Models for Hepatic Progenitor Cell Activation[1] (43660)

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Abstract. **Activation of liver progenitor cells was studied in rat liver induced to regenerate after carbon tetrachloride (CCl_4) or D-galactosamine (GalN) injury. A change in the concentration of histone-3 mRNA was used as a marker for cell proliferation and the fetal form of α-fetoprotein (AFP) mRNA as a marker for fetal hepatoblasts. γ-Glutamyltranspeptidase (GGT) and glutathione-S-transferase P were used as markers for activation of putative liver progenitor cells. After CCl_4 administration, the proliferative response was high but confined primarily to parenchymal cells. No changes in the relative expression of albumin, glutathione-S-transferase P or insulin-like growth factor-II were observed. On the other hand, the level of AFP mRNA was increased modestly and predominantly in the nonparenchymal cell (NPC) fraction. After GalN administration, proliferation of NPC began within 24 hr, primarily in the portal area around the bile ducts. Activated cells were bile "duct-like" in appearance, had scant cytoplasm, and a pale, oval-shaped nucleus. On Day 2, they formed rows and clusters, expanding from the portal zone and invading the parenchyma, as well as proliferating in regions of focal necrosis. On Days 3 and 5, NPC expressing histone-3 mRNA expanded further, forming pseudoducts and islet-like structures (NPC structures) throughout the hepatic lobule. Proliferating NPC were positive for GGT. Some GGT-positive cells on Days 3 and 5 were also positive for fetal AFP mRNA. Expression of fetal AFP mRNA lagged behind that of GGT by 24 hr, was highest on Day 5, and then declined. Expression of albumin mRNA and glucose 6-phosphatase decreased during the first 48 hr after GalN administration and then resumed. These findings indicate that after GalN injury, the liver responds with activation of putative progenitor cells that proliferate and then differentiate through the hepatocyte lineage, whereas the regenerative response after CCl_4 administration is primarily through proliferation of preexisting hepatocytes.** [P.S.E.B.M. 1993, Vol 204]

Observations from models of liver carcinogenesis in the rat have strongly suggested the existence of liver progenitor cells ("oval" cells) that proliferate under certain conditions. The proliferation of oval cells was demonstrated mainly in carcinogenic models (e.g., azo-dyes such as 2-acetylaminofluorene), when normal liver regeneration was impaired by inhibition of hepatocytes to enter the cell division cycle. The origin of oval cells is unknown. It has been suggested that they may be derived from ductular epithelial cells lining the canals of Hering or from stem cells located in the periportal region. These cells express traits specific for differentiated hepatocytes and fetal hepatoblasts, e.g., α-fetoprotein (AFP), albumin, γ-glutamyltranspeptidase (GGT), cytokeratin 19, adult and fetal isoenzyme forms of aldolase, pyruvate kinase and lactic dehydrogenase, and other liver-specific proteins, such as α-1-acid glycoprotein. They also have different developmental potentials (for reviews, see Refs. 1–6).

Attempts to follow the fate of proliferating oval cells after discontinuance or a short period of carcinogen treatment have led to contradictory conclusions. Some authors suggested that they may differentiate into hepatocytes (7–12), others proposed their atrophy (13–15). On the other hand, substantial evidence has been

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presented that oval cells can undergo transition to hyperplastic nodules and neoplastic transformation after prolonged carcinogen treatment and that oval cell lines can produce hepatocellular carinomas upon inoculation into recipients (11, 16–19).

However, very little data have been reported on the significant activation of a facultative liver stem cell population during liver regeneration, making its existence still controversial. Two well-defined models of liver regeneration are carbon tetrachloride (CCl_4) and D-galactosamine (GalN) injury. Both agents cause extensive liver damage and hepatocyte necrosis through different mechanisms. CCl_4 intoxication provokes changes in the plasma membrane, impairment of protein and lipoprotein synthesis, uncoupling of oxidative phosphorylation, inhibition of lipid transport, and accumulation of fat in hepatocytes (20–23) as a result of free radical formation (CCl_3) (24). At the structural level, acute CCl_4 toxicity is manifested by vast centrilobular necrosis with comparatively low inflammation but leaving intact the periportal zone. GalN, introduced into rats at a concentration higher than 20 mg/100 g body wt, causes complete consumption of all uridine nucleotides. As a consequence of uridylate "trapping," the synthesis of RNA is blocked, followed by inhibition of protein, glycoprotein, glycolipid, glycolipoprotein and phosphoprotein synthesis, and changes in plasma membranes, leading to hepatocyte death (25–27). Ultrastructural changes, observed after GalN administration, resemble acute hepatitis in the human: focal hepatocyte necrosis, inflammation, and proliferation of nonparenchymal cells (27–29).

In our present research, we have exploited these two models of liver regeneration to study activation of putative hepatocyte progenitor cells. We analyzed changes in expression of markers specific for immature fetal hepatocytes, e.g., AFP and GGT or glutathione-*S*-transferase P ($GST\text{-}Y_P$), and mature hepatocytes, e.g., albumin and glucose 6-phosphatase (G6Pase), in parenchymal cells (PC) and nonparenchymal cells (NPC) over the time course of liver regeneration. The results of these experiments lead to the suggestion that under some conditions of liver regeneration, there is activation of the putative hepatocyte progenitor cells, low after CCl_4 and high after GalN injury.

Materials and Methods

Animals and Treatment. Male Sprague-Dawley, Lewis, or Fischer 344 rats (180–200 g; Charles River Laboratories, Wilmington, MA) were used. Animals received standard laboratory chow and water *ad libitum*. CCl_4 was introduced through gastric gavage at a single dose of 0.5 ml/100 g body wt, and mixed 1:1 (v/v) with mineral oil 3 days before sacrifice. GalN was introduced intraperitoneally at a single dose of 70 mg/100 g body wt dissolved in 0.14 M NaCl. One to 5 days later, the animals were sacrificed and pieces of liver were fixed in formalin for histological analysis or frozen in 2-methylbutane at −70°C.

Isolation and Purification of Parenchymal and Nonparenchymal Liver Cells. Liver cells were isolated after standard collagenase perfusion and fractionated and purified consecutively through a Nycodenz (Accurate Science, Westbury, NY) density gradient and centrifugal elutriation (Beckman J2–21 centrifuge), as described previously (30).

Northern Blot Analysis. Total RNA was isolated from PC and NPC, electrophoresed in a denaturing gel, blotted to a nylon membrane, and hybridized to the respective multiprime random DNA-labeled probes as described previously (30). The DNA fragments used as probes were: (i) a 516-bp fragment of the rat albumin cDNA sequence, pGEM4ZALB, cloned in our laboratory; (ii) a 750-bp fragment of rat AFP cDNA pRAF87, originally cloned by Jagodzinski *et al.*, a gift from Dr. T. Sargent; (iii) a 204-bp fragment of rat histone-3 cDNA, pGH3, a gift from Dr. A. Skoultchi, Albert Einstein College of Medicine, Bronx, NY; (iv) a 718-bp fragment of human $GST\text{-}Y_p$ cDNA, a gift from Dr. I. Listowsky, Albert Einstein College of Medicine; and (v) an 833-bp fragment of human insulin-like growth factor-II cDNA, pGEM3ZhIGFII, a gift from Dr. D. Yang, Albert Einstein College of Medicine.

In Situ Hybridization. *In situ* hybridization was performed essentially as described previously (30). The following plasmids were used for preparing the riboprobes for hybridization: (i) pGH3, as described above; (ii) 345 bp from rat albumin cDNA, pGALB 345, described by Shalaby and Shafritz (31); and (iii) 700 bp from rat fetal AFP cDNA, pBAF700, kindly provided by Dr. N. Fausto, Brown University, Providence, RI.

Histochemistry. The histochemical reaction for GGT was performed according to the method of Rutenberg *et al.* (32). The sections were counterstained with hematoxylin and mounted in Gel/Mount (Biomedica Corp., Foster City, CA).

Results

Changes in Gene Expression of PC and NPC during Liver Regeneration Induced by Carbon Tetrachloride. The steady state level of liver-specific mRNAs and histone-3 mRNA was analyzed 3 days after the injury, when, as shown previously, the concentration of AFP mRNA in the liver reaches its maximum (33). RNA from fractionated PC and NPC was isolated and fractionated on a denaturing gel and the relative concentration of mRNA was determined after hybridization to DNA-specific probes.

In normal (quiescent) liver, the level of histone-3 mRNA in PC was so low that it was barely detectable (Fig. 1). On the other hand, the expression of histone-3 mRNA was conspicuous in the NPC fraction, reflect-

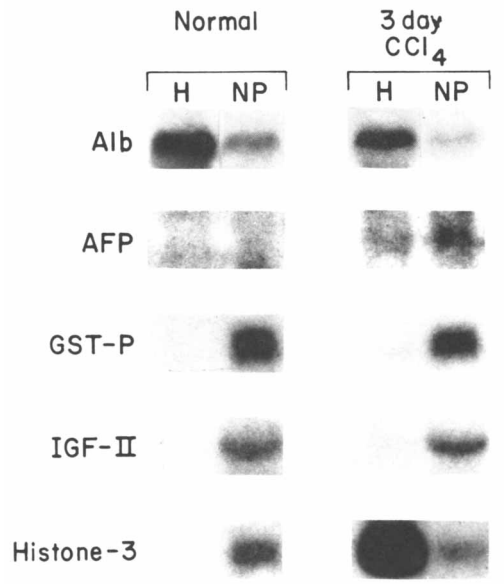

Figure 1. Changes in the steady state level of specific mRNAs in PC and NPC of rat liver after CCl_4 administration. Ten micrograms of total RNA from each cellular fraction were electrophoresed under denaturing conditions and transferred to a nylon membrane and the blots were probed with specific cDNA clones as indicated (see Materials and Methods for experimental details).

ing a higher proliferative activity. Three days after CCl_4 administration, the level of histone-mRNA in the PC fraction increases abruptly, but it did not change in the NPC fraction. These results indicate that the bulk of NPC are not activated to proliferate after CCl_4 administration.

We tested two other liver-specific genes, expressed in fetal but not in adult hepatocytes, that serve as markers for NPC: AFP and GST-Y_p. While GST-Y_p mRNA did not show changes in its expression, AFP mRNA was increased in the PC fraction and considerably more in the NPC fraction, suggesting mild activation of putative progenitor cells. As expected, the steady-state level of albumin mRNA in PC was still slightly decreased on Day 3, since the normal level is reached only after 7 days following CCl_4 administration (33). The low level of albumin mRNA expression in NPC is most probably due to slight contamination of the NPC fraction with PC.

In the course of these experiments, we made the interesting observation that insulin-like growth factor-II is expressed only in the NPC fraction. This has not been reported previously in the adult liver. The level of mRNA for this protein was not increased.

Changes in Gene Expression of PC and NPC during Rat Liver Regeneration Induced by D-Galactosamine. Changes in the level of mRNAs in liver-specific genes was studied by *in situ* hybridization to ^{35}S antisense riboprobes. Histone-3 mRNA expression was used to follow the proliferative response of the different cell types. Expression of albumin and AFP mRNA detected by *in situ* hybridization, and G6Pase and GGT, demonstrated by histochemistry, were used as a tool to follow the differentiation potential of NPC cells during liver regeneration induced by GalN.

Changes in Histone-3 mRNA Expression after GalN Injury. In normal liver, very few hepatocytes were positive for histone-3 mRNA, reflecting a very low proliferative activity of adult hepatocytes (34). In contrast, groups of NPC in the portal regions expressing histone-3 mRNA could be found much more frequently (Fig. 2). Initially, 24 hr after GalN administration, periportal sinusoidal cells and single or small groups of NPC with the appearance of bile duct-like cells began to proliferate in the portal and periportal zones, as noted by expression of histone-3 mRNA. The reaction in mature bile duct epithelial cells was much lower and occasional single small hepatocytes were also labeled (Fig. 2).

Two days after GalN injury, the number of histone-3 mRNA-labeled cells in the periportal zones and in the sinusoids increased. Most of the NPC, expanding in rows from the portal triads into the parenchyma and from the areas of scattered inflammation infiltrated by white blood cells, became strongly positive for histone-3 mRNA. These cells exhibited pale, oval-shaped nuclei and scant cytoplasm. Bile duct epithelial cells were also labeled. Small and large hepatocytes scattered throughout the parenchyma also expressed histone-3 mRNA (Fig. 3).

On Day 3, clusters and duct-like structures of NPC cells expanded from the portal zone and from regions of parenchymal inflammation and invaded the parenchyma, reaching maximum expression of histone-3 mRNA. In contrast, the portal regions showed less histone-3 mRNA labeling. At this time, PC also reached maximum labeling for histone-3 mRNA. Five days after the injury, proliferative activity decreased. Periportal histone-3 mRNA expression continued to decrease and fewer labeled hepatocytes surrounded the remnants of proliferated NPC (Fig. 3).

Changes in Expression of Albumin mRNA after GalN Injury. After GalN injury, the message for albumin, the most abundantly expressed liver-specific gene, decreased abruptly, reaching minimum by Day 2 (Figs. 4 and 5, dark and bright field). In regions of focal necrosis or NPC infiltration, expression of albumin mRNA was completely shut off (Fig. 4). One day later, albumin mRNA began to accumulate in areas where the parenchyma was in the process of restoration. Some NPC in clusters and pseudoducts also expressed albumin mRNA (Fig. 5). On Day 5, the liver gradually returned to normal expression of albumin mRNA, as evidenced by reduction in the perimeter of areas where albumin mRNA was not expressed (Fig. 4, dark field). At this time, it was relatively easily to identify cells

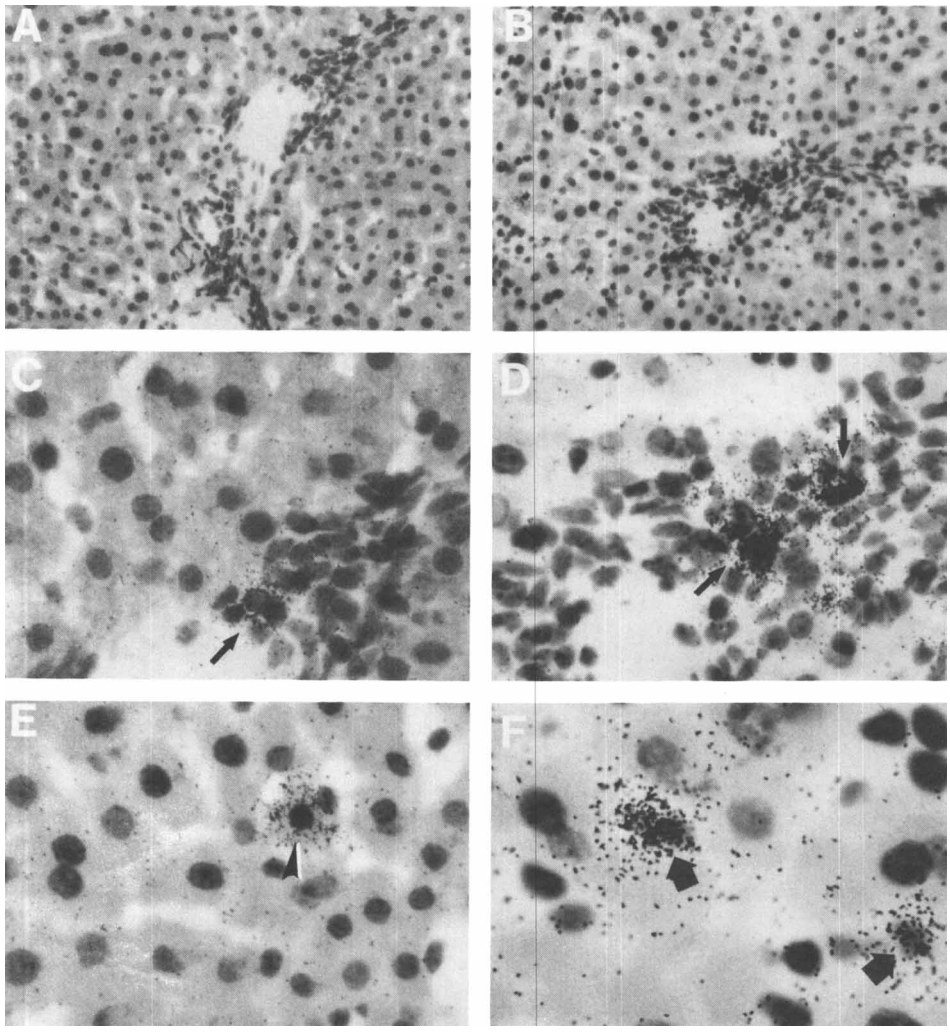

Figure 2. Detection of histone-3 mRNA by *in situ* hybridization in liver sections of rats treated with GalN. Portal and periportal zones: A, B, C, and D; parenchyma; E and F, control animals: A, C, and E. Occasionally single hepatocytes are expressing histone-mRNA (arrowhead in E); higher proliferative activity is observed in NPC in the portal area (arrow in C). One day after GalN injury: B, D, and F. The proliferative response begins in the portal region in NPC adjacent to the bile ducts (arrow in D). Activated sinusoidal cells also express histone-3 mRNA (bold arrow in F) (hematoxylin and eosin staining; original magnification A and B ×200; C, D, and E ×600; I ×1000).

within the NPC clusters expressing albumin mRNA (Fig. 5).

Changes in Expression of AFP mRNA after GalN Injury. In normal liver, fetal AFP mRNA was occasionally expressed in single hepatocytes scattered throughout the lobule (Fig. 6). One day later, expression of AFP was completely shut off and no cells labeled for AFP mRNA could be identified. On Day 2, occasionally single cells or small groups of NPC in the portal zone showed fetal AFP mRNA expression, although the majority of early proliferating NPC were negative. On Day 3, fetal AFP message appeared in NPC that formed rows, clusters, or duct-like structures in the portal zone and in adjacent parenchyma. These cells again exhibited scant cytoplasm and pale, oval-shaped, homogeneously stained nuclei. It should be mentioned that not all cells in these NPC structures expressed AFP mRNA. Single, scattered hepatocytes, positive for AFP mRNA, were also identified, mostly in the vicinity of proliferating periportal NPC. On Day 5, fetal AFP mRNA expression reached maximum, but it was present mostly in NPC that had moved away from the periportal zone into the parenchyma. Fetal AFP mRNA was also detected in single hepatocytes within the parenchyma, usually as part of duct-like structures or within/adjacent to proliferating NPC structures.

Histochemical Detection of GGT. As shown previously, GGT is expressed in normal liver only in mature bile ducts cells in the portal zone (35, 36). We found also a few scattered granulocytes expressing GGT (results not shown). On Day 1 after GalN administration, single cells in the region of inflammation, identified as eosinophils (because of high peroxidase activity), became strongly GGT positive (Fig. 7). On Days 2 and 3, proliferating oval cells within the portal areas began to express GGT, and as they expanded from the portal

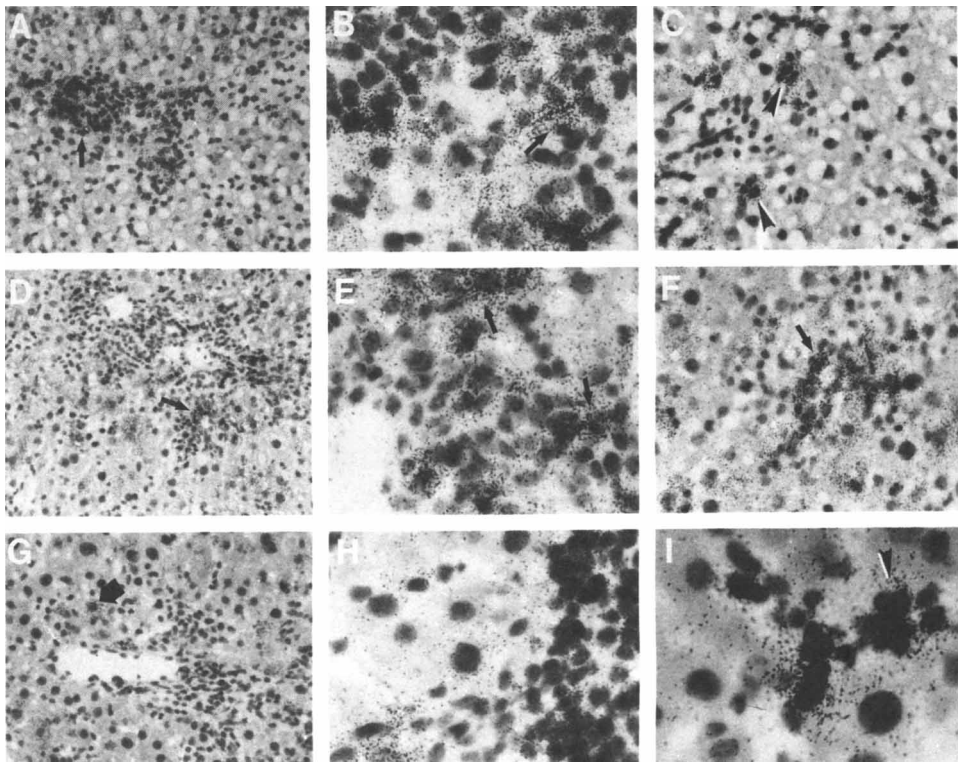

Figure 3. Detection of histone-3 mRNA in liver sections of rats treated with GalN (continued). Portal and periportal zones: A, B, D, E, G, and H; parenchyma: C, F, and I. Day 2: A–C. High expression of histone-3 mRNA in the portal region in cells forming rows, clusters, and pseudoducts (NPC structures), arrows in A and B. High expression in parenchyma, mainly in the sinusoidal spaces, as shown by arrowheads in C. Day 3: D–F. High expression of histone-3 mRNA in periportal NPC structures, arrows in D and E. NPC structures in parenchyma expressing histone-3 mRNA, arrows in F. Day 5: G–I. The proliferative response in NPC in the portal region decreases; still high expression of histone-3 mRNA is observed in hepatocytes (bold arrow in G) and sinusoidal cells in the parenchyma (arrowhead in I) (hematoxylin and eosin staining; original magnification A, D, and G ×200; C and F ×400; B, E, and H ×600; I ×1000).

regions into the parenchyma, they formed rows, sheets, clusters, and pseudoductular structures of GGT-positive cells. GGT staining in NPC was diffusely scattered throughout the cytoplasm. Some of the GGT-positive cells in NPC structures were larger with more round-shaped nuclei, their morphology being intermediate between oval cells and hepatocytes. On Day 5, hepatocytes adjacent to the periportal regions or in the vicinity of GGT-positive clusters of NPC began to express GGT. In some of these hepatocytes, the cytoplasm was diffusely stained, whereas in others, cytoplasmic staining for GGT was fading and enzyme activity was localized to the canalicular membrane surface (Fig. 7).

These results clearly show that at the beginning of liver regeneration, a subpopulation of cells in the NPC compartment are the most likely candidate as liver progenitor cells. They are expressing GGT and, as the regeneration progresses, some of these GGT-positive cells change their morphologic appearance toward the hepatocyte lineage. These cells are larger, with a more round-shaped nucleus, and are considered transitional cells (Days 3–5). Maturation of transitional cells into hepatocytes is evidenced by the appearance of GGT-positive hepatocytes on Day 5, with very young hepatocytes expressing GGT throughout the cytoplasm, whereas maturing hepatocytes express the enzyme at the canalicular surface of the cell. Fully differentiated hepatocytes no longer express GGT (as determined by this histochemical method).

Discussion

In these studies we used two models of liver regeneration, CCl_4 intoxication and GalN injury, to evaluate the role of putative liver progenitor cells in restoration of parenchymal mass. The cellular fraction/compartment under observation was that of the NPC, the most likely candidate to contain a subpopulation of hepatocyte progenitor cells. We found that 3 days after CCl_4 induced hepatic necrosis, the level of fetal AFP mRNA in the NPC fraction increased, consistent with earlier observations (33, 37, 38). Our results provide further information that the activation of AFP mRNA expression occurs primarily in NPC, i.e., in the putative progenitor cell population within in this compartment. It should be noted, however, that activation of the hepatocyte progenitor cells after CCl_4 administration was relatively low compared with the GalN model. This was also reflected in the low activation of histone-3 and

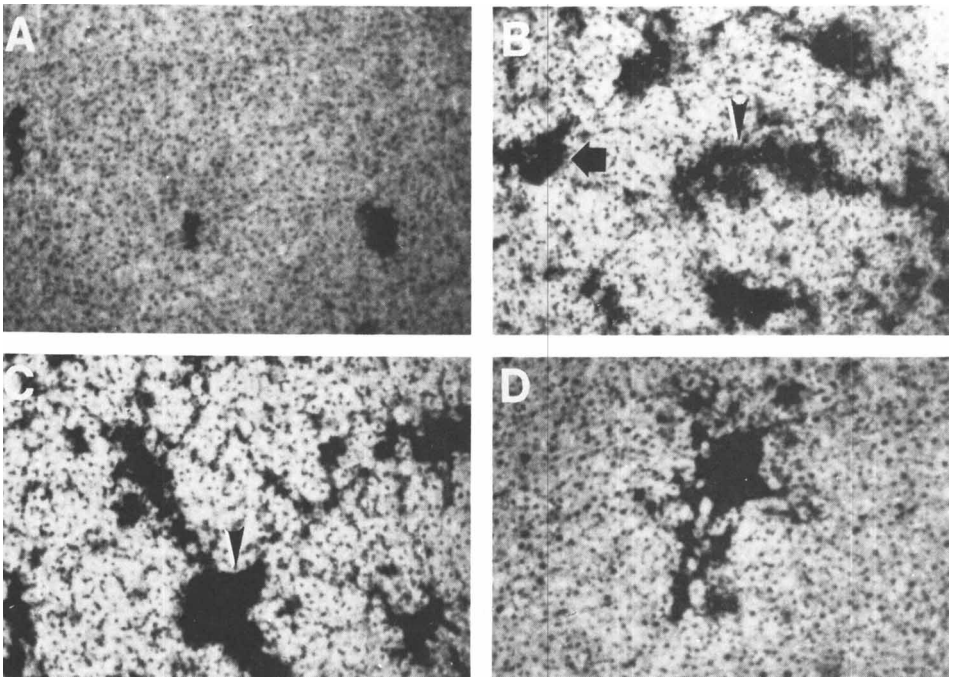

Figure 4. Detection of albumin mRNA by *in situ* hybridization in liver sections of rats after GalN treatment (dark field analysis). Overview of the liver lobule. Control: A. High expression of albumin mRNA throughout the lobule, except in the portal zone. Day 2: B. Lack of albumin mRNA expression in areas of focal necrosis (bold arrow) and in the portal region (arrowhead). Day 3: C. Regions of focal necrosis decrease; still there is no expression of albumin mRNA in portal zones engaged in NPC proliferation. Day 5: D. The liver is regaining its normal structure and areas around the portal zone, not expressing albumin mRNA, are decreasing (original magnification A–D ×100).

GTS-Y_p mRNA expression in these cells. These findings could be explained either by a dose effect of CCl_4 or the fact that CCl_4 does not block proliferation of surviving hepatocytes, whereas GalN prevents these cells from dividing.

To determine the precursor-product relationship between the putative progenitor cells and mature hepatocytes, we followed the kinetics of changes in the expression of specific liver genes, i.e., the level of AFP mRNA and GGT enzyme activity in proliferating NPC. These two markers are expressed in fetal hepatoblasts, but not in adult hepatocytes. Cells in the NP compartment that was the subject of our attention began to proliferate 1–2 days after GalN injury. In the course of 2–3 additional days, these cells formed typical "NPC structures": rows, clusters, and pseudoducts of cells resembling those comprising the bile ducts. These cells were small, with scant cytoplasm and a pale blue-staining, oval-shaped nucleus. We designate these cells progenitor cells. These progenitor cells did not appear to originate from the mature bile ducts: they began to proliferate before the bile duct cells, became positive for GGT on Day 2, and 1 day later began to express AFP mRNA, a marker that was not expressed by bile duct cells. On Day 5, we were able to distinguish morphological changes in some of the putative progenitor cells, i.e., the cells in direct continuity and physically adjacent to AFP mRNA positive oval cells in duct-like NPC structures, as well as larger cells with more round-shaped nuclei (transitional cells), expressing AFP mRNA. By double-label *in situ* hybridization experiments, we were able to demonstrate simultaneous expression of AFP and albumin mRNA in these cells (data not shown).

If mature hepatocytes were dividing immediately after GalN injury, leading to proliferation of new cells, then maximal histone-3 mRNA labeling of these hepatocytes should have been observed within 2–3 days after GalN administration. However, we observed maximum histone-3 mRNA labeling of hepatocytes on Day 5, long after the appearance of histone-3-labeled NPC and transitional cells. Moreover, the expression of AFP mRNA and GGT was observed in NPC before maximal histone-3 mRNA labeling of hepatocytes. Therefore, our results suggest that NPC that proliferate, mature, and differentiate into PC after GalN liver injury are not derived from preexisting hepatocytes, but rather from a separate population of progenitor cells (facultative "stem-like" cells) residing in the periportal region.

Initially, Kuhlmann and Wuster (39) and then Lemire *et al.* (40) found expression of AFP/AFP mRNA in bile duct cells after GalN treatment. On the basis of [^{3}H]thymidine experiments, tracing the label in the course of liver regeneration upon GalN treatment, Lemire *et al.* (40) concluded that duct epithelials cells can generate both oval cells and small hepatocytes.

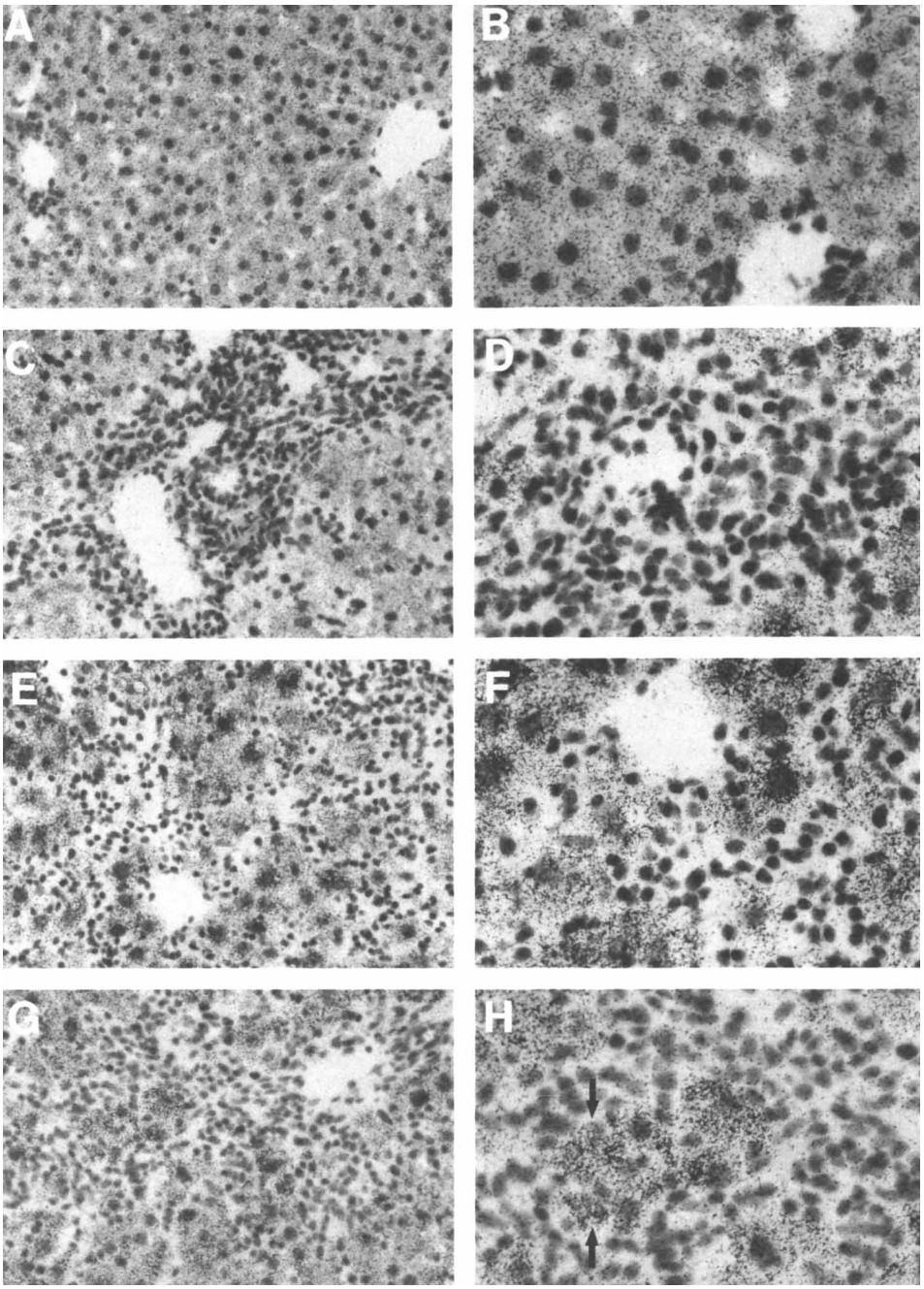

Figure 5. Detection of albumin mRNA by *in situ* hybridization in liver sections of rats after GalN treatment (bright field analysis). Controls: A and B. High expression of albumin mRNA in hepatocytes. Day 2: C and D. Day 3: E and F. No expression of albumin mRNA in NPC structures and in areas of focal necrosis and inflammation. Day 5: G and H. Appearance of cells in NPC structures that are larger and with more round-shaped nuclei, expressing albumin mRNA (arrows in H) (hematoxylin and eosin staining; original magnification A, C, E, and G ×200; B, D, F, and H ×400).

Tournier *et al.* (41) detected higher expression of AFP mRNA in proliferating bile "duct-like" cells but not in mature bile duct cells. Our results suggest that hepatocyte progenitor cells most probably do not originate from bile duct cells, but represent a separate population of cells with a morphologic appearance similar to bile duct cells, that are localized in the portal zone in the neighborhood of bile duct cells.

We found that the degree of activation of liver progenitor cells depends on the agent causing hepatocyte death. Liver damage caused by CCl_4 and GalN triggers different mechanisms of response. The first cause pericentral necrosis, with low to moderate inflammation, and little involvement of the portal zone. The second causes focal spotty necrosis, high inflammation, and initiation of a proliferative response in the portal

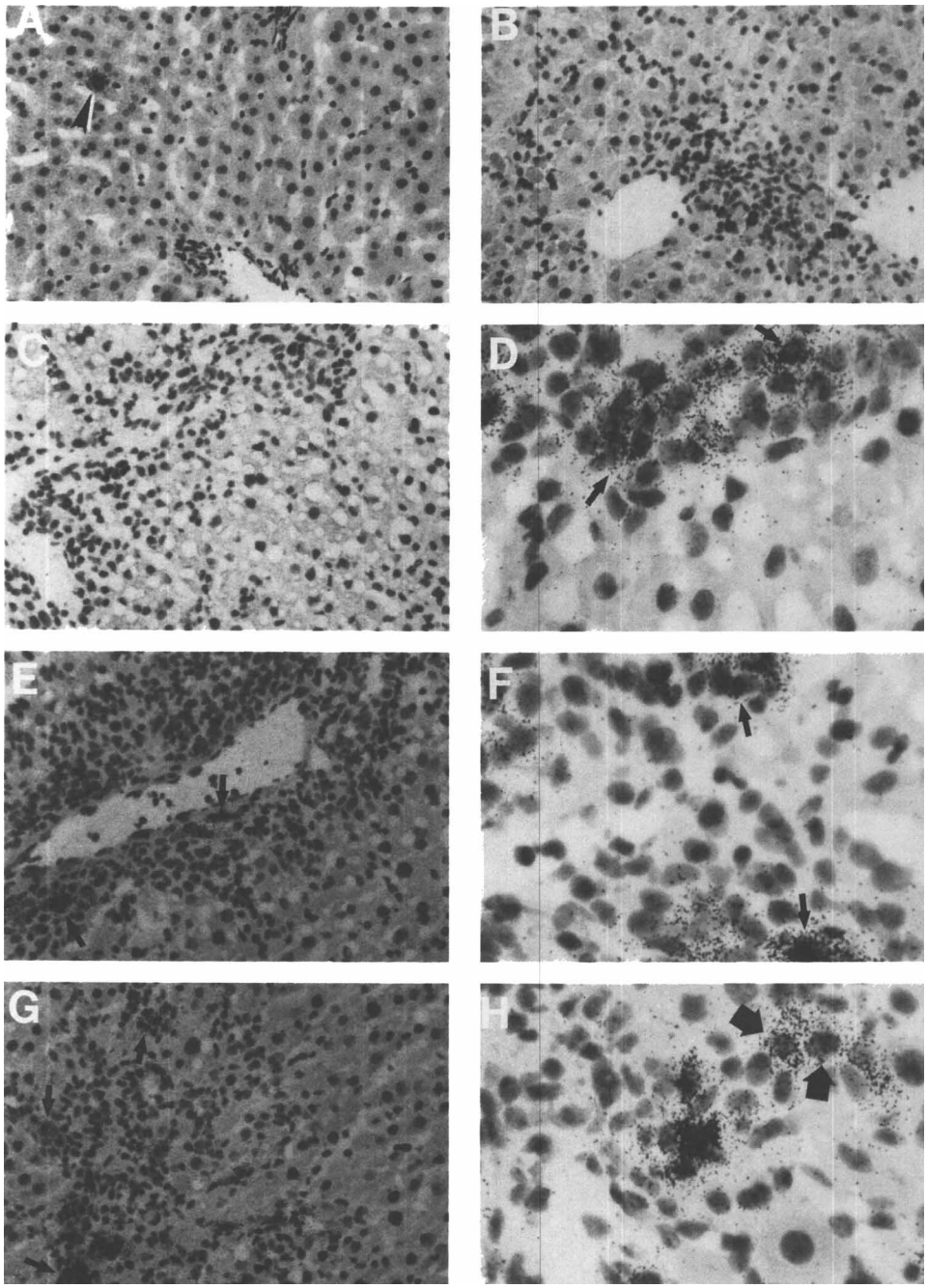

Figure 6. Detection of fetal AFP mRNA by *in situ* hybridization in liver sections of rats after GalN treatment. Portal and periportal region. Control: A. Occasional single hepatocytes are expressing fetal AFP mRNA (arrowhead). Day 1: B. No expression of fetal AFP mRNA. Day 2: C and D. Usually, the entire liver lobule is not expressing AFP mRNA (C). Occasionally, high expression of fetal AFP mRNA is visible in NPC in the portal zone around the bile ducts (arrows in D). Day 3: E and F. Some cells in proliferating NPC structures in the periportal region are expressing fetal AFP mRNA (arrows). Day 5: G and H. Expression of AFP mRNA also occurs in NPC structures in the parenchyma and in single hepatocytes in close vicinity to these structures (bold arrows in H) (original magnification A, B, C, E, and G ×200; D, F, and H ×600).

zone. We assume that specific signals in the GalN model lead to a typical and high activation of liver progenitor cells. The nature of these signals is still largely unknown.

There are several factors that may play a role in the activation of liver progenitor cells: (i) The remaining hepatocytes have a limited ability and a delay in the response to proliferation, because of the inhibition of RNA/DNA synthesis. Our results and those of others (25, 26, 40) show that hepatocytes enter the S phase almost 2 days after GalN injury. (ii) There is an extensive inflammatory response with polymorphonuclear and mononuclear infiltration in areas of focal necrosis (29, 42, 43) which may activate progenitor cells by cytokines and/or other factors released from these cells. (iii) There are other hepatic cells involved in the re-

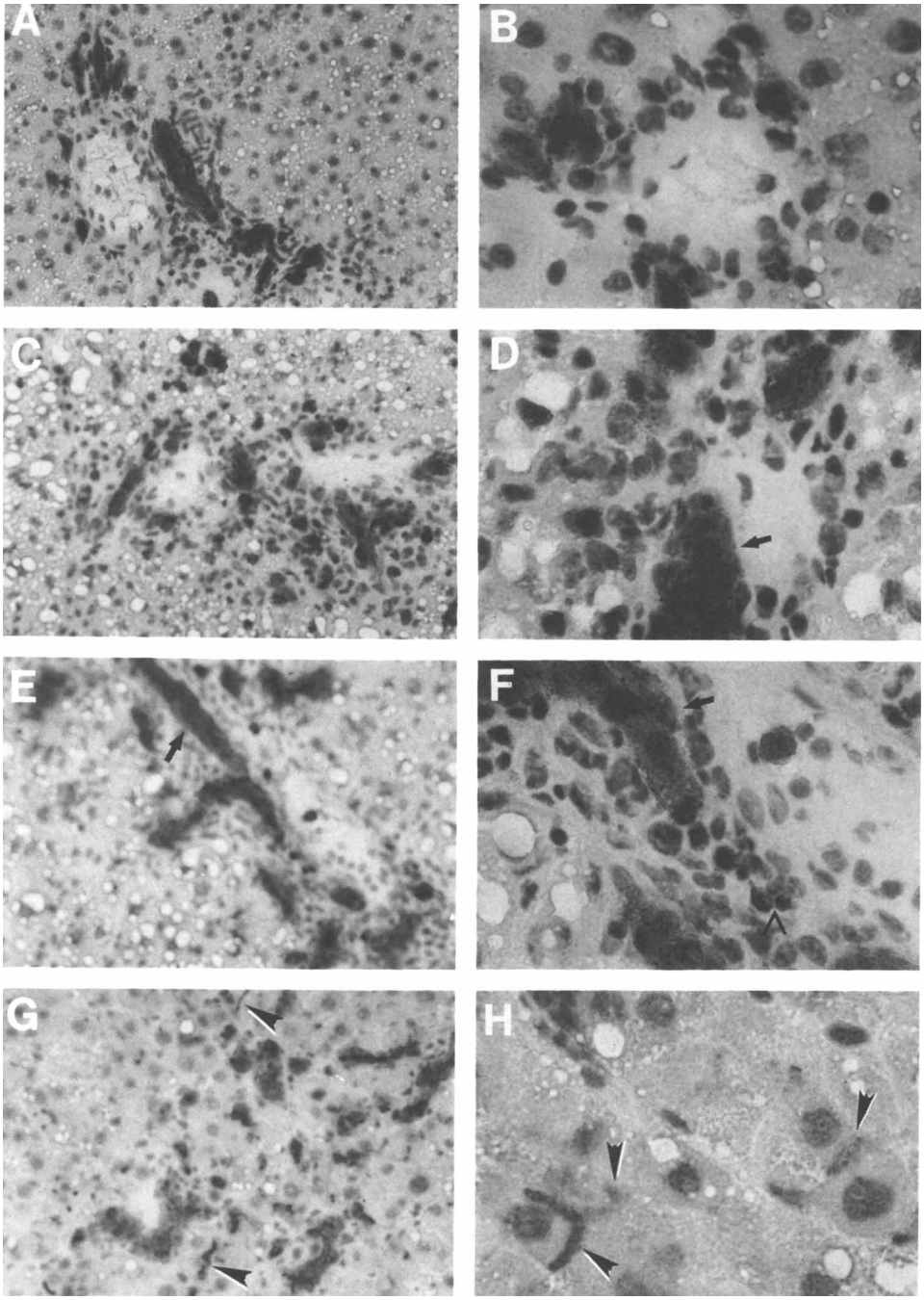

Figure 7. Histochemical expression of GGT in liver sections of rats after GalN treatment. Portal and periportal zones. Day 1: A and B. GGT activity is confined to bile duct cells. Single polymorphonuclear cells are also expressing GGT. Day 2: C and D. Increased numbers of labeled cells in the periportal region forming NPC structures (arrow in D). Day 3: E and F. GGT-positive cells form a network expanding from the portal zone into the parenchyma (arrows in E and F). An eosinophil expressing GGT is shown by an arrow tip (F). The staining is diffusely scattered throughout the cytoplasm. Mature hepatocytes do not express GGT. Day 5: G and H. Hepatocytes adjacent to the periportal regions or in the vicinity of NPC structures also express GGT activity at the canalicular membrane surface (arrowheads) (counterstained with hematoxylin; original magnification A, C, E, and G ×200; B, D, F, and H ×600).

sponse to GalN injury (sinusoidal lining cells, Kupffer cells, Ito cells, etc.) with their specific paracrine factors as signals for hepatocyte progenitor cells activation. In fact, Kinoshita *et al.* (44) observed a marked increase in HGF mRNA in NPC from the liver of GalN-treated rats. Thorgeirsson and co-workers recently observed parallel activation of oval cells and Ito cells during liver regeneration and differentiation (45). We observed, as early as 24 hr after GalN injury, the activation and proliferation of sinusoidal cells that continued during the entire process of liver regeneration (unpublished results). (iv) The site of initiation of GalN injury in the

periportal region ensures proximity between proliferating hepatocyte progenitor cells and Ito cells and would be consistent with the close contact and attachment between these cells via multiple intercellular junctions, as observed during early stages in the induction of hepatocarcinogenesis (46).

What are the paracrine and autocrine factors participating in activation of liver progenitor cells? Where do they come from? How and under what circumstances are the signals for activation of liver progenitor cells transduced? What is the mechanism for this complex activation? These intriguing questions remain the subject of future investigation.

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