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Activation, Proliferation, and Differentiation of Progenitor Cells into Hepatocytes in the D-Galactosamine Model of Liver Regeneration

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Rat liver regeneration was studied from 24 hours to 8 days after a single intraperitoneal injection of D-galactosamine (GalN). Morphological changes in the liver were analyzed in parallel with sequential changes in expression of histone-3 mRNA (a marker of cell proliferation), fetal α-fetoprotein (AFP) mRNA and γ-glutamyl transpeptidase (GGT) (markers of fetal hepatocytes), and albumin mRNA and glucose-6-phosphatase (G6Pase) (markers of adult hepatocytes). Proliferation of nonparenchymal epithelial cells (NPC), detected in situ by [^{3}H]thymidine labeling or histone-3 mRNA expression, began after 24 hours primarily in the portal area around the bile ducts. After 2 days, histone-3 labelling intensity increased in rows and clusters of NPC which expanded from the portal zone and invaded into the parenchyma. On days 3 and 5, NPC expressing his-3 mRNA expanded further, forming pseudo-ducts and islet-like structures (NPC structures). Proliferating NPC were positive for GGT. Some GGT positive cells were also positive for the fetal form of AFP mRNA, which lagged behind GGT by 24 hours and peaked on day 5. On day 3, some cells with the appearance of NPC expressed albumin mRNA. Double label in situ hybridization for fetal AFP and albumin mRNAs and dual histochemistry for GGT and G6Pase showed simultaneous expression of these markers in NPC on day 5. Other cells expressing fetal AFP mRNA or GGT on day 5 had a morphological appearance between NPC and hepatocytes (transitional cells). Proliferation of hepatocytes began on day 2, reached maximum on day 5 and then declined. Proliferating hepatocytes did not express fetal AFP mRNA or GGT. These findings indicate that after GalN injury, the liver responds by activation of progenitor cells that proliferate and then differentiate into mature hepatocytes. Adult hepatocytes can also proliferate after GalN injury, but these hepatocytes do not undergo dedifferentiation/redifferentiation during regeneration of the hepatic lobule. (Am J Pathol 1993, 143:1606–1620)

Under classical conditions of liver regeneration after partial hepatectomy, various liver cell types proliferate through different lineages. However, during this process, only limited information is available concerning the possible activation of liver progenitor cells and the potential contribution of such progenitor cells to the restoration of hepatocyte mass.[1–3] In 1956, Farber[4] used the term "oval cells" to describe a population of liver epithelial cells induced early in the hyperplastic response following administration of the carcinogenic agents ethionine, 2-acetylaminofluorene (2-AAF) or 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB). These cells proliferate around ducts and vessels in the portal area and resembled the cells of the bile duct; they were small and oval shaped and had scanty, lightly basophilic cytoplasm and pale blue-staining nuclei. From various studies, Farber and coworkers[5] and others[6,7] concluded that after removal of carcinogenic agents, oval cells atrophy and are not progenitors of normal or transformed hepatocytes. Nonetheless, using short term carcinogen treatment, other investigators have sug-

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gested that oval cells may, under certain circumstances, differentiate into hepatocytes or neoplastic hepatocytes.[8–14] In addition, epithelial cell lines, derived from the liver and resembling oval cells, can acquire phenotypic characteristics of hepatocytes[15,16] and under certain conditions can also produce hepatocellular carcinomas when inoculated into appropriate hosts.[17–19]

The origin of oval cells is still controversial. It has been suggested that they originate from the ductular epithelial cells/canals of Hering[20] or from stem cells located in the periductular spaces.[21] These cells express traits specific for bile duct epithelial cells (eg, γ-glutamyl-transpeptidase (GGT) and cytokeratin-19), fetal hepatoblasts, eg, α-fetoprotein (AFP), glutathione-S-transferase P, and fetal isoenzyme forms of aldolase, pyruvate kinase, and lactic dehydrogenase, and hepatocytes (eg, albumin and α_1-acid glycoprotein) and, therefore, have bipotential developmental capability (for reviews see refs. 22–27).

Proliferation of oval cells is also observed in the noncarcinogenic model of D-galactosamine (GalN) induced liver injury.[28–30] When GalN is introduced into rats at a concentration of 20 mg/100 g body weight, a series of specific metabolic reactions occurs in the hepatocyte with complete consumption of uridine nucleotides and UDP-glucose, and accumulation of slowly metabolizing UDP-hexosamines and UDP-N-acetylhexosamines. This consumption of uridine nucleotides, or uridylate "trapping," leads to a block in RNA synthesis, followed by inhibition of protein synthesis. At higher doses, GalN causes hepatocyte necrosis and an intense inflammatory response.[31–33]

After GalN injury, the liver responds by activation of nonparenchymal cells that are not the target of UTP trapping. Originally, Kuhlmann and Wurster[34] reported that AFP was expressed in bile duct cells following GalN injury. Subsequently, Tournier et al[35] demonstrated that AFP mRNA was present in periductular-like epithelial cells but not in mature bile ducts, and that AFP mRNA expression peaked at day 4 following GalN administration. Finally, on the basis of [^{3}H]thymidine labeling studies, Lemire et al[30] concluded that duct epithelial cells can generate both oval cells and small hepatocytes in response to GalN. However, these previous studies have not fully identified and characterized the cell types involved and the precise sequence of events occurring during restoration of parenchymal cell mass following GalN-induced liver injury.

In the present study, we used GalN hepatitis as a noncarcinogenic model to study the activation, proliferation and differentiation of putative hepatocyte progenitor cells in the liver. To document maturation and differentiation of these putative progenitor cells through the hepatocyte lineage, we followed the kinetics of proliferation over time during the regenerative process of different liver cell types and the kinetics of expression of two "early" genes (GGT and AFP) expressed in differentiating but not in mature hepatocytes, and two "late" genes (albumin and G6Pase) expressed specifically in differentiating and/or mature hepatocytes but not in bile duct epithelial cells. The results of this analysis lead to the conclusion that a subpopulation of oval cells proliferate during liver regeneration with the potential to differentiate into mature hepatocytes. Adult hepatocytes also divide following GalN injury, but do not express GGT or fetal AFP mRNA. Thus, in GalN-induced liver injury, hepatocytes derived from two separate pathways, one utilizing progenitor cells and the other utilizing preexisting mature hepatocytes, participate in reconstitution of the hepatic lobule.

Materials and Methods

Animals

Male Lewis (Charles River Breeding Laboratory, Wilmington, MA) or Fischer 344 (Taconic Farms, German Town, NY) rats, weighing from 180 to 220 g, were maintained at 22 C and given standard laboratory chow and water *ad libitum*. GalN, dissolved in 0.14 M NaCl, was introduced intraperitoneally at 10:00 am in a single dose of 70 mg/100 g body weight. One to 8 days later, the animals were killed and pieces of liver fixed in formalin for histological analysis or frozen in 2-methylbutane at −70 C for cryostat sectioning.

DNA Labeling

[^{3}H]methyl-thymidine (ICN Radiochemicals, Irvine, CA, specific radioactivity 67 Ci/mmol) was injected intraperitoneally. 24 hours after GalN administration at a dose of 100 μCi/100 g body weight. Animals were killed by cervical dislocation under ether anesthesia 10 hours later. Paraffin-embedded or frozen sections were processed for autoradiography and exposed for 30 to 45 days.

In Situ *Hybridization*

In situ molecular hybridization was performed essentially as described previously.[36] The following re-

combinant plasmids were used: pGH3, 204 nucleotides from rat histone-3 cDNA, cloned into pGem 3Z, received from Dr. A. Skoultchi, Albert Einstein College of Medicine; pGalb 345, 345 nucleotides from rat albumin cDNA, described previously,[37] and pBAF700, 700 nucleotides from rat AFP cDNA encompassing the 5′-region and representing only the fetal form of AFP mRNA, cloned into pBSM13+ (kindly provided by Dr. N. Fausto, Brown University, Providence, RI). Two riboprobes, sense and antisense, were transcribed from each of these plasmids after their linearization with appropriate restriction enzymes.

For *in situ* hybridization with double label, [^{35}S]-UTP (specific radioactivity > 1000 Ci/mmol, Amersham Corp., Arlington Height, IL) was used for labeling of the sense or anti-sense riboprobes of AFP and digoxigenin-UTP (Boehringer, Mannheim Biochemicals, Indianapolis, IN) was used for labeling of the two albumin riboprobes. Digoxigenin RNA was transcribed according to the protocol of the manufacturer. Prehybridization and hybridization was performed as described, except for use of 0.1% sarcosyl-0.02% SDS instead of 0.1% SDS. Five-μ-thick frozen sections were hybridized with 10^6 cpm/slide of ^{35}S-labeled AFP probe and 20 to 50 ng/slide of digoxigenin-labeled albumin probe for 16 hours at 45 C. Sense strand probes, synthesized from the AFP and albumin sequence containing plasmids, respectively, were used as controls during hybridization. After hybridization, the slides were washed three times with 50% formamide-4X SSC-5 mM DDT for 15 minutes at 50 C, 2 times with 2X SSC for 10 minutes at room temperature, treated for 30 minutes at 37 C with 20 μg/ml of RNase A in RNase buffer and then washed 2 times for 10 minutes at room temperature in RNase buffer. Residual unhybridized label was removed during a one-hour wash with 0.2X SSC at 50 C.

The slides were rinsed in buffer 1 (0.1 M Na–maleate-0.1 M NaCl, pH 7.5) and incubated for 1 hour in 2% blocking reagent (Boehringer), dissolved in buffer 1. Digoxigenin-AP antibody (Boehringer) was diluted 1:500 in blocking reagent and applied to the tissue sections. After 2 hours of incubation, the slides were washed 2 times for 15 minutes in buffer 1, rinsed in 100 mM Tris/HCl-100 mM NaCl-50 mM $MgCl_2$, pH 8.3, and incubated for 3 hours with the substrate HistoMark red (Kirkegaard and Perry Laboratories) according to the manufacturer's protocol. The reaction was stopped by rinsing in 10 mM Tris/HCl-1 mM EDTA, pH 8.0; the slides were dehydrated, exposed to autoradiography for 10 days counterstained with hematoxylin and permanently mounted.

Histochemistry

γ-glutamyltranspeptidase was demonstrated *in situ* on frozen sections after fixation in acidic ethanol (99:1 = 96% ethanol: glacial acetic acid) for 10 minutes at −20 C and rinsing with ice-cold ethanol. This method of fixation gave excellent assessment of the localization of GGT. The histochemical reaction was performed according to the method of Rutenburg et al[38]

Glucose-6-phosphatase activity (G6Pase) was revealed on unfixed, dried frozen sections as described,[39] with minor modifications: the sections were incubated directly in substrate solution (10 mM G6P, 40 mM Tris-maleate buffer, pH 6.5, 150 mM sucrose, 3.6 mM lead nitrate) for 15 minutes at 37 C. After incubation, the slides were washed in 0.3 M sucrose and immersed for 30 seconds in 1% ammonium sulfide in 0.3 M sucrose, rinsed 2 or 3 times in 0.3 M sucrose, and finally rinsed briefly in 0.15 M NaCl. Simultaneous detection of G6Pase and GGT activities *in situ* was performed in two steps. First, the reaction for G6Pase was developed, then the sections were fixed in acidic ethanol at −20 C and processed as above for demonstration of GGT. The sections were counterstained with hematoxylin and mounted in Gel/mount (Biomedica Corp., Foster City, CA).

Results

Morphological Changes in the Liver after GalN Treatment

Early morphological changes observed in the liver 24 to 48 hours after GalN injury were similar to those previously described.[28,29,40] Focal hepatocyte necrosis with acidophilic degeneration was conspicuous on day 1 and reached a maximum on day 2 (Figure 1). Regions of inflammation infiltrated by polymorphonuclear and mononuclear cells were scattered throughout the portal and periportal spaces and within the parenchyma in regions of hepatocyte necrosis (Figure 1B). At this time, the hepatocyte plates were completely disorganized and central veins not discernible (Figure 1C). While the number of granulocytes (comprised of neutrophils and eosinophils) decreased by day 3, clusters of mononuclear cells could still be seen on days 5 and 6 (Figure 1E). Proliferation of nonparenchymal

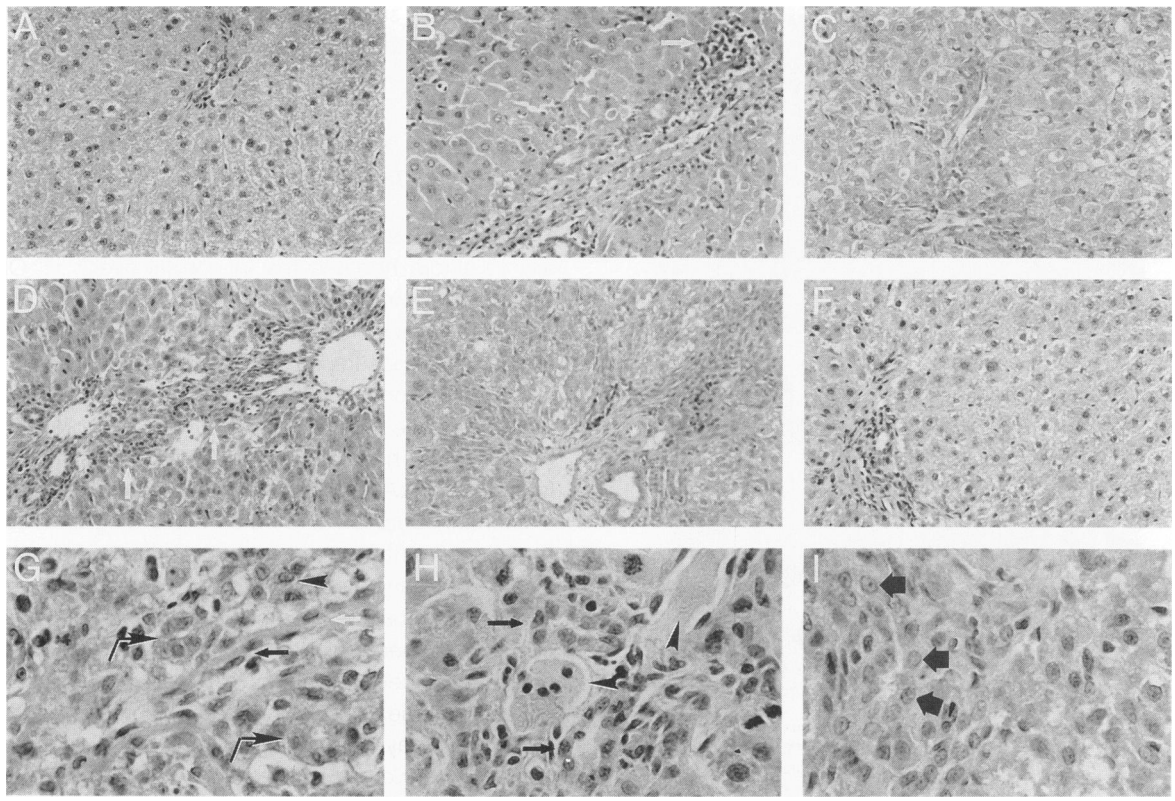

Figure 1. *Morphological changes in the liver after GalN treatment. Portal region* (**A–F**), *periportal region* (**G–I**). **A**: *Control liver.* **B**: *Day 1 after GalN treatment; inflammation in the portal region with infiltration by granulocytes and mononuclear cells. Area of focal hepatocyte necrosis is shown by arrow.* **C** *and* **G** *Day 2; hepatic plates are completely disorganized. Proliferation of NPC begins in the portal zone and extends into the parenchyma forming rows* (*arrow*, **G**), *clusters or sheets* (*arrowhead*, **G**) *and duct-like structures* (*bent arrows*, **G**) *of small cells with scant cytoplasm and pale, oval shaped nuclei* (*NPC structures*). **D** *and* **H**: *Day 3; Necrotic areas begin to recede and are invaded by proliferating NPC* (*arrows*, **D** *and* **H**) *but still many acidophilic necrotic areas are present* (*arrowheads*, **H**). **E** *and* **I**: *Day 5; restoration of liver parenchyma. Some cells within NPC structures appear larger with more round shaped nuclei and increased cytoplasmic compartment* (*blunt arrows*, **I**). **F**: *Day 8; the liver lobule almost regains its normal architecture. Hematoxylin and eosin staining. Original magnification*, **A–F**, ×*200*; **G–I**, ×*400*.

cells (NPC) began in the periportal spaces on day two and gradually invaded into the lobule (Figure 1G). By day 3, proliferating NPC formed discrete structures, comprised of rows, clusters, and duct-like structures of small cells with scant cytoplasm and oval shaped, pale, and homogeneously stained nuclei (oval cells) (Figure 1H).

NPC structures were most evident in regions directly adjacent to the portal spaces. At this time (day 3), the necrotic areas began to resolve and were invaded by proliferating NPC structures. By day 5 after GalN treatment, the liver began to restore its normal lobular structure (Figure 1, E and I). Regeneration of the liver parenchyma was marked by the appearance of large hepatocytes (beginning on day 3 and reaching a maximum on day 5) between clusters of small hepatocytes and a gradual resolution of NPC structures (Figure 1E). By day 8, the liver almost fully regained its normal architecture (Figure 1F).

Regenerative Response of the Liver after GalN Injury

The proliferation of different cell types in the liver after GalN injection was studied either by *in situ* hybridization with [^{35}S]his-3 probe or by *in vivo* incorporation of [^{3}H]methyl-thymidine, marking S phase synthesis of histones and DNA, respectively. One day after GalN administration, a proliferative response was detected in all nonparenchymal cell types within the portal and periportal regions and in adjacent sinusoidal spaces. However, by [^{3}H]-methyl-thymidine labeling, the major reaction was in nonparenchymal epithelial cells located directly adjacent to, but not within, mature bile ducts (Figure 2A and B).

In studies of his-3 mRNA expression, in control sections (day 0), there was always labeling of a few hepatocytes scattered throughout the hepatic lobule

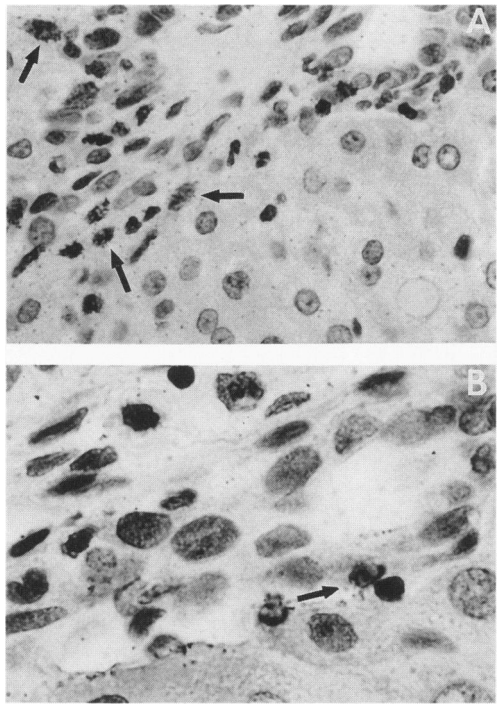

Figure 2. *[3H]methyl-thymidine labeling of cells in portal and periportal region 24 hours after GalN injury.* **A**: *NPC directly adjacent to bile ducts and within neighboring sinusoidal spaces are labeled (arrows).* **B**: *Mature bile duct cells do not incorporate the radioisotope. Arrow points to labeled NPC directly adjacent to bile duct. H and E staining. Original magnification:* **A**, *×600;* **B**, *×1000.*

(Figure 3B). Higher activity was observed in the periportal zones, where some nonparenchymal cell types were also his-3 mRNA positive (Figure 3A). On day 2, the number of his-3 mRNA positive NPC in the portal and periportal zones and in the sinusoids increased and reached maximum (Figure 3C). At the same time, cells expanding in rows from the portal triads into the parenchyma (Figure 3C) and within areas of focal inflammation (Figure 3D) were strongly positive for his-3 mRNA. On day 3, clusters and duct-like structures of NPC in regions of inflammation reached maximum labeling for his-3 mRNA (Figure 3E). In contrast, the portal zone itself became less labeled. Movement of the proliferative wave from the portal area into the parenchyma was well documented by dark field analysis (Figure 4A-H). By day 5, proliferative activity in NPC in the portal spaces (his-3 mRNA expression) ceased (Figure 3G), although residual activity remained in the parenchyma (Figure 3H). Activation of hepatocytes also began on day 2, reached a maximum on day 3, and then gradually declined. His-3 mRNA was localized both in small and large hepatocytes (Figure 3, F and H). While on days 2 and 3, proliferating hepatocytes were scattered throughout the parenchyma, on day 5, they mainly surrounded the remnants of proliferating NPC structures (Figures 3 and 4).

Expression of AFP and Albumin mRNAs after GalN Treatment

The fetal form of AFP mRNA is expressed occasionally in single hepatocytes in normal liver.[41,36] On day 1 following GalN administration, no cells showed expression of fetal AFP mRNA, even after prolonged exposure of the slides (Figure 5A). On day 2, occasional single or small groups of NPC in the portal zone showed fetal AFP mRNA expression (Figure 5B). Three days after GalN administration, fetal AFP mRNA appeared in NPC that formed rows, clusters, or duct-like structures expanding from the portal areas into the parenchyma (Figure 5C) and also in resolving necrotic areas (Figure 5D). Fetal AFP mRNA positive cells showed scant cytoplasm and oval shaped, pale, and homogeneously stained nuclei (oval cells). It should be noted that not all cells in these NPC structures expressed AFP mRNA. On day 3, single fetal AFP mRNA positive hepatocytes were also identified, usually in the vicinity of proliferating NPC structures (data not shown).

On day 5, fetal AFP mRNA expression was much more abundant. Expression was most prominent in clusters of NPC in periportal and midlobular zones, as well as in areas of resolving inflammation (Figure 5, E and F). AFP mRNA was also detected in single cells with the appearance of hepatocytes, sometimes in the parenchyma but very often in hepatocytes adjacent to NPC clusters or as part of NPC duct-like structures (Figure 5F). Expression of fetal AFP mRNA was still above control levels on day 8, when frequently single or double NPC or hepatocytes were labeled (data not shown). Increased AFP mRNA expression over proliferated bile duct-like structures following GalN injection was previously reported by Tournier et al[35] and Lamire et al[30] In these studies, the peak of this expression occurred at day 4 or 5, respectively.

mRNA for albumin, the most abundantly expressed liver specific gene, decreased abruptly after GalN treatment, reaching a minimum by day 2. In large periportal regions, as well as in areas of focal necrosis, albumin mRNA was not expressed (Figure 6B). However, after day 3, albumin mRNA was expressed in NPC within the periportal spaces (Figure 6C). Occasionally, on day 3, albumin mRNA positive cells with a larger, more round shaped, and

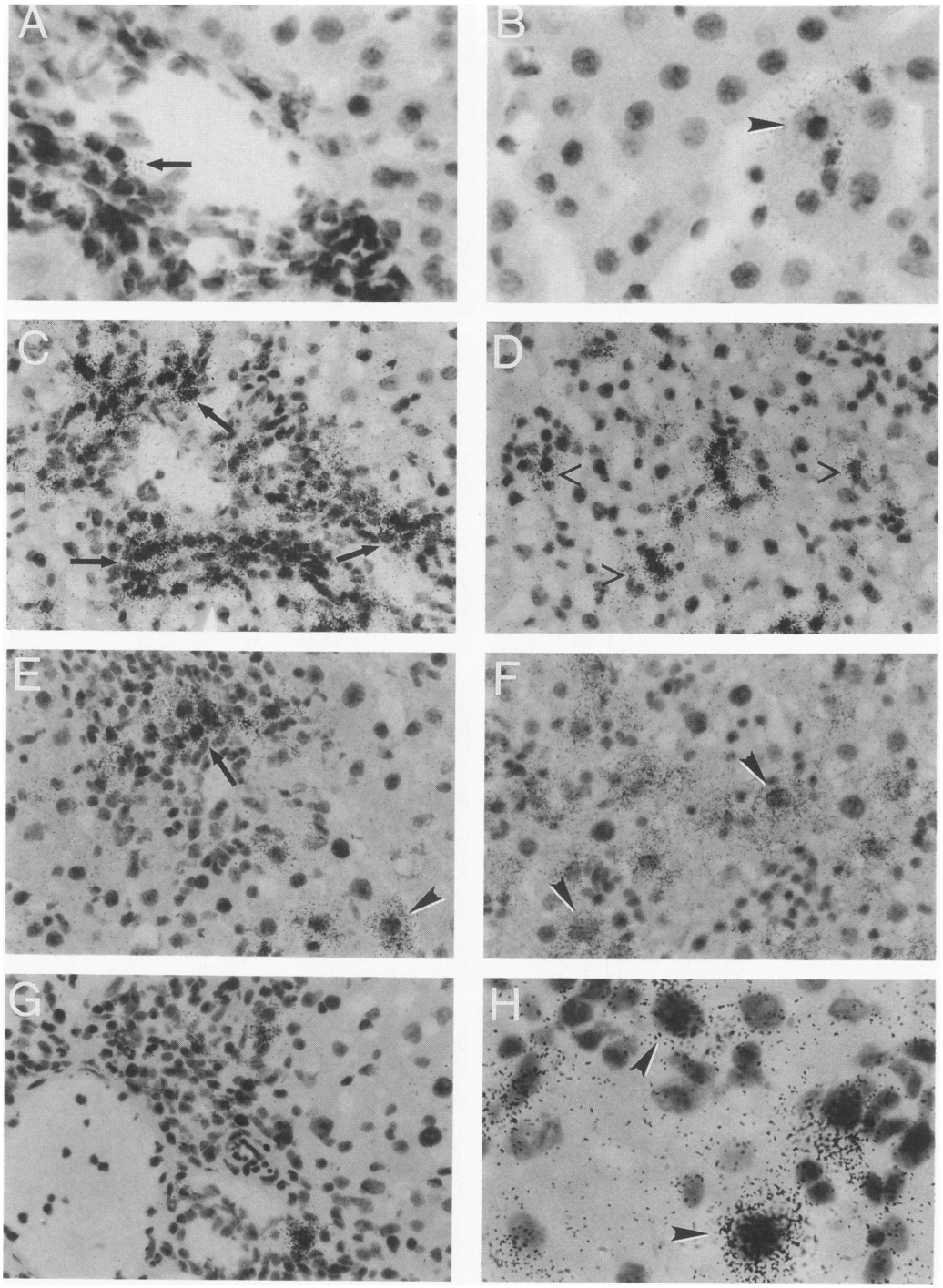

Figure 3. In situ *hybridization with ^{35}S-labeled histone-3 mRNA antisense riboprobe in liver sections after GalN injury.* **A**, **C**, **E**, *and* **G**: *portal and periportal zones;* **B**, **D**, **G**, *and* **H**: *midlobular zone.* **A**: *Control (day 0); his-3 mRNA labeling of NPC in the portal zone (arrow).* **B**: *Control (day 0); labeled hepatocyte surrounded by sinusoidal cells (arrowhead).* **C**: *Day 2; large numbers of his-3 mRNA labeled cells are present in typical NPC structures in the periportal region (arrows).* **D**: *Labeling of sinusoidal cells is also increased (arrow tips).* **E** *and* **F**: *Day 3; labeling in the portal region and increased labeling of NPC structures expanding into the parenchyma (arrows); labeling of hepatocytes reaches maximum (arrowheads).* **G**: *Day 5; proliferative wave in the periportal region recedes.* **H**: *Labeled hepatocytes in the vicinity of NPC structures (arrowheads). Original magnification:* **C–G**, ×*200*; **A**, **B**, ×*600*; **H**, ×*1000.*

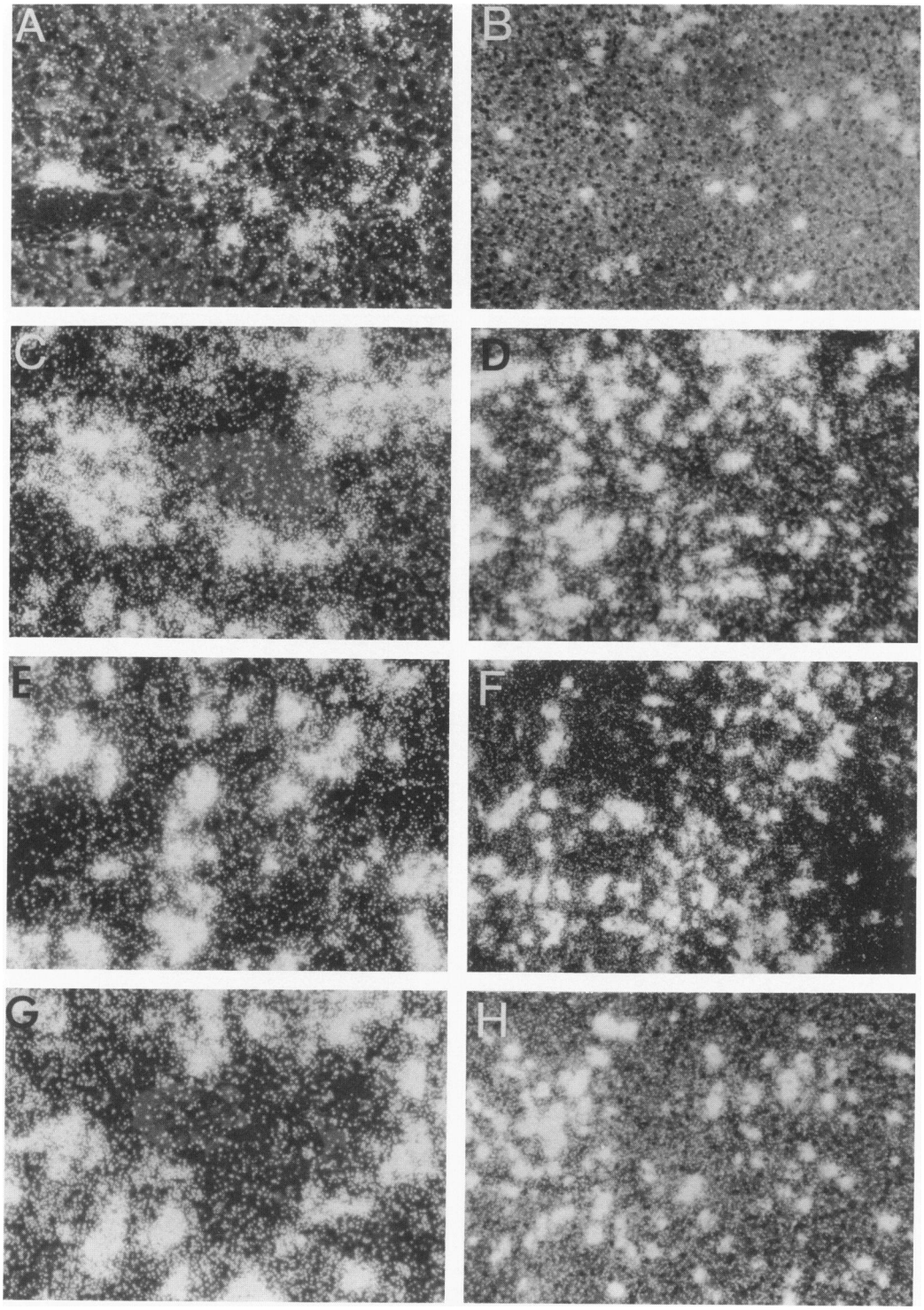

Figure 4. *Dark field analysis of* in situ *hybridization for [^{35}S]histone-3 mRNA antisense riboprobe.* **A**, **C**, **E**, *and* **G**: *portal (noted by the letter P) and periportal zones, original magnification, ×200;* (**B**, **D**, **G**, *and* **I**) *midlobular zone, original magnification ×100. Day 1* (**A** *and* **B**); *labeling of sinusoidal cells in the periportal and midlobular zone and single or small groups of NPC in the periportal region. Day 2* (**C** *and* **D**); *maximum labeling of NPC structures in the periportal region and parenchyma and of sinusoidal cells throughout the liver lobule. Day 3* (**E** *and* **F**); *decreased labeling of cells in the portal region (P) and still high activity in the midlobular zone, where hepatocytes reach maximum labeling. Day 5* (**G** *and* **H**); *labeling of cells in the portal region (P) is reduced. Residual activity is present in NPC structures and hepatocytes in areas of resolving focal necrosis.*

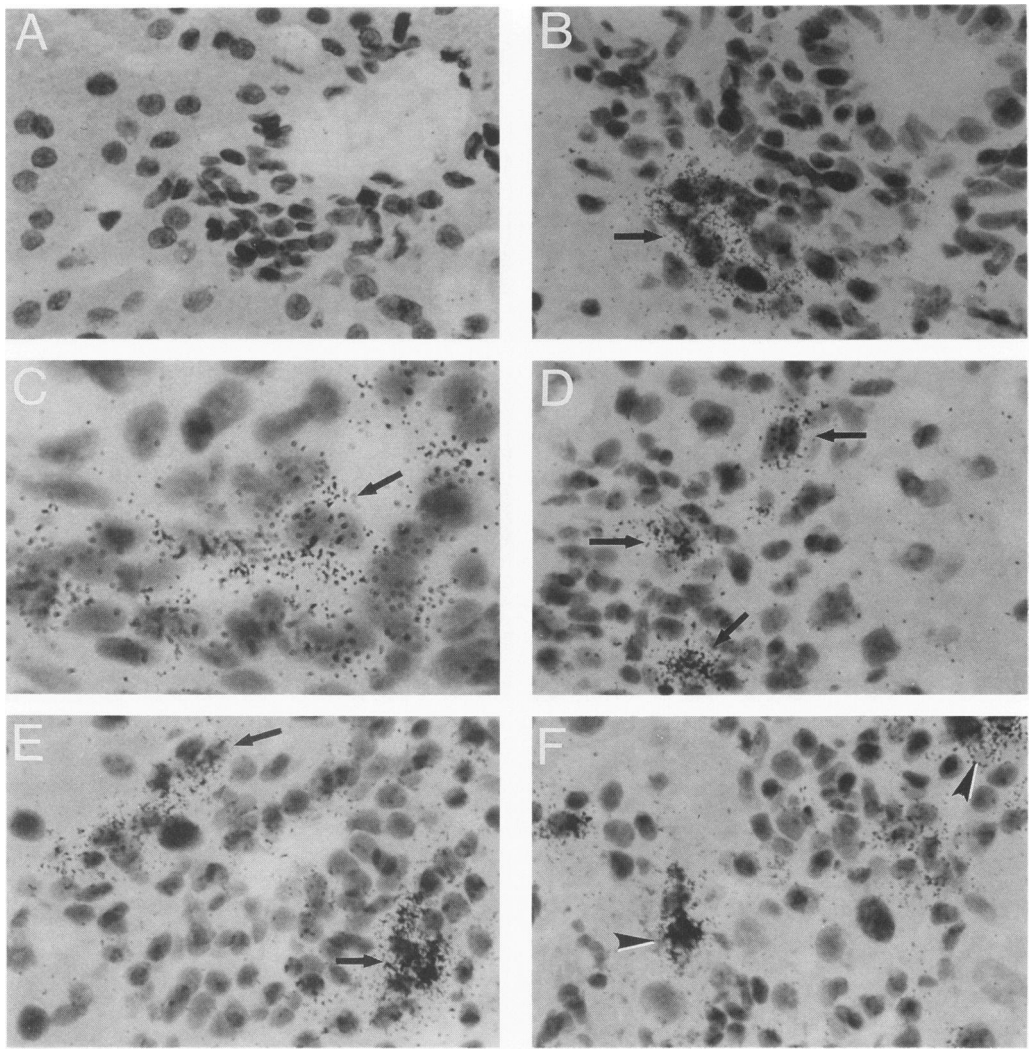

Figure 5. In situ *hybridization for ^{35}S-labeled AFP mRNA antisense riboprobe in liver sections after GalN treatment. Portal and periportal zone* (**A**, **B**, **C**, *and* **E**); *midlobular zone* (**D** *and* **F**). *Day 1* (**A**); *no labeling of cells in the portal region. Day 2* (**B**); *a group of NPC positive for fetal AFP mRNA* (*arrow*). *Note that preexisting bile ducts are not labeled. Day 3* (**C**); *labeling of NPC structures in the periportal region* (*arrow*). *Day 3* (**D**); *labeling of single or groups of NPC in an area of focal inflammation* (*arrows*). *Please note that not all NPC structures express fetal AFP mRNA. Day 5* (**E**); *labeling for fetal AFP mRNA is higher in NPC structures that have moved away from the periportal zone into the parenchyma* (*arrows*). **F**: *Day 5; single hepatocytes* (*arrowheads*) *in the vicinity of NPC structures are also labeled. Original magnification*: **A**, **B**, **D**, **E**, **F**, ×*600*, **C**, ×*1000*.

darker staining nucleus were identified within proliferating NPC structures (Figure 6C, blunt arrow). These cells had the morphological appearance of transitional cells. By day 5, some cells expressing albumin mRNA within proliferating NPC structures had the morphological appearance of hepatocytes (Figure 6D). At this time, the basic liver structure was beginning to reform.

Histochemical Expression of GGT

In normal liver, GGT is confined to the bile duct epithelium in the portal triad (Figure 7A). One day after GalN treatment, only single cells in the region of inflammation were GGT positive, and these cells were identified as eosinophils by their high peroxidase activity (data not shown). On day 2, proliferating NPC in the portal areas also became GGT positive (Figure 7B). On day 3, GGT positive cells formed a prominent network, expanding from the periportal regions into the parenchyma (Figures 7C and D). On day 5, hepatocytes in close vicinity to GGT positive clusters of NPC also expressed GGT (Figure 7E). In some of these cells, the whole cytoplasm was diffusely stained, whereas in others cytoplasmic staining was much less and enzyme activity was localized to the canalicular surface of parenchymal cells (Figure 7F).

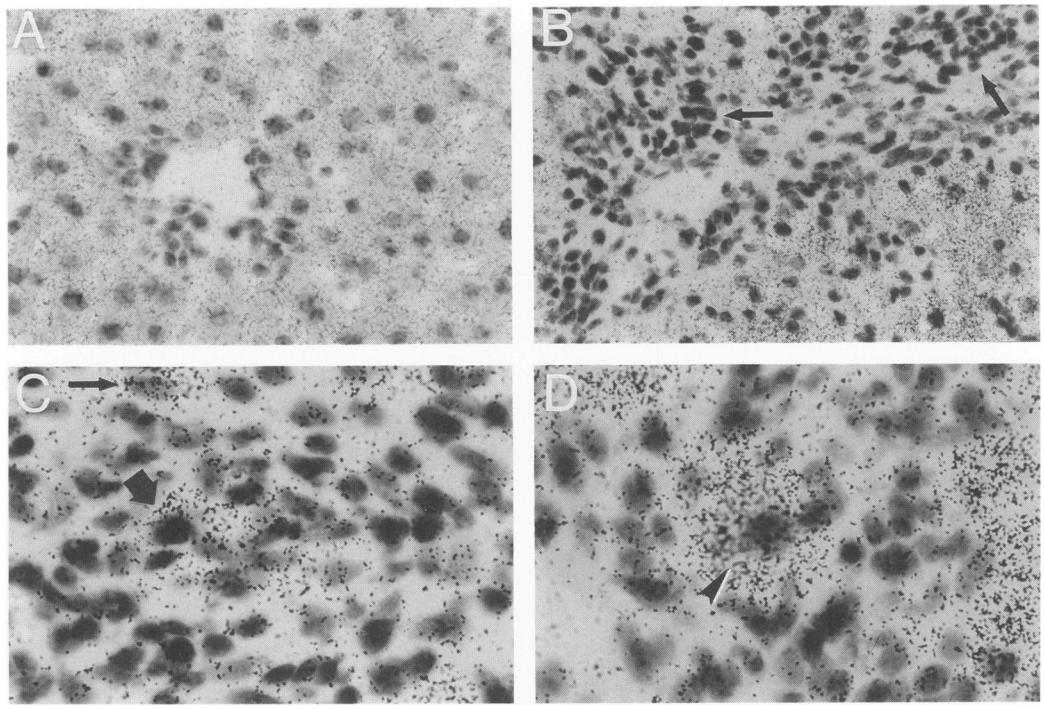

Figure 6. In situ *hybridization for ^{35}S-labeled albumin mRNA antisense riboprobe in liver sections after GalN treatment. Portal and periportal zone.* **A**: *Control; marked labeling of hepatocytes but no signal in NPC.* **B**: *Day two; proliferating NPC within the portal region are negative for albumin mRNA (arrows). High expression remains in surviving hepatocytes.* **C**: *Day 3, albumin mRNA appears in some NPC in the periportal regions and in areas of resolving focal necrosis (arrow). One labeled epithelial cell within this area, which we refer to as a transitional cell, has a larger, more round shaped and darker stained nucleus and a small rim of eosinophilic cytoplasm (blunt arrow).* **D**: *Day 5; expression of albumin mRNA in an hepatocytes within an NPC structure (arrowhead). Original magnification:* **A**, ×400; **B**, ×200; **C** *and* **D**, ×600.

Simultaneous Detection of AFP and Albumin mRNAs

To determine whether a precursor-product relationship exists between NPC and hepatocytes expressing fetal AFP mRNA, we performed double labelling experiments, using for *in situ* hybridization digoxigenin-labelled albumin riboprobe (identified by Histo-Mark red color) and [^{35}S]-fetal AFP riboprobe (detected by autoradiography). On day 5 following GalN administration, we found expression of both fetal AFP mRNA and albumin mRNA not only in cells with the appearance of hepatocytes (Figure 8A), but also in a number of oval cells within NPC structures (Figure 8A). We also found both mRNAs in cells that were larger than oval cells and had a more round-shaped, darker stained nucleus and increased cytoplasmic compartment, ie, transitional cells (Figure 8B). A control slide at day 5 following GalN administration, hybridized with sense strand riboprobes for AFP and albumin (Figure 8C), showed no autoradiographic grains or red color, indicating the very low level of background in these experiments. On day 8, double label was detected mainly in cells with the appearance of hepatocytes (data not shown). This result demonstrates that part of the NPC are on a lineage pathway to differentiate into hepatocytes.

Histochemical Expression of G6Pase and Double Staining for GGT and G6Pase

Glucose-6-phosphatase (G6Pase) was used as an hepatocyte specific marker to follow the fate of hepatocytes after GalN induced liver injury and to follow the progression of cellular differentiation through the hepatocyte lineage. In parallel with albumin mRNA, G6Pase was expressed in all hepatocytes (Figure 8D), but was reduced dramatically 24 hours after GalN administration (data not shown). Overall expression of G6Pase continued to decline until day 2. Regions of necrosis and inflammation showed no enzyme activity, nor did rows or clusters of proliferating NPC. By days 3 and 5, a restoration of G6Pase was observed in parenchymal cells (Figure 8, E and F). At higher magnification with double histochemical staining for GGT and G6Pase, simultaneous expression of both enzymes was noted in some NPC and in hepatocytes usually within or adjacent to ductular-like

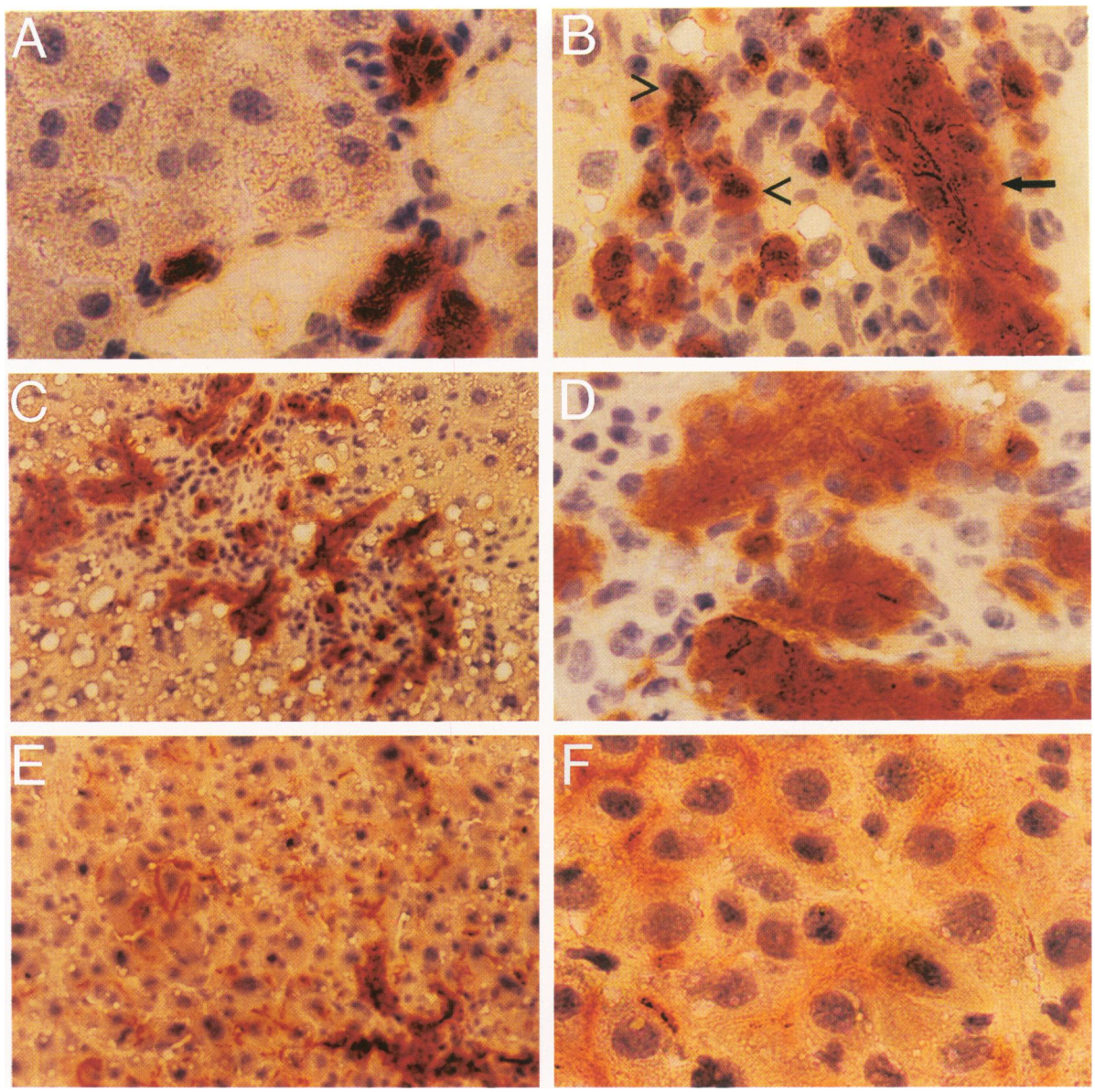

Figure 7. *Histochemical expression of GGT in the portal and periportal zone after GalN injury.* **A:** *Control; GGT activity is confined to mature bile duct cells.* **B:** *Day 2; increased numbers of GGT labeled cells are seen in the periportal region that form NPC structures (arrow). A few granulocytes are also GGT positive (arrow tips).* **C** *and* **D:** *Day 3; GGT positive cells form a prominent NPC network, expanding from portal regions into the parenchyma. GGT staining is distributed diffusely throughout the cytoplasm. Some GGT positive cells in NPC structures have the morphological appearance of transitional cells. Mature hepatocytes do not express GGT.* **E** *and* **F:** *Day five; hepatocytes adjacent to periportal regions or in the vicinity of GGT positive NPC clusters also express GGT. In some of these cells, the cytoplasm is diffusely stained, whereas in others, cytoplasmic staining is reduced and enzyme activity is localized at the canalicular membrane surface. Original magnification:* **A**, **B**, **D**, *and* **F**, ×600; **C** *and* **E**, ×200.

structures (Figure 8F). These results are consistent with the conclusion drawn above that some NPC behave like hepatocyte progenitor cells and follow the lineage progression observed during hepatocyte differentiation.

By comparing the timing of AFP and GGT expression in NPC, it becomes evident that the expression of AFP lags behind that of GGT by one day. In addition, a smaller number of cells express fetal AFP mRNA, although it is difficult to compare directly results of histochemical staining with *in situ* hybridization. However, it is clear that cells progressing through the hepatocyte lineage and showing transient fetal AFP mRNA expression continue to express GGT at the canalicular surface of differentiating hepatocytes (cf Figures 5, 7, and 8).

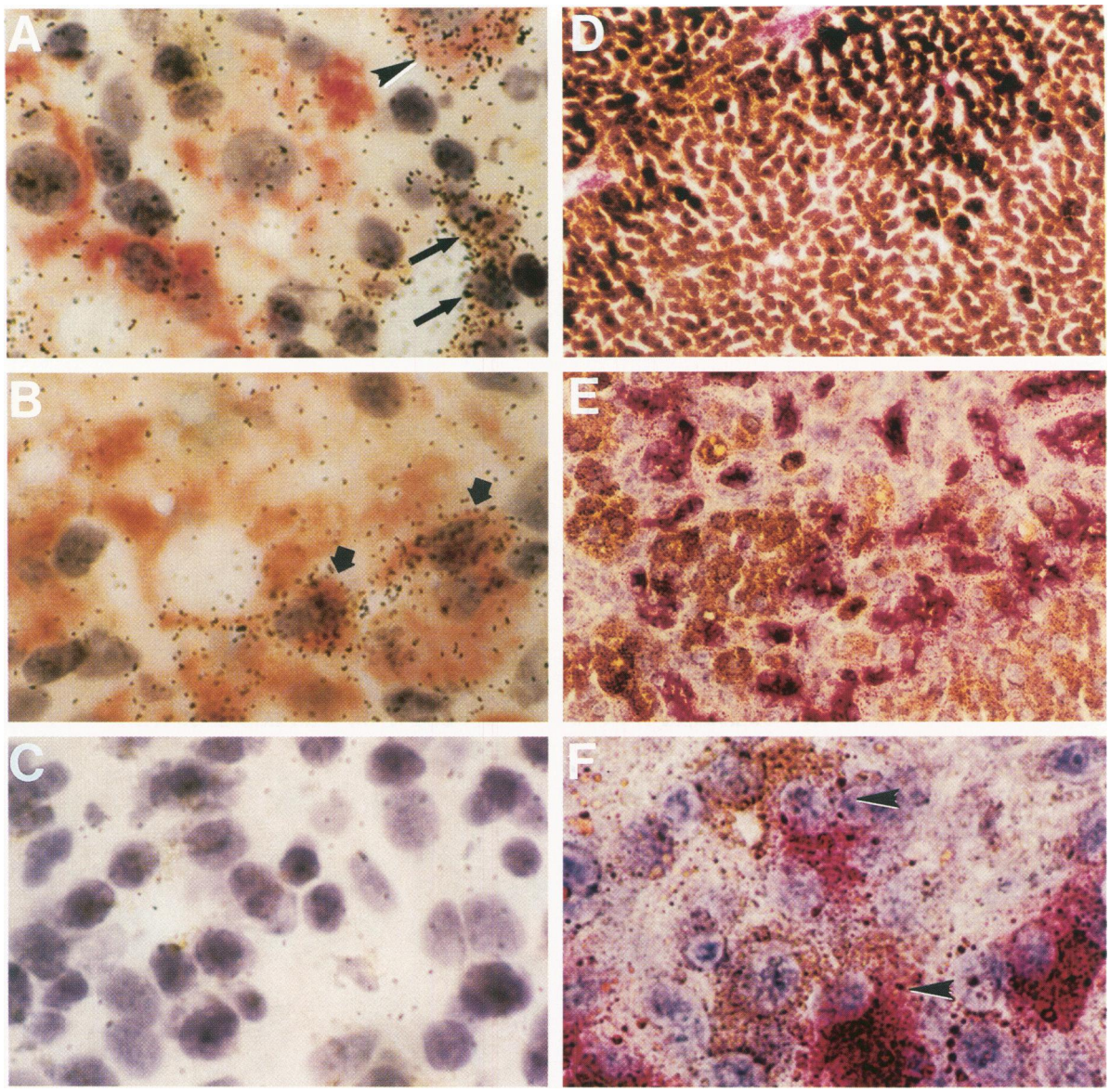

Figure 8. *Simultaneous detection of fetal AFP mRNA and albumin mRNA by* in situ *hybridization and GGT and G6Pase by histochemistry.* ^{35}S*-labeled fetal AFP mRNA (autoradiographic grains) and digoxigenin-labeled albumin mRNA (red). Counterstaining with hemotoxylin.* A, B, *and* C: *Day 5 after GalN treatment; dual hybridization with AFP and albumin antisense strand probes (*A *and* B*) and AFP and albumin sense strand probes (*C*). Not only hepatocytes (arrowhead,* A*) but also transitional cells (blunt arrows,* B*) in pseudoduct-like structures adjacent to proliferating NPC express fetal AFP and albumin mRNAs. NPC express predominately fetal AFP mRNA (arrows,* A*). Control hybridization with AFP and albumin sense strand probes (*C*) shows no autoradiographic grams or red color, respectively.* D, E, *and* F: *Dual histochemistry for GGT and G6Pase.* D: *Control (day 0); mature bile duct cells positive for GGT and hepatocytes strongly positive for G6Pase.* E *and* F: *Day 5 after GalN injury; appearance of dual staining in some cells which are either transitional cells or small hepatocytes (arrowheads,* F*). Original magnification:* A, B, *and* C, *×1000;* D *and* E, *×200;* F, *×1000.*

Discussion

In our studies, we used the GalN model of liver injury to identify of a subclass of liver NPC that possibly represents liver progenitor cells. There were two main reasons for choosing this model: 1) GalN causes selective hepatocyte death and impairs the proliferative capability of residual hepatocytes for nearly 48 hours, which triggers a signal(s) for NPC proliferation and 2) This model is not carcinogenic. In our analysis, we use the term nonparenchymal cells (NPC) to designate the compartment of cells of endogeneous origin that after GalN treatment proliferate from the portal areas and expand into the parenchyma forming a network of GGT positive cells in the liver lobule. These cells have been alternatively referred to as oval cells.[5,12,14,30,35]

The approach used previously to suggest a precursor-product relationship between oval cells and hepatocytes *in vivo* was to trace the transfer of radiolabeled thymidine incorporated from oval cells to hepatocytes.[5,7,13,14,30] This approach, however, has certain disadvantages in the case of extensive tissue damage, including possible reutilization of the labeled compound and marked dilution of labeled cells during the regeneration process.

In the present study, we took an alternative approach and followed serial changes in various molecular, enzymatic, and structural features of different cell types during liver regeneration after GalN injury; their morphological appearance, lobular localization, proliferative activity, and expression of specific genes in the hepatocyte lineage. We followed the kinetics of resurgence of two liver specific markers expressed in fetal hepatocytes (GGT activity and fetal AFP mRNA) and two specific markers unique to hepatocytes (albumin mRNA and G6Pase activity). These markers were expressed in the NPC compartment that proliferates and expands from the portal region into healing necrotic areas in the liver. With this approach, we showed that a subpopulation of small nonparenchymal cells (NPC) with scant cytoplasm, oval shaped, pale and homogeneously stained nuclei (oval cells) that proliferate significantly after GalN injection, increase in size and differentiate through the hepatocyte lineage. During this period, these cells recapitulate the sequential liver specific gene expression program observed during normal development. This was conclusively demonstrated by simultaneous detection of GGT and G6Pase, as well as fetal AFP and albumin mRNAs, in single cells progressing through the differentiation program. The NPC compartment proliferating after GAlN injury is heterogeneous and by our analyses contains at least two types of liver epithelial cells: bile duct/canalicular cells expressing GGT and progenitor cells expressing GGT and fetal AFP mRNA. This conclusion stems from our analysis of the relative number of cells expressing GGT activity and fetal AFP mRNA, their localization, and the time course of GGT and AFP mRNA expression during GalN induced liver regeneration.

Our results show that activation and differentiation of the putative liver progenitor cells requires several days (illustrated schematically in Figure 9). The first event after GalN injury is activation of liver progenitor cells: his-3 mRNA appears first in many single duct-like epithelial cells (activated progenitor NPC) in the periportal regions, while mature bile

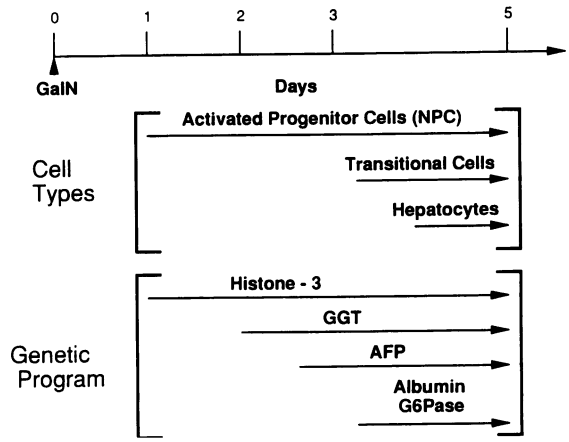

Figure 9. *Sequence of events during differentiation of liver progenitor cells through the hepatocyte lineage.*

duct epithelial cells are only occasionally labeled. After they proliferate, activated NPC begin to express AFP, which indicates that they were previously in a more or less dormant state. This conclusion is based on two observations: 1) In liver sections of control animals or in sections taken one day after the injury, we found very few duct-like cells expressing fetal AFP mRNA and no such cells in mature bile ducts and 2) The synthesis of AFP mRNA lags behind that of his-3 mRNA by at least 24 hours. Precise timing of the activation of the second marker, GGT, cannot be determined because mature bile-duct cells are positive for this marker. In addition, our data cannot discriminate whether progenitor cells in the periportal region express GGT activity before or after their activation. In any case, the expression of GGT in the expanded periportal zone is well documented on day 2 following GalN administration and precedes AFP mRNA expression by approximately 24 hours (Figure 9).

Based on the above considerations, we conclude that NPC proliferation is the first event in a cascade that leads to initiation of maturation (a state of competence), when progenitor cells express the early fetal liver marker GGT (day 2). One day later, some of the activated cells begin to express AFP, indicating that they are now committed to progress through the hepatocyte lineage. Two days later (day 5), all NPC that are committed to undergo differentiation through the hepatocyte lineage express AFP mRNA and GGT. Some of these cells begin to express "later" hepatocytes specific genes, eg, albumin mRNA and G6Pase. These cells are still much smaller than mature hepatocytes but have a larger, more round-shaped and darker stained nucleus and increased

cytoplasmic compartment (transitional cells). Transitional cells have also been observed and isolated from rat liver at early stages of chemical carcinogen treatment.[42,43] The morphological and biochemical characteristics of transitional cells are intermediate between bile duct epithelial cells and hepatocytes, showing heterogeneous expression of albumin, AFP, G6Pase, and GGT.[42–44] In fact, Scoazec et al[45] have reported simultaneous expression of AFP and albumin in carcinogen-induced oval cells.

Our results raise the question of whether expression of GGT and AFP in hepatocytes reflects retrodifferentiation/redifferentiation and not a terminal differentiation of activated progenitor cells. We think that the former possibility is unlikely for the following reasons: 1) Expression of his-3 mRNA in hepatocytes begins on day 2, when not even single hepatocytes are positive for GGT or AFP mRNA. This suggests that hepatocytes first would have to divide and then become reprogrammed, which is not the sequence of events predicted during retrodifferentiation/redifferentiation.[46,47] 2) If the normal sequence of events during hepatocyte proliferation is DNA synthesis, followed immediately by serial changes in specific gene expression (reflecting hepatocyte dedifferentiation), we should have seen a substantial number of hepatocytes expressing GGT and AFP mRNA on day 3 and the bulk of hepatocytes expressing these markers on days 4 and 5. However, on day 3, we saw only single hepatocytes expressing AFP mRNA or GGT. In fact, on days 4 and 5, hepatocytes expressing fetal markers were confined mainly to the area within or immediately adjacent to NPC structures. 3) Analysis of AFP mRNA expression during liver regeneration after partial hepatectomy or carbon tetrachloride toxicity (where activation involves primarily hepatocytes) showed that fetal AFP mRNA expression follows hepatocyte proliferation. In these models of liver regeneration, AFP mRNA expression is moderate (2 to 2.5 times above background), transient and can be attributed to the limited proliferation of the NPC compartment.[41,48–51] On the other hand, activation of AFP expression after GalN injury is 60 to 100 times higher than control levels.[35,41] Therefore, the appearance of fetal AFP mRNA during liver regeneration in all models probably reflects activation of liver progenitor cells. We conclude further that: 1) hepatocytes that proliferate after GalN injury do not recapitulate the liver developmental/differentiation program; and 2) in the GalN model of liver regeneration, we have identified two pathways for restoration of hepatocyte mass: one utilizing liver progenitor cells and the other using mature hepatocytes. At present, however it is not possible to give a precise assessment of the contribution of each pathway toward restoration of liver parenchyma.

The signals governing the activation, proliferation and differentiation of liver progenitor cells are largely unknown. Cell death and/or inactivation of hepatocyte function appears to play an essential role in this process. We have found that lower doses of GalN do not produce typical activation of NPC but do cause proliferation of hepatocytes. GalN hepatitis triggers a vast inflammatory response with polymorphonuclear and mononuclear infiltration in regions of focal hepatocyte necrosis. Endogeneous and exogeneous macrophages and T lymphocytes are known to be activated.[40,52] This suggests a possible role of cytokines and/or other factors released from these cells as participants in liver progenitor cell activation.

Thorgeirsson and coworkers[53] have reported parallel activation of NPC and Ito cells during liver regeneration induced by 2-AAF and partial hepatectomy. In other related models of rat liver hyperplasia/neoplasia,[54] there is close contact between nonparenchymal epithelial cells and Ito cells. Twenty-four hours after GalN administration, we have observed a proliferative response in other liver nonparenchymal cell types, including Kupffer cells, endothelial lining cells and Ito cells. During liver regeneration, expression of a variety of growth factors, oncogenes, cytokines, extracellular matrix components and autocrine/paracrine factors are known to be modified.[25,55–57] How all these factors and cell types participate in the liver regenerative process, under what circumstances they are activated, and precisely how they induce restoration of liver mass remain intriguing questions for future investigation.

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