Cell Proliferation and Oncogene Expression After Bile Duct Ligation in the Rat: Evidence of a Specific Growth Effect on Bile Duct Cells

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The proliferative response of the rat liver was measured after temporary or permanent total biliary obstruction (BDO) and in different regions after selective ligation of the lobar ducts draining the right 60% of the hepatic mass. The results were compared with those after 70% partial hepatectomy (PH). Cell proliferation was assessed globally by measuring DNA synthesis and stratified to the separate cell populations with cytostaining techniques that allowed distinction of hepatocytes, duct cells, and nonparenchymal cells (NPCs). In selected experimental groups, gene expression was determined using techniques that allowed distinction of hepatocytes, duct cells, and nonparenchymal cells (NPCs). In selected experimental groups, gene expression was determined using techniques that allowed distinction of hepatocytes, duct cells, and nonparenchymal cells (NPCs).

Abbreviations: BrdU, 5 bromo-2'-deoxyuridine; NPC, nonparenchymal cells; PH, partial hepatectomy; TGF, transforming growth factor; BDO, bile duct obstruction.

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The proliferative response of the rat liver was measured after temporary or permanent total biliary obstruction (BDO) and in different regions after selective ligation of the lobar ducts draining the right 60% of the hepatic mass. The results were compared with those after 70% partial hepatectomy (PH). Cell proliferation was assessed globally by measuring DNA synthesis and stratified to the separate cell populations with cytostaining techniques that allowed distinction of hepatocytes, duct cells, and nonparenchymal cells (NPCs). In selected experimental groups, gene expression was determined using techniques that allowed distinction of hepatocytes, duct cells, and nonparenchymal cells (NPCs). In selected experimental groups, gene expression was determined using techniques that allowed distinction of hepatocytes, duct cells, and nonparenchymal cells (NPCs).

The proliferation of the obstructed side was similar to the mixed duct cell/hepatocyte response after total BDO, but this almost exclusively involved duct cells on the freely draining side. In contrast to the findings after BDO, livers after PH regenerated maximally at 24 hours rather than 48 hours, had a predominantly noninflammatory hepatocyte as opposed to duct cell response, and had marked expression of the prothrombin and TGFα genes but only weakly and late of c-erb-B2 messenger RNA. The results show that the liver responds as a whole and in a biologically intelligent way to the nature of the injury inflicted on any part of it. It further implies the presence of humoral communications and control networks that assure organ homeostasis and relate this to total body homeostasis. (Hepatology 1995;21:1070-1078.)

The kinetic and morphological features of the proliferative response to bile duct ligation in rats1,8 as well as those to 70% hepatectomy2,8 have been extensively studied. With the hypothesis that the two response patterns reflect different mechanisms, we have compared the changes including those of oncogene expression in both experimental models. The results have confirmed our hypothesis and have shown the uncanny coordination of the whole liver response to injury to any portion.

MATERIAL AND METHODS

Chemical and Biological Reagents

Fraction V Bovine Albumin, sodium phosphate, 5 bromo-2'-deoxyuridine (BrdU), ethylenediaminetetra-acetic acid disodium salt, lauryl sulfate sodium salt, and ethidium bromide were purchased from Sigma Chemical Company (St. Louis, MO). Vectastain ABC kit PK 4002, for cell proliferation determination, was purchased from the Vectors Laboratories (Burlingame, CA); [3H]-thymidine (50 to 80 Ci/mmol), from Du Pont-New England Nuclear (Boston, MA); and Aquasol (scintillant solution), from Amersham Corp. (Arlington Heights, IL). Cyclosporine and FK 506 were gifts from Sandoz Pharmaceuticals Inc. (East Hanover, NJ), and the Fujisawa Pharmaceutical Company Ltd. (Osaka, Japan), respectively. RNAzol was purchased from Biotecx, Houston, TX; labeling kits for cDNA probes, from Boehringer Mannheim Co. (India-
napoli, IN; and cytokeratin 19 specific monoclonal antibody (CK19) for immunohistochemistry studies and anti-5-bromo-2'-deoxyuridine monoclonal antibody, from Dako Corporation (Carpinteria, CA). Silicone tubes for surgical technique were purchased from Portex Ltd. (Hythe, Kent, UK).

**Animals**

Male Fischer rats (F-344) weighing 180 to 220 g were purchased from Zivic Miller Laboratories (Zelienepole, PA). All the animals were maintained in a temperature- and light-controlled room (light from 6:30 AM to 6:30 PM) for at least 1 week before being used. They received food and water ad libitum.

**Surgical Procedures**

Surgical procedures were performed between 8 and 10 AM, under 40 mg/kg nembutal anesthesia administered intraperitoneally.

**Temporary Bile Duct Obstruction.** The common bile duct was isolated and transected 1 cm below the lowest tributary, taking care not to damage the pancreatic ducts. The proximal and distal duct ends were then cannulated and connected through a silicone tube loop (inner diameter 0.28 mm; outer diameter 0.61 mm). The loop was externalized through the abdominal wall and secured with two 6–0 silk threads. The central part of the externalized tube was tied off with a 6–0 silk, causing total obstruction, which was relieved by deligation from 0 to 48 hours later, restoring free bile flow through the tube and into the distal duct. The animals were killed at 48 hours for tissue collections.

**Permanent Total Bile Duct Obstruction.** After the same dissection, the common duct was ligated and transected, with the identical technique used by Accatino et al.2

**Regional Bile Duct Obstruction.** The bile duct branches of the right lateral, right and left medial, and caudate lobes were doubly ligated and divided, leaving intact the biliary drainage of the left lobe (Fig. 1), which constitutes approximately 40% of the rat liver mass. The absence of ductal cross-communication between the obstructed and nonobstructed lobes was proved with methylene blue injections (Fig. 2). The animals were killed 2 days later for tissue collections.

**Regional Bile Duct Obstruction Plus Immunosuppression.** Cyclosporine and FK 506 have been shown to augment regeneration after partial hepatectomy10-12 and portacaval shunt.13 The mechanisms of these hepatotropic effects are not known. However, they may be by immune modulation of the nonparenchymal cells (NPC). Thus, exactly the same experiment as that just described was performed except that the rats were pretreated for 4 days before operation and the first 2 days afterward with cyclosporine (10 mg/kg/day orally) or FK 506 (1 mg/kg/day intramuscularly).

**Partial Hepatectomy.** Seventy percent partial hepatectomy (PH) was performed as described by Higgins and Anderson.14 Thirty animals (three for each group) underwent 70% PH and were killed after 12, 24, 36, 48 hours, and 3, 4, 5, 6, 7, and 8 days.

**Sham Operation.** Twenty control rats underwent laparotomy only. Ten animals were killed at 0 hours and 10 at 48 hours after the surgery.

**EXPERIMENTAL DESIGN**

The groups are summarized in Table 1. In essence, the proliferative and gene expression response to total biliary obstruction (group 3) and the duration of obstruction necessary to evoke these responses (group 2) were defined. In addition, the effect of lobar biliary obstruction on the obstructed versus the drained portion of the liver was determined in untreated (group 4) and immunosuppressed animals (group 5). The results were compared with those in rats after sham operations (group 1 and specific group controls) and with those after 70% hepatectomy (group 6).

**Experimental End Points**

All analyses were of liver fragments obtained at killing from multiple lobes. The specimens for studies of gene expression or DNA synthesis were snap frozen and stored at −80°C.

**Assessment of Cell Proliferation.** The animals whose DNA synthesis was measured chemically by thymidine incorporation were given an intraperitoneal injection of 200 µCi/kg 3H-thymidine 2 hours before killing. Frozen samples were analyzed as previously reported.15

Animals studied histopathologically for localization of the proliferating cells were injected intraperitoneally with 120 mg/kg BrDU 2 hours before killing. Liver samples, 5 mm thick, from the different lobes were fixed in buffered formalin, imbedded in paraffin, sectioned at 4 µm, and stained with hematoxylin-eosin and trichrome.16 BrDU incorporation was detected with a monoclonal anti-BrdU antibody at a 1:20 dilution.17-19 The reaction product was developed by 3-amino-9-ethylcarbazole (AEC).20 Bile duct epithelial cell proliferation was determined in 10 randomly selected portal areas in which the number of nuclear labeled and unlabeled epithelial cells was counted. At least 500 cell counts were obtained from each liver lobe. To measure hepatocyte proliferation, 25 parenchymal fields of 1,000 hepatocytes were counted, from which the percent of BrdU-positive cells was determined.
The number of inflammatory cells was counted in the regional bile duct obstruction experiment 7 days after surgery. In addition, cytokeratin 19 expression (C-K 19, mol wt 40,000 in the catalogue of Moll et al.) was used as a specific marker of bile duct cells.

**Gene Expression Determination.** These studies were performed with previously described methods. Briefly, total cellular RNA from frozen hepatic tissue samples was extracted by RNazol using the manufacturer's procedure. Total RNA (20 μg) was subjected to electrophoresis in a 1% agarose gel and then transferred to Zitabind nylon membrane (Whatman Co., Maidstone, UK) overnight in 20× standard saline citrate. After transfer, the blot was fixed by ultraviolet light (short wave, 254 nm). Complementary DNA probes were labeled with 32P with a random primed labeling kit. Prehybridization and hybridization were performed at 70°C with Church buffer (1% bovine serum albumin 7% sulfate sodium salt, 0.5 mol/L sodium phosphate, 1 mmol/L ethylenediaminetetra-acetic acid). The membranes then were washed, air-dried, and autoradiographed at −70°C. To check for equivalent transfer of RNAs from agarose gel to membrane, the gel was stained with ethidium bromide after capillary transfer and examined; little RNA was found to remain in any lane. The probes used were c-raf (purchased from American Type Culture Collection, Rockville, MD); transforming growth factor-β1 (TGF-β1) (gift from R. Derynk; Genetech, San Francisco, CA); prothrombin (gift from S. J. F. Degen, University of Pittsburgh, PA); c-erb-B2 (gift from Wataru Yasui, Hiroshima University, Japan); rodent TGFα (gift from G. Lee, University of North Carolina). The TGF-alpha and β and c-raf probes were selected because they have been frequently used to study post-hepatectomy regeneration. The c-erb-B2 probe was selected because this gene, like c-raf, has been specifically associated with cholangiocarcinoma. For internal controls, we used 28S ribosomal RNA (Oncogene Science, Inc., Uniondale, NY), and human cyclophilin G (gift from C. T. Walsh, Harvard University, Cambridge, MA).

**STATISTICAL ANALYSIS**

Statistical analysis was performed using a two-way ANOVA (Epistat software) available on IBM (Danbury, CT) PC and assuming statistical significance only with a P < .05. Data were expressed as (M ± SD).

**RESULTS**

**Total Bile Duct Obstruction**

**Standard Histopathology.** The livers of sham-operated rats were normal. Livers with biliary obstruction had a light infiltration of inflammatory cells in the portal areas by 2 days. By day 7, there were signs of intensive proliferation and formation of new bile ducts (Fig. 3), which were surrounded by thin fibrous connective tissues linking some portal areas, as has been reported. The hyperplastic ducts as well as the normal ducts and ductules in the sham-operated livers were positive for cytokeratin 19, whereas hepatocytes were always negative (data not shown) as reported by Alpini et al.

**FIG. 2.** Evidence of lack of ductal cross-communication between obstructed and non-obstructed lobes proved with methylene-blue injection into the common bile duct. Right lobe obstructed and left lobe nonobstructed.

**FIG. 3.** Rat liver 7 days after common bile duct ligation. Proliferation of bile ducts and nonparenchymal liver cells, that form bridging connection between portal areas. (Trichrome stain; original magnification ×100.)
DNA Synthesis. A crescendo of DNA synthesis began at 24 hours, was maximal at 2 days, and receded almost to baseline by day 6 (Fig. 4).

BrdU Incorporation. The increased DNA synthesis could be seen in the nuclear labeled BrdU-positive cells to be mainly a reflection of duct cell proliferation rather than of hepatocytes (Fig. 5). In the animals of group 3, 17% of bile duct cells were labeled at the peak of the response at 2 days compared with 1% at the outset. A much smaller response, reaching 4.8% at 2 days, was seen in the hepatocytes of these livers (Fig. 6, top panels).

These results were strikingly different than those following 70% PH (Fig. 6, lower panels). With PH, a bile duct response also promptly occurred, but it was short-lived compared with bile duct obstruction (BDO). In addition, the percentage of BrdU-positive hepatocytes was more than 5 times greater than after BDO (Fig. 6).

**Required Time of Obstruction.** In the animals of group 2, biliary obstruction for 12 hours or even 24 hours did not cause significant increases in BrdU-positive cells in the livers obtained at killing at 2 days. However, with obstruction for 36 hours that was then relieved or with continuous obstruction throughout the 48-hour duration of the experiment, the duct cell proliferation (>15% of total) was similar to that found in the animals of group 3 (Fig. 7).

**Gene Expression.** Hepatic gene expression was different after BDO than after 70% PH, and the differences had a physiologic correlation. The features that appeared to be most typical of BDO during the first 48 hours were lack of expression of TGFα, weak and delayed prothrombin gene expression, but with prompt...
appearance of c-erb-B2 messenger RNA. In contrast, the typical response to PH was strong expression of TGFα and prothrombin by 12 hours but without the appearance of c-erb-B2 messenger RNA.

Essentially no increase of TGFα, a potent hepatocyte mitogen, was detectable during the first 48 hours after BDO when DNA synthesis was maximum, whereas TGFα was markedly increased after PH (Fig. 8). Elevated levels of c-erb-B2, an oncogene associated with cholangiocarcinoma, were clearly expressed from 48 to 96 hours after BDO, but appeared only at 72 hours after PH (Fig. 9). c-raf, a proto-oncogene associated with hepatocytes proliferation after PH, and also with cholangiocarcinoma, increased significantly 48 to 96 hours after BDO, whereas it was slightly detectable after PH (Fig. 9). Interestingly, the expression of the hepatocyte-specific prothrombin gene was enhanced during the increased DNA synthesis and mitoses after PH, but not after BDO (Fig. 10). TGF-β1 messenger RNA expression was similar both after PH and BDO (Fig. 11) and was similar to that previously reported for PH.

**Regional Bile Duct Obstruction**

**Standard Histopathology.** The obstructed liver lobes in the rats of group 4 at 7 days had early changes similar to those in the totally obstructed livers of group

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<th>kb</th>
<th>TGFα</th>
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<td>4.5-4.8</td>
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**Fig. 7.** Effect of total biliary obstructions for different durations on the percent of BrdU nuclear labeling of duct cells (mean ± SD) in animals killed at 2 days (n = 5 rats at each time point). *P < .005 vs. value at time 0.

**Fig. 8.** Northern blot analysis of RNA from rat liver at different times after PH and BDO, and hybridized to probes for TGFα or cyclophilin (n = 3 rats for each time point).

**Fig. 6.** Percent (mean ± SD) of hepatocytes and bile duct cells with nuclear labeling with BrdU at successive times after total bile duct obstruction (upper) and 70% hepatectomy (lower) (n = 3 rats each time point). The control value (0 time) is the mean average of five sham-operated rats. *P < .005 vs. sham controls. tP < .005 vs. value of PH rats at same time point.
3, including the appearance of inflammatory cells (Table 2). The freely draining left lobes had a normal number of inflammatory cells, but there was striking proliferation and formation of new ducts.

**DNA Synthesis.** At 48 hours, which was the time of the maximal proliferative response in the total biliary obstruction experiments previously described, DNA synthesis was significantly and almost equally elevated, in the obstructed as opposed to the freely draining lobes (Fig. 12). The magnitude of DNA response in both locations was less than half that caused by total biliary obstruction.

**BrdU Incorporation.** As in the total biliary obstruction experiments of group 3, the predominant proliferative response was that of the duct cells. This was true both in the obstructed and freely draining lobes (Fig. 13). Interestingly, increased hepatocyte proliferation occurred only in the obstructed lobes. In the drained lobes, hepatocyte proliferation was negligible although significantly more than in the sham-operated controls (Fig. 13).

**Addition of Immunosuppression.** Preoperative and postoperative treatment with cyclosporine and FK 506 did not significantly affect the results (data not shown).

**DISCUSSION**

The literature on an increasing number of hepatic growth factors has accumulated rapidly in the last two decades. However, this information has not been synthesized into a generally accepted explanation of hepatic regeneration. Because hepatocytes, biliary duct cells, and NPCs have long been known to have different kinetic orders of proliferation, a reasonable implication is that they are variably cross-regulated. NPCs have been particularly attractive candidates for such a role.

The biliary obstruction models have lent themselves well to proliferation studies of the duct cell component of the liver. These cells constitute approximately 30% of the total liver mass. Previous investigations have defined the morphologic consequences of duct obstruction, which include pronounced ductular hyperplasia, a modest hepatocyte proliferative response, and infiltration of inflammatory cells. These findings were confirmed in rat livers after common duct ligation and in liver lobes that were selectively obstructed. The duct cell and hepatocyte proliferative responses were not simultaneous but they were closely related temporally. These were compared with the changes after 70% partial hepatectomy.

With PH, the duct cells and hepatocytes both participated vigorously in the regeneration, the peak hepatocyte response being at 24 hours, with the duct cells only slightly behind. The global proliferative response to BDO or regional BDO as defined by DNA synthesis was similar to PH except that its peak was at 2 days instead of 1, with a slower return to baseline. However, the differences between the PH and BDO models were

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<th>TABLE 2. Inflammatory Cells Infiltrating Portal Tracts in Sham-Operated and Drained Versus Nondrained Lobes 7 Days After Regional Bile Duct Ligation Operated Rats</th>
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<td><strong>Sham Rat Lobes</strong></td>
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<td>Neutrophils</td>
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<td>Macrophages</td>
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<td>Lymphocytes</td>
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**NOTE.** The values, M ± SD, are the inflammatory cell numbers observed in 0.02 mm$^2$ of the portal areas.

* Significantly different from the sham and noncholestatic lobes, $P < .05$. 

FIG. 11. Northern blot analysis of RNA from rat liver at different times after PH and BDO, and hybridized to the probe of TGF-β1.
more than temporal. Cell labeling studies demonstrated that most of the heightened DNA synthesis after biliary obstruction was occurring in the duct cells, with only a minor contribution from the hepatocytes. The required stimulus in the BDO model was continuation of the obstruction for more than a day. Blockage for 24 hours or less was without significant effect at the time of killing at 2 days.

Hypotheses have been proposed to explain the hyperplastic duct cell response to biliary obstruction in humans and animals. Excluding the possibility that there are pluripotent (oval) stem cells, these fall into two general categories. In one of these generic alternatives, Slott et al. suggested that proliferating hepatocytes were able to invade the basal membrane of the obstructed duct, taking up residence in the epithelium, where they differentiated to the phenotypic characteristics of biliary duct cells. Our experiments provided no support for this concept. Instead, the primary proliferative response of the totally obstructed liver was of the duct cells, the hepatocytes being relatively minor participants. The observations in the regional biliary obstruction experiments were considerably more damaging to the ectopic hepatocyte hypothesis. Here, the duct cell proliferation in the ostensibly healthy freely draining lobes was of the same magnitude as in the lobes with ligated ducts. In both the draining and cholestatic lobes, the muted hepatocyte response (which was slightly greater in the obstructed lobes) presumably was in response to partial obstruction to the injury caused unilaterally by the duct ligation. This response to partial obstruction was only about half of that caused when the entire biliary system was occluded, as would be expected with the proportionately less complete damage to the parenchyma.

In an alternative general hypothesis explaining the ductular hyperplasia after biliary obstruction, Buyssens has proposed a contributory or regulatory role of NPC by-products of the lipoxygenase pathway, which are chemotactic for and activate polymorphonuclear leukocytes as well as multiple cytokines. A histopathologic basis for such a mechanism was evident in that there was a striking leukocyte inflammatory reaction that appeared to include multiple NPC lineages in the obstructed liver tissue, whether this obstruction was of the whole organ or only part of it. However, in the partial obstruction experiments the vigorous duct cell proliferation in the freely draining lobes that did not have a significant inflammatory reaction showed that the local presence of NPCs was not a direct paracrine requirement for stimulation of duct cell hyperplasia, while not ruling out a humorally transmitted effect of cytokines from leukocytes on the contralateral side. The role of these immunologically capable NPCs in the events after duct ligation remains to be determined. Although the T-cell-directed drugs, cyclosporine and FK 506, both augment the proliferation associated with hepatectomy and Eck's fistula, these immunosuppressive agents did not affect the outcome of the regeneration after regional biliary obstruction. If a stem cell actually exists, it could be accommodated by this paradigm.

Whether or not an immune component was involved,
the regional biliary obstruction experiments showed the intelligence with which one part of the liver communicates with another. This has been noted before in auxiliary liver transplant models and in “double liver” preparations in which one liver fragment is provided a physiologic advantage such as splanchnic venous blood that is rich in hepatotrophic factors. Under such circumstances, there is increased proliferation by the “disadvantaged” liver or fragment, but this is minor in the long run compared with the compensatory hyperplasia and hypertrophy in the advantaged hepatic tissue. The coregulation between the coexisting livers or liver fragments results in the eventual atrophy of the damaged liver fragment, regeneration of the healthy one, and maintenance of the original total hepatic mass. Although slower in evolution than with regional portal blood deprivation, this also is the well-known outcome weeks or months after lobar duct ligation in animals and humans.

The specificity of the cross-talk between liver fragments was evident in the regional duct ligation experiments. Here, the acute primary insult to the duct cells in one part of the liver was promptly sensed and responded to by duct cell hyperplasia in the affected as well as the unaffected liver. This consequence could reflect a unique humorally transmitted signal from a stimulus such as but not limited to increased intraductal hydrostatic pressure, in which inflammatory cells could play an intermediary role. Alternatively, the possibility cannot be ruled out that there are separate and highly specific growth factors other than cytokines governing duct cell regeneration.

The dissociation of messenger RNA expression of the four growth factors studied was striking and correlated with the physiologic end points. The complete absence of TGFα expression was striking in animals whose global DNA response to BDO was essentially equivalent to that of animals with 70% PH, but with a different temporal and cell target profile. The early expression of c-erb-B2 with BDO only was of particular interest because of the association of this oncogene with obstructing cholangiocarcinoma. It will be worthwhile to do systematic studies of this oncogene in biopsy specimens of other duct-specific diseases. In addition, it will be important to determine the more complete range of gene activation caused by BDO, which may prove to be multiple, as has already been shown after PH.

REFERENCES


42. Starzl TE, Porter KA, Kashiwagi N, Putnam CW. Portal hepatotropic factors, diabetes mellitus and acute liver atrophy, hypertrophy and regeneration. Surg Gynecol Obstet 1975;141:843-858.


