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In Vivo Responses of Macrophages and Perisinusoidal Cells to Cholestatic Liver Injury

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We investigated the response of macrophages and perisinusoidal (Ito) cells (PSCs) during the development of secondary biliary cirrhosis after ligation and division of the common bile duct. Liver tissue was obtained from three groups of male Wistar rats: 1) untreated controls (n = 3); 2) common bile duct-ligated (CBDL) animals (n = 15); and 3) sham-operated controls (n = 15). Material from animal groups 2 and 3 was obtained on days 3, 7, 14, 21, and 28 after operation; in all animals 5-bromo-2-deoxyuridine was administered intraperitoneally before death. Monocytes and macrophages were detected using the monoclonal antibody ED1 and tissue macrophages using the antibody ED2. Cell proliferation within the macrophage population was demonstrated by double labeling for ED2 and incorporated 5-bromo-2-deoxyuridine. PSCs were demonstrated in tissue sections by immunolocalization of desmin; proliferating PSCs were identified by double labeling for desmin and incorporated 5-bromo-2-deoxyuridine. Evidence of phenotypic modulation of PSCs was sought using anti-α-smooth muscle actin (α-SMA) antibody. Increased numbers of ED1- and ED2-positive cells were seen in CBDL animals at all time points. Local proliferation of macrophages could be identified and reached a peak at day 3, thereafter falling toward control values. Compared with those of controls, livers of CBDL animals showed increased numbers of desmin-positive PSCs in periportal zones from day 3 on, reaching a peak at day 14 (127.8 ± 10.99 cells/0.635 mm²) and followed by a plateau. PSC proliferation peaked at days 3 and 7 (labeling indices 11.2% and 11.2%, respectively) and thereafter fell toward control values; no expansion of the PSC population was seen in sham-operated rats. Increased α-SMA-positive cells were also noted from day 3, with a peak at day 21 (231.1± 11.52 cells/0.635 mm²) and followed by a plateau. En face ***labeling experiments in days 14, 21, and 28 CBDL animals showed cells co-expressing α-SMA and desmin and cells expressing α-SMA alone. These results indicate that in response to chronic cholestatic liver injury, PSCs proliferate and undergo phenotypic modulation toward "myofibroblast-like" cells. The kinetics of the response are similar to those of the ED2-positive cell population in keeping with a hypothesis that PSC proliferation and activation may be mediated by factors released by macrophages in response to various forms of liver injury. We conclude that the responses of macrophages and PSCs to cholestatic injury are similar to those after toxin-induced hepatocyte necrosis. (Am J Pathol 1993, 142:511–518)***

Fibrosis, a common characteristic of many chronic liver diseases, is a dynamic process involving changes in both the synthesis and degradation of extracellular matrix proteins.[1–6] Although several studies have implicated hepatocytes[7,8] and bile duct epithelial cells[9] in the production of extracellular matrix proteins, and thus in hepatic fibrogenesis, most evidence suggests that the principal source of such proteins is the perisinusoidal (Ito) cell (PSC; also lipocyte or fat-storing cell).[10,11] PSCs can be readily identified in sections of rat liver by immunolabeling of the intermediate filament protein desmin.[12] This has enabled quantitative analyses of their response in animal models of both acute and chronic chemically induced liver injury.[13–17] It has been proposed that after such toxin-induced hepatocyte necrosis, there is an inflammatory response, including expansion of the Kupffer cell (KC) popula-

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tion, resulting from both local proliferation of the KCs and influx of monocytes.[4,14,15] The macrophage population releases various peptide growth factors, including transforming growth factor-β1 and platelet-derived growth factor. These are thought, in turn, to stimulate proliferation within the PSC population and subsequent "activation" and modulation toward "myofibroblast-like" cells, with resultant increased synthesis of matrix proteins.[18,19] Such "activated cells" may themselves express transforming growth factor-β1, leading to further autocrine stimulation.[20] Although the process may be transient in some forms of acute injury,[14,15,21] it persists when there is continued liver cell damage and is associated with a net accumulation of matrix proteins.

Although this sequence may explain many forms of hepatic fibrosis, some human disorders such as hereditary hemochromatosis and chronic cholestasis are associated with little hepatocyte necrosis and only a limited inflammatory response. This has led some investigators to suggest that other mechanisms may exist in the fibrosis of chronic biliary disease.[9] Common bile duct ligation (CBDL) in rats results in hepatic fibrosis and is considered to be a suitable model for studying biliary cirrhosis.[22] Although extracellular matrix protein synthesis has been well documented in this model, little is known of the KC and PSC responses. The aim of the present study was to investigate the response of these cells to CBDL and also to examine the activation and modulation of PSCs toward "myofibroblast-like" cells in cholestasis by detection of α-smooth muscle actin (α-SMA), a marker of smooth muscle differentiation.

Materials and Methods

Animal Model

Biliary fibrosis was induced in male Wistar rats (225 to 250 g) by double ligation and division of the common bile duct 3, 7, 14, 21, and 28 days before death (n = 3 per time point). Sham-operated animals served as controls (n = 3 per time point). In addition, three unoperated animals were used as day 0 controls. An intraperitoneal injection of 5-bromo-2-deoxyuridine (BrdU; 50 mg/kg body weight; Sigma Chemical Company Ltd., Poole, Dorset, UK) was given to each animal 1 hour before death by cervical dislocation. A standard laboratory diet and water were available *ad libitum* throughout the study. All animal procedures were carried out in compliance with guidelines of the Home Office and under license.

Samples of liver tissue (~10 × 5 × 2 mm) were fixed in formal sublimate (4% neutral phosphate-buffered formaldehyde in saturated aqueous mercuric chloride) for 4 hours and then processed using routine histological procedures. Paraffin sections (3 μ) were mounted on poly-L-lysine slides for immunohistochemical studies and the following conventional tinctorial stains: hematoxylin and eosin, Shikata's orcein, and Picro-Mallory trichrome technique.

Immunohistochemistry

Details of the mouse monoclonal antibodies used in this study are given in Table 1. Full details of their generation and specificities are given elsewhere.[15,23,24] For each antibody, dilutions and trypsin times were previously optimized.[25] The secondary antiserum (peroxidase-conjugated rabbit anti-mouse immunoglobulin) was obtained from Dako Ltd., UK.

Single labeling

A single-labeling technique was used for the detection of monocytes and macrophages, desmin, and α-SMA. In brief, sections were treated with methanol and hydrogen peroxide (0.5%) for 15 minutes at room temperature to inactivate endogenous peroxidase activity and then incubated with 0.1% porcine trypsin (Sigma), pH 7.8, for 5 minutes at 37 C (with ED1 and ED2 only). They were then incubated with 20% normal rabbit serum in Tris-buffered saline, pH 7.6, for 10 minutes to block nonspecific binding sites, and then incubated with primary antibody overnight at 4 C. They were then incubated with secondary antibody (1:20 in normal rabbit serum/Tris-buffered saline) for 30 minutes at room temperature, treated with 3,3′-diaminobenzidine (0.1%) in the presence of hydrogen peroxide (0.02%), counterstained with Mayer's hematoxylin, and mounted in

Table 1. *Details of Primary Antibodies*

Antibody	Source	Labeled cells	Dilution
ED1	Serotec, UK	Rat monocytes and macrophages	1:600
ED2	Serotec, UK	Rat macrophages	1:60
Anti-desmin (clone D33)	Dako Ltd., UK	PSCs	1:200
Anti-α-SMA (clone 1A4)	Sigma, UK	Activated PSCs and "myofibroblast-like" cells	1:700
Anti-BrdU (clone B44)	Becton Dickinson, UK	Cells in S-phase	1:250

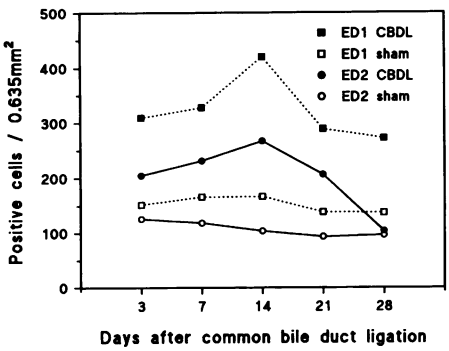

Figure 1. *Quantitation of ED1- and ED2-positive cell populations in CBDL and sham-operated animals. Each value is the mean obtained from three animals.*

DPX. In all experiments, negative controls were included when nonimmune serum or buffer was used in place of the primary antibody. All liver sections contained inherent positive control material in that α-SMA and desmin are both expressed by smooth muscle cells of hepatic artery branches. The intensity of staining for these antigens was found to be constant in all test sections.

To assess the presence of simultaneous expression of desmin and α-SMA by PSCs, serial *en face* sections were immunolabeled for each of these antigens. Corresponding areas of the labeled sections were then compared.

Double labeling

Nuclear incorporation of BrdU was detected in ED2-positive macrophages by the following double-labeling method.[25] In brief, sections were treated with methanol/peroxide as above, incubated with trypsin as above, treated with 2 mol/L hydrochloric acid for 30 minutes at 37 C to denature DNA, and incubated with anti-BrdU for 60 minutes at 37 C. They were then incubated with secondary antibody, immersed in nickel-modified 3,3′-diaminobenzidine with Tris-cobalt chloride enhancement to yield a black reaction product, incubated with ED2 antibody as before, incubated with secondary antibody, immersed in 3,3′-diaminobenzidine (0.1%) to yield a brown reaction product, and dehydrated and mounted in DPX. A reversal of the above sequence was found to give optimal results for desmin and BrdU double labeling.[25]

Quantitation

Cell numbers were assessed in 30 random high power fields per section; three sections were examined from each animal. The results are expressed per unit area equivalent to 10 high power (×400) fields (0.635 mm²). For assessment of ED1- and ED2-positive cells, only those that had been sectioned through the plane of the nucleus were counted. Desmin- and α-SMA-positive cells were counted only when a nucleus and at least one cytoplasmic process were present. Labeling indices were obtained for proliferating macrophages and PSCs in double-labeled sections by expressing the number of double-labeled cells as a percentage of the total number of ED2-positive macrophages and desmin-positive PSCs, respectively; up to 800 cells were assessed per animal. All values are expressed as the mean ± standard error of the mean. Cell counts in CBDL animals were compared with those of sham-operated controls using Student's *t*-test; $P < 0.05$ was considered significant.

Results

Morphology

At day 3 after CBDL, ductular proliferation was evident within portal tracts and at the limiting plate, although at this stage there was little apparent increase in fibrous tissue. By day 7, there was mild edema within the portal tracts, expansion of the portal areas by fibrous tissue, and more marked ductular proliferation; early septum formation was present. Cholangioles were surrounded by a mild neutrophil infiltrate. Cholestatic change in hepatocytes was more extensive, numerous mitotic figures were noted within epithelial cells, and sinusoidal cells were prominent.

In day 14 CBDL animals there was disruption of acinar architecture by frequent narrow fibrous septa linking portal areas; ductular proliferation was again more prominent and occasional bile infarcts were present. At day 21, bile duct proliferation and septum formation were further increased; occasional fine orcein-positive elastic fibers were noted in fibrous septa. By day 28 there was replacement of much of the hepatic architecture by extensive bile duct proliferation, periductular fibrosis, and septum formation; the periductal fibrous tissue and septa contained orcein-positive elastic fibers. Parenchymal nodules were present, the appearances amounting to early biliary cirrhosis.

ED1 and ED2 Immunolabeling

In control animals (day 0 and sham-operated), ED1 and ED2 immunoreactivity was present in sinusoidal

cells with the characteristic morphology of KCs; in addition, ED1 positivity was shown by small round cells within sinusoidal lumina having the morphology of blood monocytes.

After CBDL, there was an expansion of both the ED1- and ED2-positive cell populations, principally within periportal zones. Quantitative analysis showed significantly higher numbers of ED1- and ED2-positive cells in these animals compared with sham-operated controls at all time points (except ED2 on day 28). Peaks in ED1- and ED2-positive cell counts occurred at day 14 (CBDL compared with sham-operated; $P < 0.001$ for both ED1 and ED2), followed by a fall in cell numbers which, in the case of ED2-positive cells, reached control values at day 28 ($0.5 > P > 0.1$). Throughout the study the number of ED1-positive cells significantly exceeded that of ED2-positive cells ($P < 0.0001$ at each time point) (Figure 1).

Proliferation in the ED2-positive cell population was identified using the double-labeling method described (Figure 2). The peak in the labeling index occurred at day 3 (day 3: CBDL, 11.25 ± 0.58%; sham, 0.92 ± 0.72%; $P < 0.001$), followed by a gradual fall toward control values by day 28 (day 28: CBDL, 2.25 ± 0.29%; sham, 1.38 ± 0.88%; $0.5 > P > 0.1$).

Desmin Immunolabeling

In control animals, desmin immunoreactivity was present in smooth muscle cells within the media of portal tract vessels and within occasional small stellate PSCs in the acini. After CBDL there was an increase in the number of desmin-positive PSCs in periportal zones. These cells were present in the fibrous tissue surrounding proliferating bile ductules in periportal areas and were also seen in close apposition to hepatocyte rosettes within the acini and at the periphery of bile infarcts. Desmin-positive PSCs in CBDL animals were larger and showed more intense desmin immunoreactivity than those of control animals. A significant increase in the desmin-positive PSC cell population was seen at day 3 and reached a peak at day 14 (CBDL compared with sham-operated; $P < 0.001$), followed by a plateau (Figure 3).

Double labeling for desmin and BrdU showed proliferation within the PSC population; the peak in the

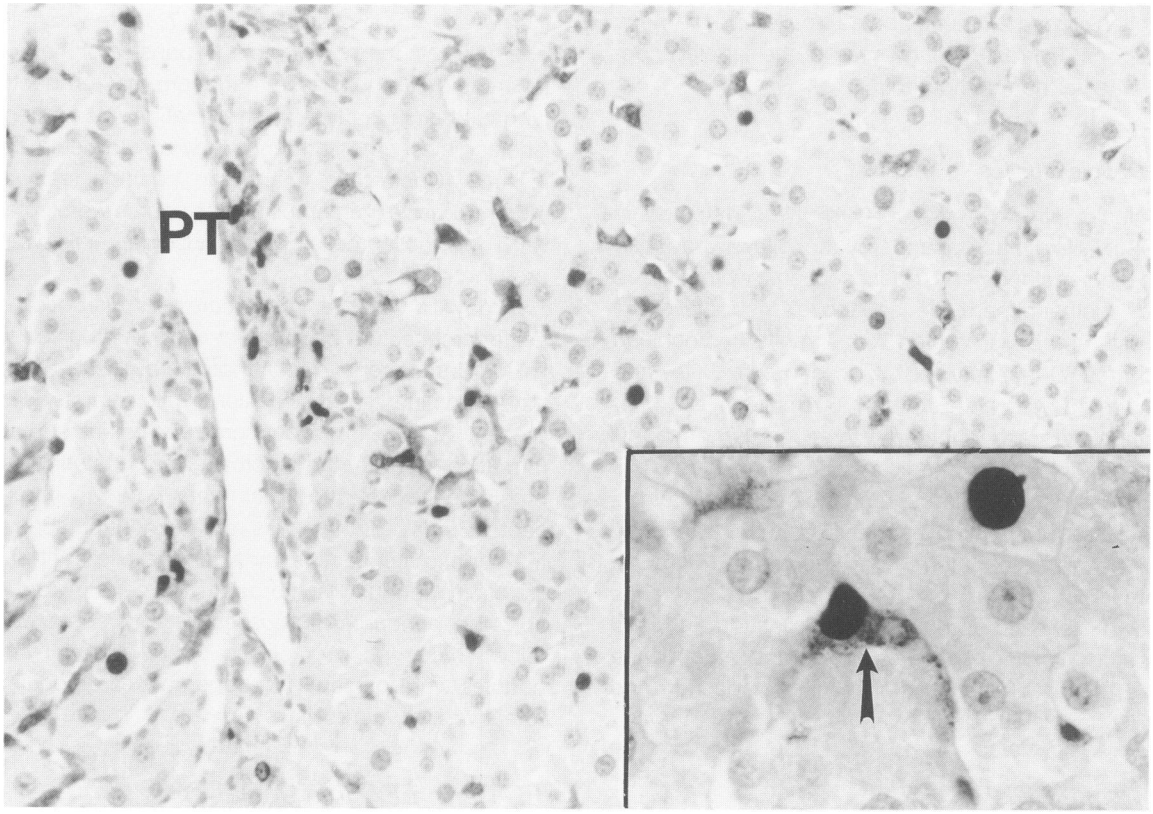

Figure 2. *ED2/BrdU double labeling in a day 3 CBDL animal. Cells in S-phase show strong nuclear staining for BrdU (×120).* Inset, *ED2/BrdU co-localization in a proliferating KC (arrow). Proliferation in an adjacent hepatocyte (ED2-negative) can also be seen. ED2/B44; indirect immunoperoxidase (×580).*

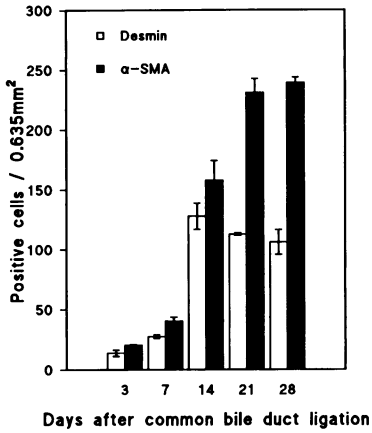

Figure 3. *Quantitation of desmin-positive PSCs and α-SMA-positive cells in CBDL and sham-operated animals. Each point is the mean ± SEM of three animals.*

labeling index occurred at days 3 and 7 (day 3: CBDL, 11.17 ± 1.17%; sham, 0.77 ± 0.29%; $P <$ 0.001; day 7: CBDL, 11.17 ± 0.5%; sham, 0.66 ± 0.33%; $P < 0.001$), followed by a fall to control values by day 28 (day 28: CBDL, 1.45 ± 0.22%; sham, 0.44 ± 0.29%; $0.1 > P > 0.05$).

α-SMA Immunolabeling

In control animals, α-SMA positivity was present in the media of portal tract vessels and in elongated cells surrounding the terminal hepatic venules; occasional small stellate immunoreactive cells were present within the sinusoids. After CBDL, there was a marked increase in the number of α-SMA-positive cells, especially within fibrous septa and around proliferating bile ductules (Figures 4 and 5), as well as around hepatocyte rosettes within the acini and at the periphery of bile infarcts. In addition the individual cells were larger than those of control animals. A

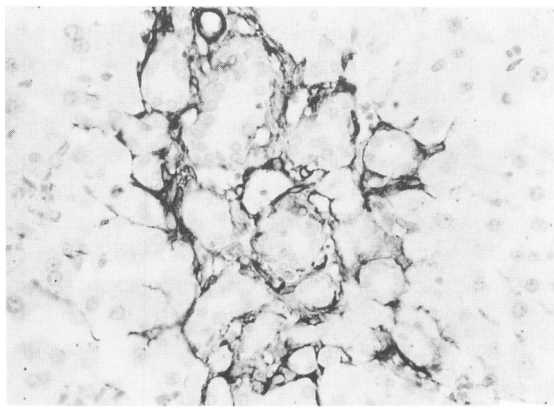

Figure 4. *α-SMA-immunoreactive cells in acinar zone 1 (periportal zone) of a day 14 CBDL animal. 1A4; indirect immunoperoxidase (×120).*

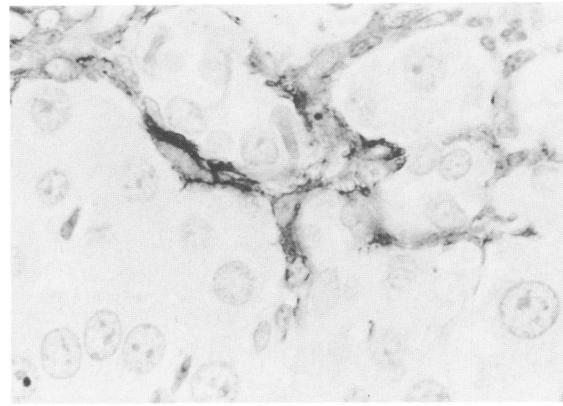

Figure 5. *α-SMA-positive cells surrounding ductular structure in a day 28 CBDL animal. 1A4; indirect immunoperoxidase (×390).*

significant increase in the number of α-SMA-positive cells was seen at day 3, with a peak occurring at day 21 ($P < 0.001$ compared with shams) and followed by a plateau (Figure 3).

Throughout the study the α-SMA-positive cells were morphologically identical to the desmin-positive PSCs. At days 21 and 28 in CBDL animals, the α-SMA-positive cell population was significantly greater than the desmin-positive cell population (day 21, $0.01 > P > 0.001$; day 28, $P < 0.001$). Immunolabeling of *en face* sections at days 3 and 7 showed the majority of these cells to be both α-SMA-positive and desmin-positive. However, at days 14, 21, and 28, in addition to many cells exhibiting dual immunoreactivity, there were large numbers of α-SMA-positive/desmin-negative cells in areas of cholestatic damage (Figure 6).

Discussion

Chronic cholestasis in man, resulting from either obstruction of the extrahepatic bile duct or disorders of the intrahepatic biliary system, commonly leads to periportal and portal fibrosis.[26] Portal-portal linkages are formed and further distortion of the hepatic architecture by associated nodular regeneration leads to the development of a micronodular and so-called monolobular pattern of cirrhosis. At the interface between fibrous septa and parenchyma, there is ductular proliferation with an associated cholangiolitis and within the immediate periportal zone there is hydropic degeneration of hepatocytes due to cholate stasis (biliary piecemeal necrosis).[26] The morphological changes induced in our adult Wistar rats by ligation and transection of the common bile duct closely resembled those seen in human chronic biliary disease, although the ductular response was

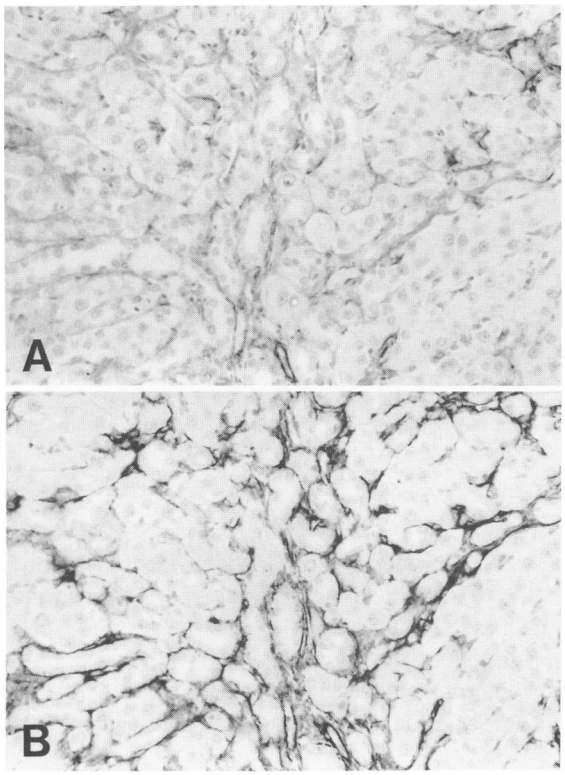

Figure 6. En face *labeling for* (**A**) *desmin and* (**B**) α*-SMA in a day 28 CBDL animal. Although some stellate-shaped cells express both proteins, numerous* α*-SMA-positive/desmin-negative cells can be identified (×120).*

more intense. Thin fibrous septa with early portal-portal linkages were identified by day 14. Abdel-Aziz et al[27] have shown that, at this stage, the process may be reversible; reanastomosis of the common bile duct in their experience led to a return to normal liver morphology and a normal distribution of extrahepatic matrix proteins (with the exception of collagen type IV). By day 28 in our experiments, fibrous septa were more marked and were associated with the deposition of elastic fibers, detected using Shikata's orcein stain. Elastin accumulation is generally a late feature in fibrogenesis and is regarded as an indicator of irreversibility. Furthermore, in these animals, there was evidence of some nodular regenerative activity, and the histological changes amounted to early biliary cirrhosis. The presence of portal hypertension was suggested by the observation of gross splenomegaly and ascites formation (data not shown). These features indicate that although biliary fibrosis may be potentially reversible in the early stages, in time there is progression to cirrhosis, with a pattern similar to that seen in some forms of human biliary disease.

Although only a sparse inflammatory infiltrate, principally in the form of a cholangiolitis, was noted in routinely processed histological sections, immunolabeling with ED1 and ED2 demonstrated a marked expansion of the monocyte/macrophage population. This occurred as early as day 3, with a peak at day 14, and persisted throughout the study, although by day 28 ED2-positive cell numbers in CBDL animals had returned to those of sham-operated controls. The number of ED1-positive cells (monocytes and macrophages) exceeded that of ED2-positive cells (macrophages only), which suggested that recruitment of monocytes contributes to the expansion of the macrophage population. However, local proliferation of KCs was evident in the double-labeling experiments, where a peak in the labeling index was noted as early as day 3. These observations indicate that a KC response occurs early in cholestatic injury and are consistent with the hypothesis that these cells are involved in mediating PSC proliferation and activation as in toxin-induced injury.[14,15,28] The stimulus for the rapid macrophage response in cholestasis remains to be established. In contrast to chemically induced damage, there is little hepatocyte necrosis apparent in the early stages after CBDL. We speculate that leakage of hydrophobic bile acids such as chenodeoxycholic acid and lithocholic acid from cholangioles and proliferating ductules at the limiting plate may be involved in stimulating monocyte influx and KC proliferation.

We have also demonstrated an expansion of the desmin-positive PSC population in periportal zones after CBDL. These cells were predominantly found surrounding proliferating bile ductules. A similar distribution of PSCs in CBDL-induced injury in rats was noted by Milani et al,[29] although no quantitative data were reported. In their study, cells exhibiting α_2(I), α_1(III) and α_1(IV) procollagen expression as detected by *in situ* hybridization using RNA probes appeared to co-localize with desmin-positive PSCs, suggesting that these cells were active extracellular matrix producers. Increased numbers of PSCs have also been identified in periportal zones of rat liver in the Solt-Farber model of hepatocarcinogenesis, where they were seen in close apposition to oval cells.[30] In the present study, we have shown that after CBDL, desmin-positive cell numbers reached a peak at day 14 and were followed by a plateau. This expansion in the cell population was due, at least in part, to local cell proliferation as evidenced by our double-labeling experiments; this phenomenon has previously been documented in chemically induced injury.[14,15,17,28]

Several studies have shown that in response to prolonged culture[18,31] or *in vitro* exposure to KC-conditioned medium or peptide growth factors PSCs

acquire phenotypic and functional characteristics of myofibroblasts[31–35] including expression of the α (smooth muscle) isoform of actin.[18,31] We have previously demonstrated that in acute carbon tetrachloride-induced liver injury, PSCs expressing this protein appear transiently in areas of injury.[21] By contrast, in the present study, we have shown that after CBDL there is a progressive and persistent increase in α-SMA-positive cells. Although in the early phases of the response many PSCs were found in *en face* labeling experiments to co-express desmin and α-SMA, by day 28 large numbers of α-SMA-positive/desmin-negative cells were identified in developing septa; by this stage α-SMA-positive cell numbers were double those of desmin-positive cell numbers. Similar observations have been made after chronic carbon tetrachloride-induced injury, although no quantitative analysis was reported in these studies.[18,19] These findings suggest that in chronic liver injury PSCs may undergo a series of phenotypic changes from "quiescent" cells (desmin-positive/α-SMA-negative), through activated PSCs or "myofibroblast-like" cells (desmin-positive/α-SMA-positive) to myofibroblasts (desmin-negative/α-SMA-positive). It remains possible, however, that an alternative explanation for our findings is concomitant proliferation of an α-SMA-positive mesenchymal cell population that is present in the normal rat liver portal tracts. Further double-labeling studies are indicated to test this hypothesis.

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