyperplastic bile ducts resulting from biliary obstruction in the rat,[50] in cultures of hepatic stem cells,[51] and in areas of some human hepatoblastomas.[52] On the other hand, Alpini et al[53] failed to immunocytochemically detect vimentin in strings of cytokeratin 19-positive cells induced in the rat by the feeding of the CDE diet. The cause of this discrepancy with the present observations is not clear but it could be related to tissue fixation because we used methacarn (a precipitative fixative) for this purpose as opposed to formalin (a cross-linking fixative) or snap-

frozen material. The reason for vimentin expression is unknown, although conceivably it could be related to the motility of the cells.

Our data support the observations of many others that HGF mRNA is more abundant in the liver after PH but extend those observations substantially by showing that the increased expression is variable within the liver. HGF mRNA-positive cells were almost twice as abundant in the periportal *versus* the centrilobular regions after 7 days, despite there being a widespread distribution of HGF-positive sinusoidal lining cells across the liver during the previous days. This focal accumulation of HGF-producing sinusoid lining cells in the immediate vicinity of oval cell activation strongly supports the involvement of local HGF production in the processes of proliferation, migration, and probably duct formation because HGF can induce tubule formation in cultures of nonparenchymal epithelial liver cells.[54] The synthesis of transforming growth factor-α also increases shortly after PH[42] and has been localized by immunocytochemistry principally to the new ductular structures.[55] Many other growth factors are probably involved in controlling regeneration,[56] one of which, parathyroid hormone-related peptide, seems particularly implicated in the growth and differentiation of reactive bile ductules.[57]

In conclusion, after PH when the liver mass cannot be renewed from existing hepatocytes, stem cell-derived progeny, commonly referred to as oval cells, proliferate and ultimately differentiate apparently into several distinct lineages. In this particular model, these oval cells are primarily organized into ducts sprouted from and continuous with intrahepatic bile ducts in the portal areas.

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