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Origin and Structural Evolution of the Early Proliferating Oval Cells in Rat Liver

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We have analyzed the histological changes in rat liver after 2-acetylaminofluorene (AAF) administration. The data demonstrate that AAF-induced oval cells were preferentially generated by proliferation of the terminal biliary ductules that we suggest constitute the primary hepatic stem cell niche. The oval cells formed ductular structures, representing an extension of the canals of Hering. This histological organization provides continuous bile drainage of the hepatocytes and uninterrupted blood flow in the sinusoids. The oval cell ductules are surrounded by a continuous basement membrane that is intermittently disrupted by processes of stellate cells that form direct cell-cell contact with the oval cells. Although both AAF treatment and bile duct ligation results in proliferation of biliary epithelial cells, the mechanism(s) responsible for the proliferation of the biliary epithelium seems to differ in the two models. In contrast to the biliary proliferation stimulated by bile ligation, AAF-induced oval cell proliferation as well as the capacity of these cells to differentiate into hepatocytes, bile epithelial cells and possibly other cell lineages can be blocked by administration of dexamethasone. ***(Am J Pathol 2001, 158:1313–1323)***

Although a substantial amount of knowledge has been accumulated throughout the last 2 decades about liver stem cells,[1,2] numerous aspects of this intriguing cell compartment remain undefined. Indeed, there are conflicting data on the exact location of liver stem cells and even the growth pattern of these cells is not completely understood. The proliferating oval cells—the progeny of the stem cells—always expand into liver parenchyme from the portal area. Furthermore, selective damage of the periportal zone reduces oval cell proliferation.[3] These observations support the notion that the stem cells must reside somewhere in the periportal region. The phenotypic resemblance between the oval cells and biliary epithelium suggests that they derive from the biliary tree, and terminal hepatic ductules (canals of Hering) that connect the most distal hepatocyte of the hepatic plate to the interlobular bile ducts are thought to harbor the hepatic stem cells.[4–7] However, there is no general agreement on this issue. In fact, potential candidates for the stem cells outside the biliary system have been proposed.[8]

In the absence of a specific marker for the hepatic stem cells, several investigators using different models have attempted to identify the stem cells by labeling the dividing cells in the early phase of oval cell expansion.[4,8–10] However, most of the experimental protocols for the activation of the hepatic stem cell compartment require a relatively long time and this may explain the divergent results. The 2-acetylaminofluorene (AAF)/partial hepatectomy (PH) model of oval cell proliferation/differentiation has been extensively used to analyze the hepatic stem cell compartment during the last few years.[11–13] We have recently modified the classical AAF/PH model[14] and demonstrated that after a single dose of AAF administration a notable cell proliferation takes place in the periportal zone and at least some of these proliferating cells are the precursors of oval cells. Therefore AAF administration provides a uniquely fast and synchronized activation of the oval cell precursors without any major disruption of the hepatic structure. We could identify dividing cells in the interlobular bile ducts after AAF treatment, whereas the exact nature of the rest of the thymidine-labeled cells could not be unambiguously defined by traditional light microscopy.[14]

Biliary cell proliferation can also be induced in rats by the ligation of the common bile duct (BDL).[15,16] This reaction is, however, morphologically and phenotypically very different from the oval cell proliferation. After BDL, proliferating biliary cells do not show any signs of differentiation into other cell types. Another difference between BDL- and AAF-induced biliary cell proliferation is illustrated by selective inhibition of oval cell proliferation by dexamethasone.[17]

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In the present work we have characterized the early cellular events in the liver during the proliferative response induced by AAF or BDL. To obtain a more detailed morphological assessment, the samples were analyzed by, in addition to traditional light microscopy, both confocal and electron microscopy. Both AAF and BDL induced an intense biliary cell proliferation. The frequency of dividing cells after AAF treatment was significantly higher in the terminal hepatic ductules. Morphological analysis revealed that the early oval cells are strictly confined to ductular structures surrounded by basement membrane, representing an extension of the canals of Hering.

Materials and Methods

Animal Experiments

Male F-344 rats (180 to 200 g) were used for all experiments and kept under standard conditions. The animal study protocols were conducted according to NIH guidelines for animal care.

AAF/PH Experiment

AAF (1.5 mg) suspended in dimethyl-cellulose was given to the rats on 4 consecutive days by gavage. Traditional 70% PH[18] was performed on the fifth day, which was followed by five additional AAF treatments. Animals were sacrificed at the described time points (at least three at each time point).

BDL

BDL was done according to Cameron and Oakley.[19] The rats were sacrificed 48 hours after the operation.

Electron Microscopy

Preparation of liver tissue for electron microscopy was done by perfusing the livers under anesthesia (35 mg/kg, Nembutal; Serva, Heidelberg, Germany) via the portal vein with phosphate-buffered saline (PBS) for 10 minutes and with 2.5% glutaraldehyde in 0.05 mol/L Na-cacodylate (pH 7.2) for 15 minutes at room temperature. Livers were cut into 1 × 3 mm pieces and immersed in 2.5% glutaraldehyde for 2 hours. The pieces were postfixed in 1% OsO4, 0.05% K-ferrocyanide in 0.05 mol/L Na-cacodylate for 1 hour, dehydrated in a graded series of alcohol, contrasted *en bloc* with 2% uranylacetate, and embedded in Spurr's mixture. Ultrathin sections were stained with lead citrate and examined on a Philips CM10 electron microscope.

Ultrastructural Analysis of 5-Bromo-2′-Deoxy-Uridine (BrdU)-Labeled Periportal Cell

BrdU (100 mg/kg) was injected intraperitoneally after two doses of AAF, 24 hours after the second treatment or 2 days after BDL. One hour after the injection, the animals were anesthetized and perfused via the portal vein with PBS for 10 minutes, followed by 4% paraformaldehyde for 20 minutes. Livers were removed, cut into 5-mm thick slices and postfixed for 24 hours. The fixed tissues were washed in PBS and immersed in 15% sucrose for 24 hours followed by 30% sucrose for another 24 hours. Specimens were frozen in isopentane cooled by liquid nitrogen.

Cryosections (15 μm thick) were mounted on microscope slides coated with parlodion. The cryosections were rinsed in PBS and incubated for 20 minutes in 3 N HCl at room temperature. After washing in PBS, the sections were incubated with monoclonal anti-BrdU antibody (diluted 1:100; Becton-Dickinson, Mountain View, CA) for 3 hours and later with biotinylated anti-mouse antibody (Vector Laboratories, Burlingame, CA) for 2 hours. The reaction was developed by an ABC reaction (Elite ABC Kit, Vector Laboratories) using diaminobenzidine as chromogen. The sections were osmificated (1% OsO4 in PBS), dehydrated in graded series of ethanol, and embedded in Epon 812. Blocks were removed by immersing the slides in liquid nitrogen. Semithin sections were slightly stained by 0.5% toluidine blue (pH 8.5), portal areas containing BrdU-labeled cells were trimmed out and unstained ultrathin sections were analyzed on a Philips CM 10 electron microscope. Labeled cells were divided into three categories based on their localization: 1) cells residing outside the basement membranes in the periportal connective tissue; 2) cells confined within the basement membrane, as part of bile ducts; and 3) cells of the canals of Hering. The cells comprising the canals of Hering were in direct contact with a hepatocyte or were separated from a hepatocyte by only one biliary cell.

Immunofluorescent Analysis

Double Labeling for Laminin-Cytokeratin and Laminin-Desmin

Cryostat sections (6 μm) were fixed in acetone (−20°C for 20 minutes) and incubated overnight with a mixture of rabbit polyclonal anti-laminin antibody (diluted 1:50, Z0097; DAKO, Glostrup, Denmark) and fluorescein isothiocyanate-conjugated mouse monoclonal antibody directed against human cytokeratin 5, -6, -8, -17, and -19 (diluted 1:10, M0859; DAKO) or anti-laminin and mouse monoclonal anti-desmin (diluted 1:50, M0724; DAKO), respectively. After washing with PBS the sections were incubated for 60 minutes with tetramethylrhodamine B isothiocyanate-conjugated anti-rabbit IgG (diluted 1:20, R0156; DAKO) for the laminin-cytokeratin double labeling or with the combination of the same tetramethylrhodamine B isothiocyanate-conjugated anti-rabbit IgG and fluorescein isothiocyanate-conjugated anti-mouse IgG (diluted 1:50, F5262; Sigma) for the simultaneous detection of laminin and desmin.

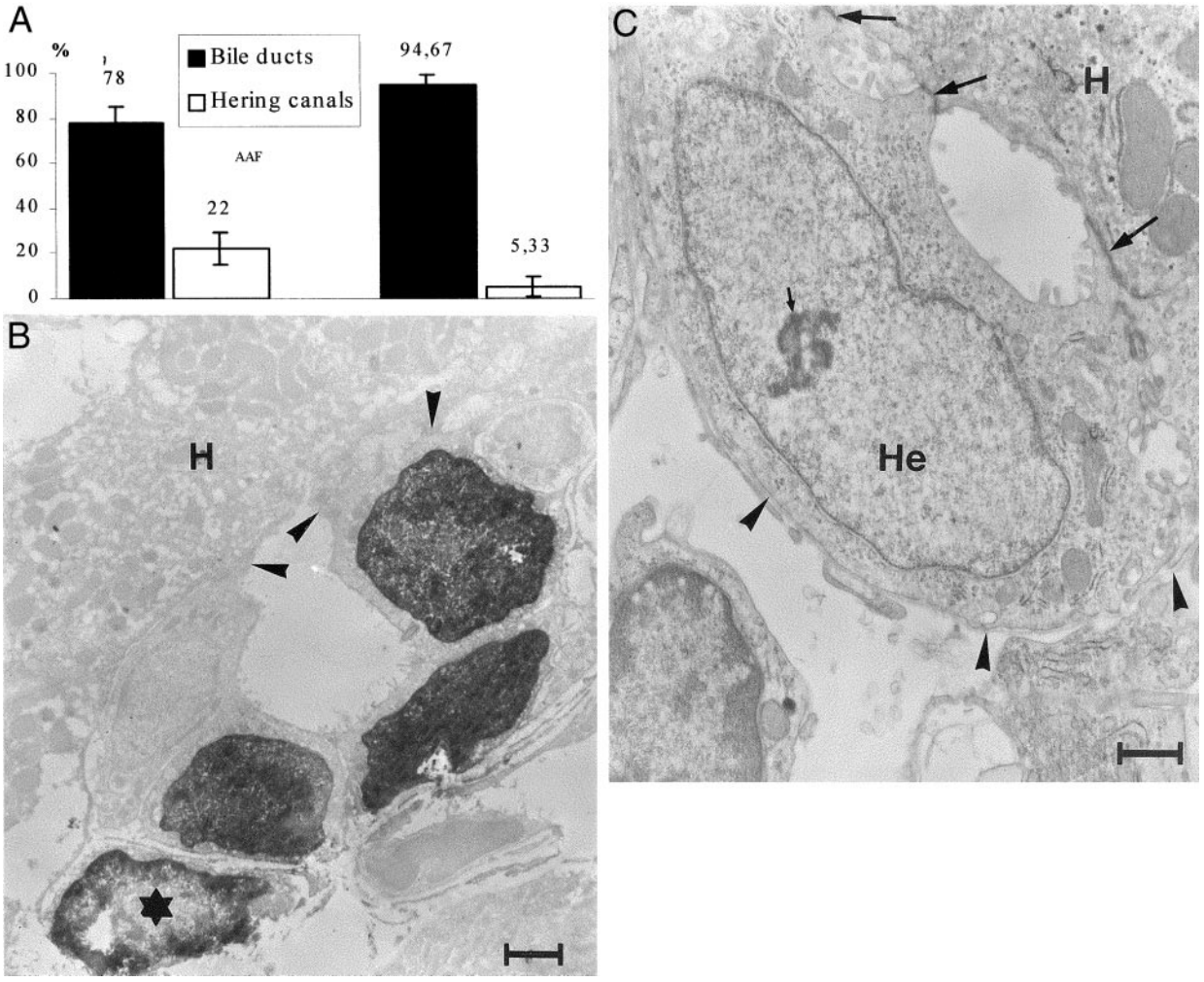

Figure 1. A: Distribution of BrdU-labeled cells, between bile ducts and canals of Hering, analyzed by immunoelectronmicroscopy. The first two columns represent the distribution after two treatments with AAF, and the second two 48 hours after BDL. Total number of labeled cells analyzed was 141 in the AAF-treated group and 143 in the BDL group. **B:** Immunoelectron micrograph 2 days after AAF treatment showing a canal of Hering consisting of four cells. Three of them are labeled by BrdU. Two cells of the canals are attached to the hepatocytes (H) (**arrowheads**). The fourth labeled cell (**asterisk**) is located in the periportal connective tissue outside the basement membrane of the terminal hepatic ductule. Scale bar, 2 μm. **C:** Activated cell in the canal of Hering (He) 2 days after AAF treatment. The nucleus is enlarged and contains euchromatin and a prominent nucleolus (**small arrow**). Continuous basement membrane is visible on the connective tissue side of the cell (**arrowheads**). The bile ductule lumen is sealed by intercellular junctions (**large arrows**) (H, hepatocyte). Scale bar, 1 μm.

Double Labeling for Laminin and α-Fetoprotein (AFP)

Liver samples were fixed in 10% formaldehyde, embedded, and cut. After deparaffinization and hydration, the sections were microwaved for 15 minutes in ethylenediaminetetraacetic acid buffer (pH 8.0), followed by incubation in 0.1% Triton X-100 in PBS for 20 minutes, and digested with Proteinase K (2 μg/ml, 15 minutes, 37°C). The sections were washed with PBS and incubated overnight with a mixture of rabbit polyclonal anti-laminin (diluted 1:20, Z0097; DAKO) and goat anti-rat AFP no. 89 antibody (diluted 1:100; generous gift from Dr. Stewart Sell, Department of Pathology and Laboratory Medicine, Albany Medical College, Albany, NY). Tetramethylrhodamine B isothiocyanate-conjugated anti-rabbit IgG (as described above), and in a second step, fluorescein isothiocyanate-conjugated anti-goat IgG (diluted 1:400, F7367; Sigma), were used as secondary antibodies. All samples were analyzed by confocal laser-scanning microscopy using the Bio-Rad MRC-1024 system (Bio-Rad, Richmond, CA).

Results

BrdU Labeling of Dividing Cells after BDL and AAF Administration

The ligation of the common bile duct resulted in a very intensive biliary cell proliferation. The cells were labeled with BrdU 48 hours after the ligation. In agreement with previous results,[20] most of the labeled cells were in the interlobular bile ducts. Immunoelectron-microscopic analysis of the localization of labeled cells revealed <6% of the BrdU-positive biliary epithelial cells in the canals of Hering (Figure 1A). Additionally, dividing inflammatory and fibroblastic cells could be found in the periportal connective tissue and a few labeled hepatocytes were also present.

Similarly to our earlier observations,[14] a single dose of AAF resulted in cell proliferation in the periportal region. However, because the number of the BrdU-labeled cells was higher after two doses of AAF, without any apparent adverse effects, we selected the 48-hour time point for the quantitative electron-microscopic analysis. Approximately 80% of the labeled biliary cells were inside the well-defined interlobular bile ducts. However, 22% of the BrdU-positive epithelial cells were located in the canals of Hering (Figure 1A). Only those biliary cells having direct connection with a hepatocyte or separated by only one interposed biliary cell from the hepatocyte were counted as lining cells of the canals of Hering (Figure 1B). It is possible that by using these strict criteria we underestimated the number of labeled cells in the canals of Hering. However, the observation that 22% of the labeled biliary cells were in these structures can be taken as preferential labeling, considering that the overwhelming majority of the biliary epithelial cells belong to the interlobular and larger bile ducts.

After AAF treatment the terminal hepatic ductules were surrounded, by an almost continuous basement membrane that terminated on a hepatocyte of the limiting plate (Figure 1C). In addition, administration of AAF also induced proliferation outside the biliary system. Mostly single cells residing in the periportal extracellular matrix were labeled. Although the exact nature of these cells could not be established by electron microscopy they most likely represent mesenchymal cells.

Immunohistochemical Analysis of the Liver 2 Days after AAF Administration

The cell density increased in the periportal space after one or two doses of AAF, but the proliferating ductules did not infiltrate the liver lobules. To determine the characteristics of the proliferating cells in the periportal space, double labeling for laminin/cytokeratin, laminin/desmin, and laminin/AFP was performed. Immunofluorescent-stained liver sections were analyzed by confocal microscopy. The cytokeratin antibody gave a weak membrane and reticular cytoplasmic staining in the hepatocytes, whereas producing a strong cytoplasmic reaction in the biliary epithelial cells. Therefore, the two cell populations could be easily distinguished. The laminin antibody, a well-established marker of the basement membrane, sharply circumscribed the bile ducts and the blood vessels, whereas the liver acini were completely negative. The terminal hepatic ductules were intensely decorated by the laminin/cytokeratin double labeling. In the appropriate plane of the section, the basement membrane stained with laminin surrounded the bile ductules and terminated on hepatocytes at the limiting plate (Figure 2A). The basement membrane extended without interruption along the biliary ducts and ductules. If the nuclei were stained by propidium iodide in combination with the double-immunofluorescent reaction, increased cell density was revealed periportally after AAF treatment (Figure 2, A and B). However, we did not observe cytokeratin-labeled cells outside the basement membrane in the periportal space. Combined laminin/AFP immunohistochemistry showed that basement membrane surrounded a few AFP-positive biliary cells after two doses of AAF (Figure 2C). Similar to the cytokeratin reaction, no AFP-stained cells were observed outside the basement membrane. Immunostaining with a desmin antibody revealed an increased number of activated stellate cells outside the basement membrane. These cells and their processes showed a very intimate connection with the proliferating ductules (Figure 2D).

Morphological Alterations after PH in AAF-treated Rats

To study later events during stem cell activation, we used the AAF/PH model and the structural alterations were analyzed 3 days after the partial hepatectomy. By this time the oval cells had extensively infiltrated the liver lobules. Electron microscopic examination revealed that the oval cells always formed ductules (Figure 3A). These growing ductules reached the sinusoids and passed along or between them. During this process the sinusoids were left intact, preserving their normal function. The basic structure of these oval cell ductules was not different from the normal canals of Hering except for the elongation. The ductules grew by the continuous proliferation of the ductular epithelial cells (Figure 3B), whereas the sides of these tubules were continuously sealed by desmosomes. Also, the ductules always terminated at a hepatocyte (Figure 3C). This hepatocyte was probably the one connected with the original canal of Hering. In this way the original connection between the bile canaliculi and the bile duct system was preserved throughout the regenerative process. The growing tubules were surrounded mostly by basement membrane that terminated on the hepatocyte. However, there were segments along these growing ductules where no structured basement membrane could be seen by electron microscope. Again no cells penetrating through the basement membrane could be observed. There were, however, plenty of proliferating cells outside the basement membrane without characteristic ultrastructural features. These cells did not show epithelial phenotype or form desmosomes, yet had a very close contact with the expanding ductular epithelial cells. Higher magnification showed that small processes of these nonepithelial cells appeared to have direct cell-cell contact with the epithelial cells (Figure 3D).

Immunohistochemical observations confirmed the electron-microscopic data. The cytokeratin-stained oval cells formed ductules that were elongations of the portally located canals of Hering. The tubular structure of these ductules with a central lumen was much more obvious in the pictures generated by confocal microscopy. The major difference between the two methods was that, although the basement membrane could not be continuously followed along these ductules ultrastructurally, the immunohistochemistry showed consistent laminin positivity around them (Figure 4A). The cylinder formed by the basement membrane had an open end plugged by a

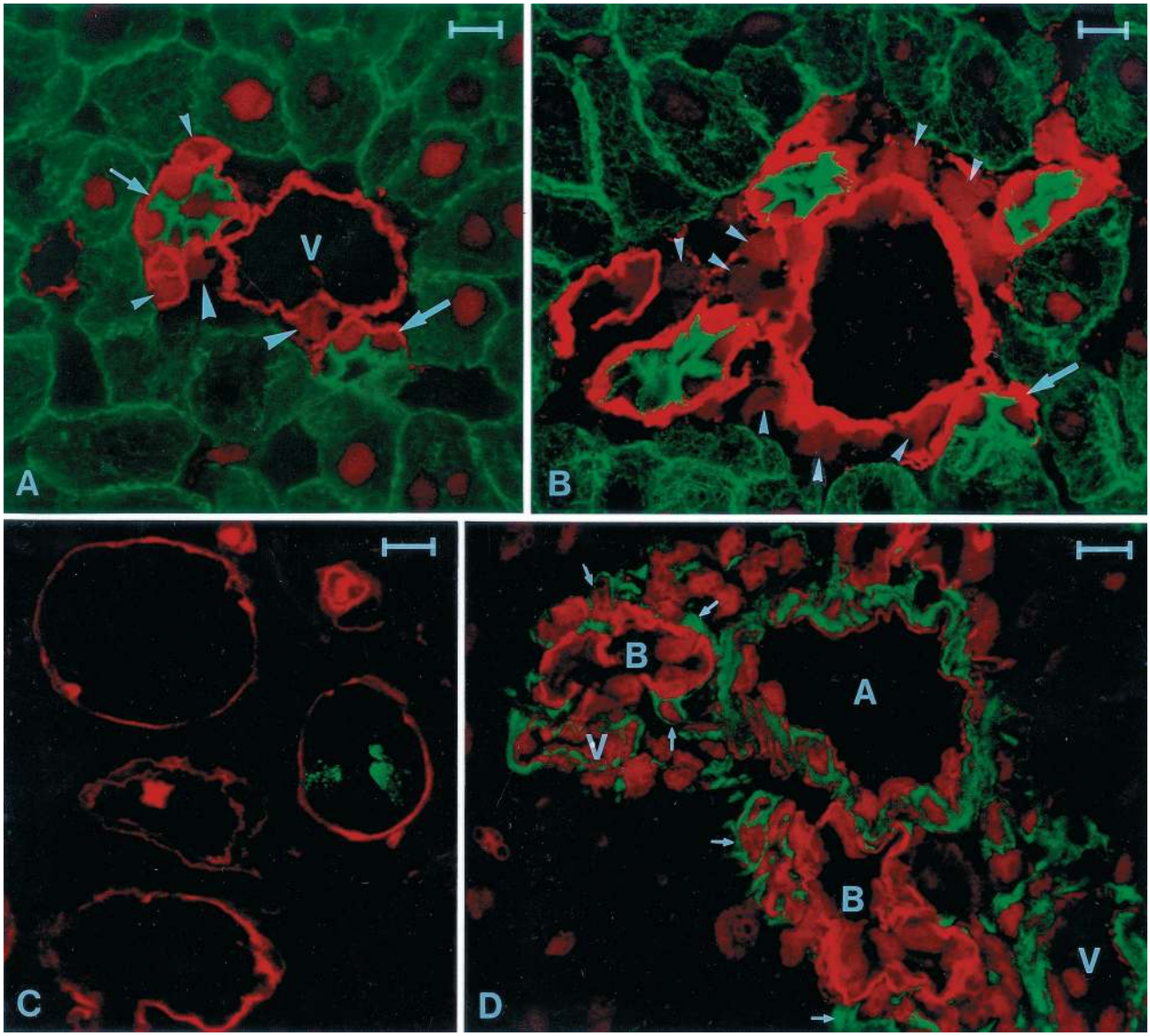

Figure 2. A: Portal area of a control liver stained for cytokeratin (green) and laminin (red). The section was additionally stained with propidium iodide to localize nuclei. Basement membrane of portal vein (V), capillaries (**small arrowheads**), bile duct (**small arrow**), and a canal of Hering (**large arrow**) are stained by the anti-laminin antibody. Nuclei of two connective tissue cells can be detected (**large arrowheads**) in the periportal area. The bile duct and canal of Hering lining cells show a stronger cytoplasmic staining for cytokeratin than the hepatocytes. Scale bar, 10 μm. **B:** Portal area stained for cytokeratin and laminin 2 days after AAF treatment. Localization of basement membranes are similar to that of control liver. Note the presence of numerous cytokeratin-negative cells in the periportal connective tissue (**arrowheads**), but there are no cytokeratin-positive cells in the periportal space outside the basement membrane. The canal of Hering (**arrow**) shows normal structure. Scale bar, 10 μm. **C:** Two days after AAF treatment AFP-positive (green) bile ductule cells are present strictly within basement membrane (red). Scale bar, 10 μm. **D:** Portal area 2 days after AAF treatment, stained for laminin (red) and desmin (green). The section was additionally stained with propidium iodide to localize nuclei, as shown in **A** and **B**. Numerous cells in the connective tissue, which proved to be negative for cytokeratin (**B**), stain positively for desmin. Some of the desmin-positive cells positioned closely to the basement membrane of the bile ducts (B) (**arrows**). A, arteriole; V, venule. Scale bar, 10 μm.

hepatocyte, and desmin-positive stellate cells accompanied the ductular structures outside the basement membrane (Figure 4B).

Ten days after the partial hepatectomy the cytokeratin-laminin immunohistochemistry did not reveal any basic change in the structure of advancing ductules, demonstrating that the oval cells still formed ductular structures extensively infiltrating into the liver parenchyma and maintained contact with hepatocytes at their origin (Figure 5, A and B). The oval cells still could be easily distinguished from the hepatocytes by the strong cytoplasmic cytokeratin staining. The proliferating oval cells were heterogeneously decorated by the AFP antibody. Similar to earlier time points, these cells were always surrounded by basement membrane and no AFP-positive cells were seen outside the laminin staining (Figure 5C). At this stage, oval cells were neither identified outside the ductular structures nor interposed between hepatocytes. The desmin reaction verified that activated stellate cells surrounded the advancing ductules and formed processes that penetrated and caused focal discontinuity of the basement membrane (Figure 5D).

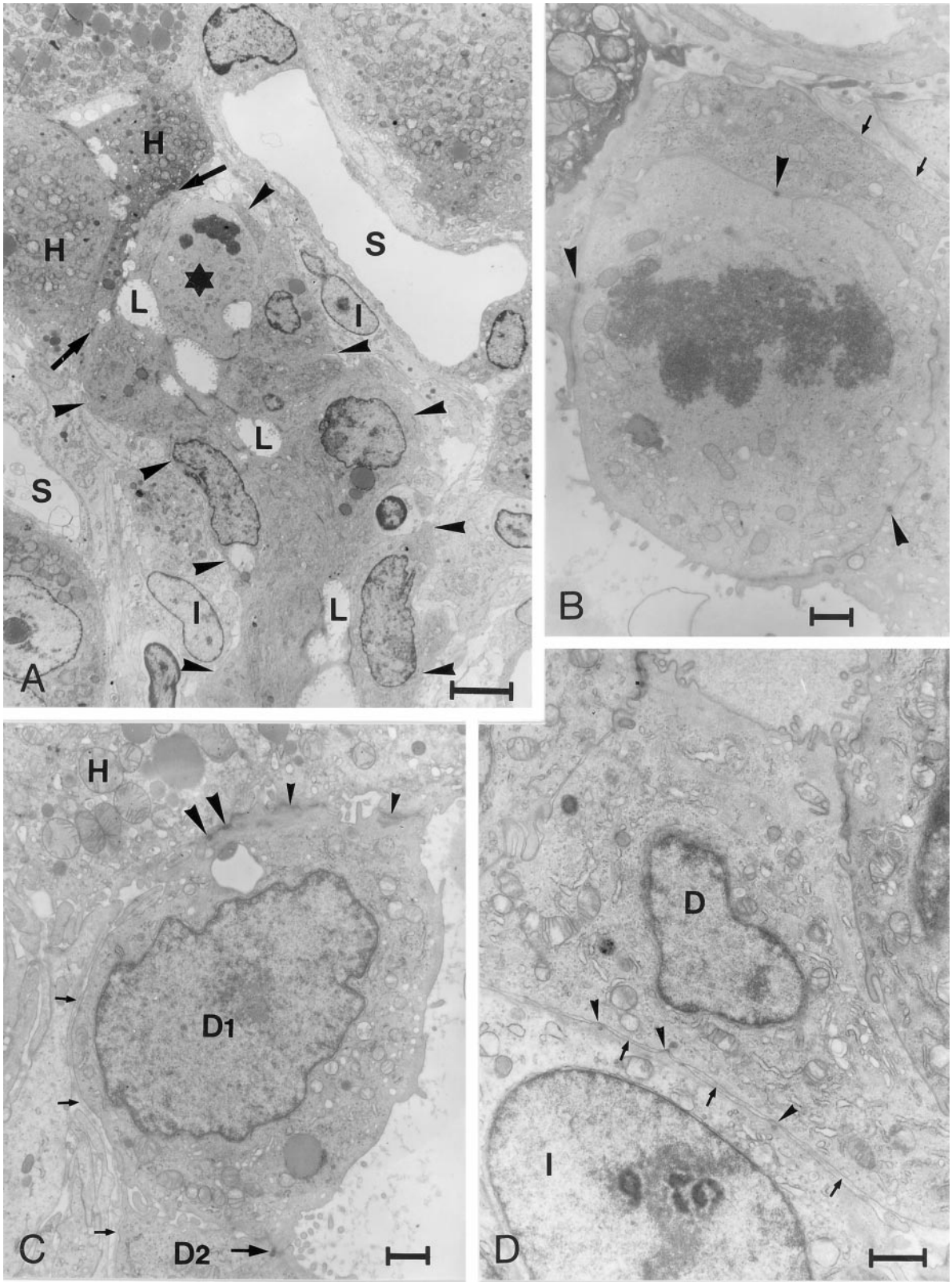

Figure 3. Ultrastructure of a portal area 3 days after PH in an AAF-treated rat. **A:** Oval cells forming a ductule (**arrowheads**; L, ductule lumen), squeeze between sinusoids (S). The ductule terminates on hepatocytes (H, **arrows**). One cell of the ductule is in mitosis (**asterisk**). Several stellate-like cells (I) are located closely to the advancing ductule. Scale bar, 5 μm. **B:** Higher magnification of the mitotic oval cell shown in **A** in a different sectioning plane. The cell is connected to the other cells forming the ductule by desmosomes (**arrowheads**). A small amount of basement membrane-like material (**arrows**) is visible under the process of a ductule cell. Scale bar, 1 μm. **C:** Detail of the ductule shown in **A**. Terminal cell of the growing ductule D1 is connected to the hepatocyte (H) by desmosomes (**arrowhead**) and tight junctions (**small arrowheads**). Desmosome (**large arrow**) is also discernible between the two ductule cells (D1, D2). Well-defined basement membrane (**small arrows**) with small gaps can be observed under the ductule cells. Scale bar, 1 μm. **D:** Ito-like cell (I) is in direct contact with the ductule cell (D), through the small gaps (**arrowheads**) of the basement membrane (**arrows**). Scale bar, 1 μm.

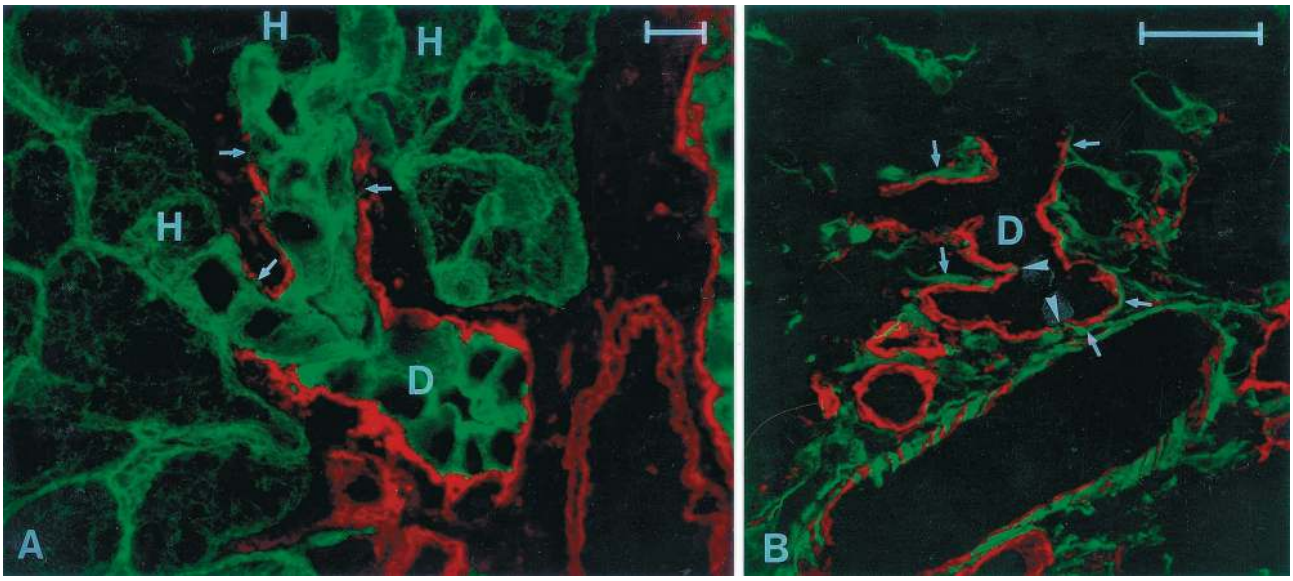

Figure 4. A: Portal area stained for cytokeratin (green) and laminin (red) 3 days after PH in an AAF-treated rat. The bifurcating ductule (D) strongly positive for cytokeratin, is surrounded by continuous basement membrane. Minute discontinuities (**arrows**) can be observed only at the vicinity of hepatocytes (H). Scale bar, 10 μm. **B:** Portal area containing a bifurcating ductule (D) stained for laminin (red) and desmin (green). The basement membrane is continuous, except minute gaps where it is penetrated by desmin-positive Ito cell processes (**arrowhead**). Numerous other desmin-positive cells are spread along the basement membrane (**arrows**). Scale bar, 50 μm.

Discussion

We have compared the BDL- and AAF-induced ductular cell proliferation in the rat liver by electron microscopy and immunohistochemistry. Both treatments resulted in the proliferation of the biliary epithelial cells. However, the distribution of the dividing biliary cells was different. The percentage of BrdU-labeled cells in the canals of Hering was significantly higher after AAF administration. Analysis of morphological changes in the AAF/PH model revealed that proliferating oval cells always formed ductules that were elongated and tortuous extensions of the pre-existing canals of Hering. These oval cell ductules were always surrounded by laminin and terminated at hepatocytes located at the limiting plate. These ductular structures were always accompanied by activated and proliferating stellate cells.

Studies similar to our own have been done by several investigators[8,13,21–24] using the AAF-induced oval cell reaction. The difference between these studies and ours is that we examined earlier time points of the model to observe the first proliferating cells under conditions in which the original histological structure of the liver is minimally affected. Our results support the notion of oval cells being derived from the biliary epithelium, strongly indicating that liver stem cells are located in the biliary system.

Among other candidates for liver stem cells are the so-called "nondescript" periductular cells. Sell and Salman[8] observed that the first proliferating cells after AAF feeding in rats maintained on a choline-deficient diet are periductal and the ductular cells follow them 1 or 2 days later. These periductal cells are located outside the basement membrane and contained no ultrastructural features that identified them. Interestingly, the increase in the number of thymidine-labeled periductular cells was equal to or greater than the labeled ductal cells. Studying a similar model to that of Sell and colleagues[21] (but without the choline-deficient diet), we have also observed periductal proliferating cells. Cytokeratin, however, decorated strictly laminin-surrounded cells in the periportal area, ie, no epithelial cells were seen outside the basement membrane. AFP is an extensively used marker of the oval cells in the rat liver[25,26] and the presence of AFP also indicates a hepatocytic directed commitment of the cells. AFP staining in some of the biliary epithelial cells after two doses of AAF supports the hypothesis that these cells are the precursors of the oval cells. In addition, the persistent lack of AFP staining outside the basement membrane throughout the experimental period strongly argues against the potential periductal origin of the oval cells. According to studies in other stem cell systems,[27] in which a high cell production rate is required, the stem cells fulfill this task via a dividing transit or amplification cell population. The cells of this compartment constitute an intermediary or transition population between the stem cells and the mature functional compartment, and they usually acquire one or more differentiation markers. Traditionally the oval cells are thought to represent this amplification compartment in this experimental model.[1] If the nondescript periductal cells studied by Sell and colleagues[21] were the stem cells, we would not expect them to proliferate at the same rate as the dividing transit compartment represented by the oval cells. Another possibility might be that the periductal cells are (alone or together with the ductular cells) the amplification compartment. These cells are, however, cytokeratin- and AFP-negative, and do not show any phenotypic or morphological (even at the ultrastructural level) sign of differentiation. Furthermore, the nondescript periductal cells would be expected to penetrate the basement mem-

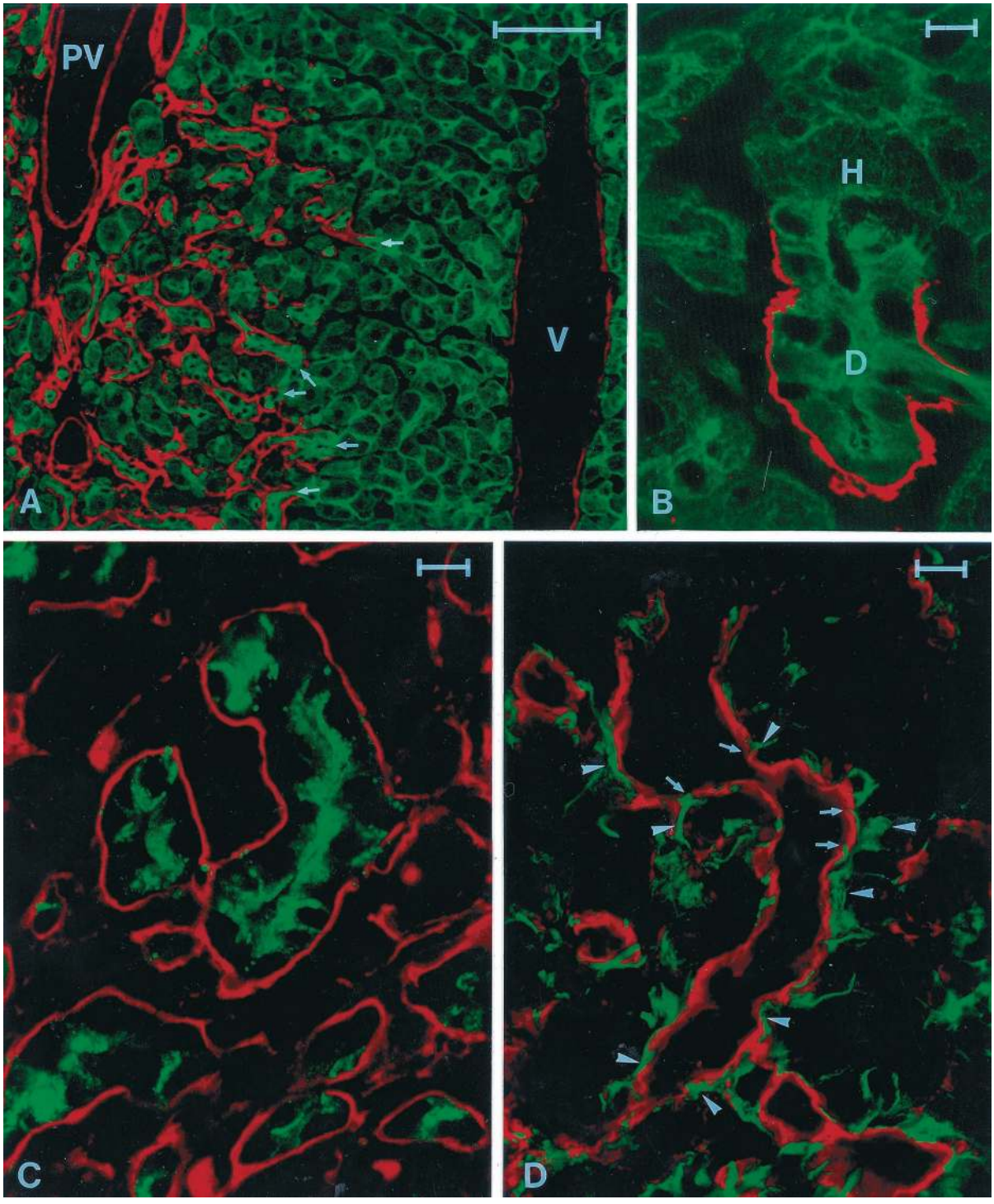

Figure 5. Immunohistochemistry of the liver 10 days after partial hepatectomy in the AAF/PH-treated rat. **A:** Low-power micrograph of a liver lobule stained for cytokeratin (green) and laminin (red). Numerous ductules surrounded by laminin are infiltrating the liver parenchyme toward the central vein (V). Several U-shaped laminin-stained structures, with open endpoints toward the central vein (**arrows**), can be seen at the border of the oval cell infiltration. (PV, portal vein). Scale bar, 100 μm. **B:** High magnification of a ductule (D) at the infiltration border. Oval cells are attached to a hepatocyte (H). Laminin staining encloses only the ductule, and it does not extend over the hepatocyte (H). Scale bar, 10 μm. **C:** Tortuous oval cell built ductules in the liver lobule stained for laminin (red) and AFP (green). The ductules are surrounded by laminin and AFP staining is observable only inside the basement membrane. Scale bar, 10 μm. **D:** Similar ductules as in **C** stained for laminin (red) and desmin (green). Numerous desmin-positive cells are spreading along the basement membrane (**arrowheads**). At several places processes of these cells puncture the basement membrane (**arrows**), but these processes never extend over the basement membrane. Scale bar, 10 μm.

brane in great numbers during the process of differentiation, but neither Sell and Salman[8] nor we observed any sign of this. Therefore, our data do not support the stem cell nature of the periductal cells. There are, however, plenty of desmin-positive stellate cells actively proliferating around the ductules in the AAF/PH model. Although we have not precisely quantitated the number of stellate cells, it seems to be approximately equal to that of oval

cells. It is therefore possible that at least some of the periductal proliferating cells described by Sell and Salman[8] might be stellate cells.

Several studies have supported possibilities of the stem cells being located in the biliary system. These include terminal hepatic ductules;[4,5,22,28] all biliary epithelial cells,[29–32] and a very primitive looking cell, referred to as a "basal cell" inside the bile duct.[23,24] The basal cells proliferated 2 to 3 days after the partial hepatectomy in the livers of rats treated according to Solt and colleagues[33] carcinogenesis schedule. We also saw these small, intraepithelial cells in our sections, but they never incorporated BrdU or showed morphological signs of proliferation. The reason for this difference is not altogether clear. Novikoff and colleagues[23] and Novikoff and Yam[24] used the DEN-initiated Solt-Farber model, whereas we avoided DEN administration. Although the oval cell reaction is similar, the histological changes are more complex in the Solt-Farber model according to our experience. Anilkumar and colleagues[34] also described divergent histological reactions in the two models. It is however evident that oval cell proliferation can be induced without the participation of the basal cells in our experimental model. Therefore, the biliary epithelial cells can function as facultative liver stem cells in the AAF/PH experimental model. We can, of course, not exclude the possibility that the basal cells represent an even more ancient stem cell population that is activated by a more drastic, carcinogenic protocol and may be responsible, eg, for the frequently observed metaplastic hemopoiesis in hepatocarcinogenesis experiments.[35,36] Recently Petersen and colleagues[37] and Theise and colleagues[38] have provided evidence that hemopoietic stem cells can give rise to oval cells and hepatocytes. The basal cells may perhaps represent a common precursor for the two systems. Although these observations have a dramatic impact on our view of stem cell biology, there is a general agreement that under most circumstances the liver regenerates from cell populations confined to the liver. These cells are the focus of this study and the participation of hemopoietic cells in liver regeneration is not addressed here.

The notion of which segment(s) of the biliary tree harbors the stem cells is still controversial. Although a substantial amount of data indicate that the liver stem cells are confined to the terminal hepatic ductules, arguments have been made suggesting that any component of the biliary tree can give rise to oval cells.[29–32] We have previously shown that chronic dexamethasone treatment is able to prevent the oval cell proliferation triggered by the AAF/PH protocol while not at all inhibiting the BDL-induced proliferation of the larger, mostly interlobular bile ducts.[17] The preferential BrdU labeling of cells in the canals of Hering after AAF administration, suggested that selective inhibition of cell proliferation in these cells might be achieved by dexamethasone. Contrary to expectations dexamethasone completely inhibited the AAF-induced biliary cell proliferation regardless of their location.[17] These results fail to provide a functional confirmation that the terminal ductules are the exclusive sources of the oval cells. Furthermore, the proliferations of morphologically and topologically identical biliary cells were differently regulated by dexamethasone. These data suggest that there are at least two different mechanisms regulating proliferation of the biliary epithelium, each providing functionally different progeny. Although the dexamethasone-sensitive pathway provides cells with stem cell potential, the dexamethasone-resistant pathway produces only biliary epithelial cells. These results may, at least in part, be analogous to the regulation of hepatocyte cell cycle induced by the partial hepatectomy and primary mitogens.[39] It is well established that these proliferative models have different biological potentials; eg, hepatocyte proliferation induced by partial hepatectomy has carcinogenic promoting capacity whereas the other one induced by direct mitogens has none.[40] There are already observations indicating the differential regulation of these two biliary reactions. Mice harboring congenitally defective SCF/c-kit system retain an intact proliferative response after BDL,[41] whereas the oval cell proliferation is remarkably suppressed in rats with deficient c-kit kinase activity.[42]

The fact that hepatocytic differentiation occurs in the pancreas[43,44] and extrahepatic bile ducts[45] also argues against the restricted occurrence of multipotential stem cells in the canals of Hering. Our observation that AAF targets preferentially the cholangioles while BDL targets the larger bile ducts can be explained by topological factors. The primary stimulus for biliary proliferation after BDL is the increased intraductal pressure.[15] The pressure is probably higher in the interlobular bile ducts, than in the ductules. Differential expression of drug metabolizing enzymes by different segments of the biliary tree[46] may provide an alternative or additional explanation for the differential response to AAF. Potten[47] described a hierarchy of the stem cells in the small intestine glands. Depending on the severity of injury, more and more resistant cells participate in the repair. Additionally, this hierarchy is related to the topography of the cells. A similar arrangement cannot be excluded in the biliary system. This notion is supported by the well-known heterogeneity of cholangiocytes.[48,49]

Experiments using injection of pigmented gelatin medium and related substances into the biliary tree have demonstrated that the majority of oval cells are part of a ductular reaction.[10,13,50] However, together with these observations, occurrence of isolated oval cells, sometimes located between pre-existent fully mature hepatocytes, have been described.[13] There is also conflicting assessments on the continuity of the basement membrane around the ductules.[24,50] To address this problem we studied earlier time points and complemented the electron microscopy with confocal laser microscopy. The confocal microscopy provided a much better overview of the histological reaction in addition to allowing simultaneous use of more than one marker. The laminin/cytokeratin double staining decorated very clearly the biliary ductules that would otherwise be difficult to recognize. There was a distinct continuous laminin staining around the canals of Hering that terminated at hepatocytes located at the limiting plate.

The traditional light microscopic view of the oval cell reaction is very complicated. The confocal microscopy, however, clearly revealed that the oval cells always form ductules surrounded by basement membrane that originate from the canals of Hering and terminate on a hepatocyte. As these oval cell ductules grow, they become tortuous, but they appear not to lose contact with their terminating hepatocyte, as was described by Betto and colleagues[51] in Long-Evans rats. To accomplish this task, it seems that the canal of Hering is ideally situated and therefore may provide the stem cell niche in the liver. Furthermore, this arrangement allows for continuous bile drainage throughout this complex reaction. The preservation of the original contact between the liver plate and the extending biliary ductule, which is composed of oval cells, may be extremely important for the maintenance of the liver architecture. We hypothesize that the disruption of the contact between the ductules and hepatocytes may occur in chronic interface hepatitis resulting in aimless ductular proliferation, followed by fibrosis, and finally reorganization of the liver structure resulting in cirrhosis.

The basement membranes frequently play an important role in the regenerative process. In certain tissues (eg, kidney tubules) the integrity of the basement membrane is required for the regeneration because it provides a track for the dividing epithelial cells.[52] The situation is probably different in the liver. There is no structured basement membrane along the liver plates. The fact that the regular basement membrane is sometimes missing ultrastructurally around the ductules, while they are always surrounded by laminin according to our immunohistochemical data, may indicate that it is in statum nascendi, providing a substrate for proliferation and migration. This process is similar to that observed during angiogenesis.[53] Activated stellate cells are always intimately associated with these ductules. Sometimes the processes of the stellate cells brake through the basement membrane and form direct cell-cell contact with the ductular epithelial cells. This connection that has not been described before may form the structural basis for the intensive cross talk between these two cell types.

In conclusion, although it has been known that most of the oval cells are organized into ductular structures sprouting from pre-existing bile ductules, this was not generalized to every oval cell and the development of these ductules was obscure. We suggest that the oval cell-formed ducts are simply extensions of the biliary ductules. The connection between the last ductular biliary cell and the corresponding hepatocyte is maintained for an extended period of time. The oval cells are preferentially generated by proliferation of the terminal biliary ductules that we suggest constitute the primary hepatic stem cell niche. However, the stem cell potential of the larger biliary ducts cannot be excluded. In fact, there seems to be two independently activated and regulated mechanisms for the proliferation of the biliary epithelium. One of these that can be blocked by dexamethasone results in progenies with the capacity for both differentiating into hepatocytes and possibly other cell lineages. The ductules are composed of oval cells and surrounded by continuous basement membrane that is intermittently disrupted by processes of stellate cells that form direct cell-cell contact with the oval cells.

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