

# Growth Factor and Procollagen Type I Gene Expression in Human Liver Disease

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**Background/Aims:** Growth factors have been implicated in the pathogenesis of liver fibrosis, a major determinant of the clinical course of chronic liver disease. The aim of this study was to study the relationship of growth factor expression to inflammation and fibrosis in a variety of human liver diseases. **Methods:** We studied by *in situ* hybridization the expression of transforming growth factor (TGF)  $\beta$ 1, platelet-derived growth factor (PDGF) A and PDGF-B, and procollagen type I (pro-I) messenger RNAs (mRNAs) in liver diseases of various etiologies. **Results:** Pro-I mRNA was expressed by mesenchymal cells at sites of inflammation and scarring, where TGF- $\beta$ 1 immunoreactivity was often found, and by perisinusoidal cells. TGF- $\beta$ 1 and PDGF-A mRNAs were expressed mainly by mononuclear cells and proliferating ductular cells. TGF- $\beta$ 1 mRNA was also expressed by perisinusoidal cells. PDGF-A gene expression was more common than that of PDGF-B. Pro-I and TGF- $\beta$ 1 expression correlated with both ductular proliferation and tissue inflammation, whereas PDGF-A and PDGF-B only correlated with ductular proliferation. **Conclusions:** Our data suggest that TGF- $\beta$ 1 and PDGF are involved in human liver inflammation and fibrosis. The expression of growth factor mRNAs in proliferating ductular cells may indicate a role for these cells in liver fibrogenesis and may help explain the pathophysiology of conditions such as biliary atresia progressing to fibrosis despite the absence of marked inflammation.

Liver fibrosis results from architectural remodeling and progressive deposition of extracellular matrix (ECM) components such as proteoglycans, fibronectin, and collagens.<sup>1</sup> Fibrosis is a major determinant of the clinical course of chronic liver disease. Active fibrogenesis is frequently preceded by and associated with inflammation. Accordingly, it is often proposed that fibrogenic growth factors and cytokines released by inflammatory cells promote the proliferation of fat-storing cells, which are considered the main cellular source of matrix proteins in the liver.<sup>2,3</sup> Recently, specific polypeptide growth factors, including members of the transforming growth fac-

tor (TGF) gene family such as TGF- $\beta$ 1 and TGF- $\beta$ 2 and platelet-derived growth factors (PDGFs), have been implicated in the pathophysiology of liver fibrosis.<sup>4,5</sup> Both families of growth factors are potent chemoattractants for inflammatory cells and fibroblasts and induce the synthesis and deposition of ECM proteins.<sup>6-13</sup> Several reports have suggested a role for these growth factors in fibrogenesis in different clinical conditions.<sup>14-21</sup> TGF- $\beta$ 1 has also been implicated in the pathogenesis of two major animal models of hepatic fibrosis, including carbon tetrachloride-induced rat liver fibrosis and murine schistosomiasis.<sup>22</sup> Moreover, increased expression of hepatic TGF- $\beta$ 1 messenger RNA (mRNA) is associated with elevated hepatic levels of procollagen type I (pro-I) mRNA<sup>14,15</sup> and with increases in serum levels of the aminoterminal propeptide of procollagen type III in patients with chronic liver disease.<sup>14</sup> TGF- $\beta$ 1 has also been shown to increase synthesis of procollagens and fibronectin in cultured human fat-storing cells.<sup>23</sup>

Elevated levels of PDGF-like molecules have been detected in the liver and ascites from patients with active alcoholic liver disease.<sup>24</sup> These are potentially important observations because several studies have shown a synergistic effect of TGF- $\beta$ 1 and PDGF in the induction of DNA synthesis, mesenchymal cell proliferation, and ECM deposition in animal models of wound repair.<sup>25-27</sup> Similar results have been reported in cultured rat liver fat-storing cells,<sup>28</sup> which, as noted, are thought to play a central role in the development of liver fibrosis. However, no studies of human liver disease to date have compared *in situ* TGF- $\beta$ 1 and PDGF gene expression with that of a presumptive target gene, namely that encoding pro-I, and with the presence of tissue inflammation. Accordingly, we evaluated the expression of pro-I, TGF-

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*Abbreviations used in this paper:* DPI, ductular proliferation index; ECM, extracellular matrix; HAI, histological activity index; HPF, high-power field; PDGF, platelet-derived growth factor; pro-I, procollagen type I; TGF, transforming growth factor.

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$\beta$ 1, PDGF-A, and PDGF-B genes in patients with end-stage liver diseases of various etiologies and varying degrees of inflammatory activity. We used *in situ* hybridization to determine the cellular distribution of type I collagen and growth factor mRNAs. This approach avoids the problems inherent in biochemical determinations that average mRNA or protein abundance and allows the detection of potentially important spatial relationships between cytokine production and collagen gene expression.

## Materials and Methods

We studied formalin-fixed, paraffin-embedded liver tissue from hepatectomy specimens obtained from 37 patients who underwent liver transplantation for end-stage liver disease of various etiology and type and from 2 normal donors. Patient characteristics are shown in Table 1. The histological diagnosis of cirrhosis was made according to internationally accepted criteria.<sup>29</sup> Histological activity was graded according to Scheuer<sup>30</sup> and by using the histological activity index (HAI) described by Knodell et al.<sup>31</sup> Ductular proliferation, defined as an increase in the number of ductular structures in portal tracts, fibrous septa, and periportal and perinodular areas was scored on a scale from 0 to 4 on the basis of semiquantitative assessment of relative areas involved in at least 10 portal tracts and fibrous septa and relative periportal/perinodular areas per sample. The scale used, termed the ductular proliferation index (DPI), was scored as 0, no ductular proliferation; 1, <25%; 2, 25%–50%; 3, 50%–75%; 4, >75%. This scoring system was used together with HAI for statistical analysis. Both DPI and HAI scores were calculated on semiserial sections of the same paraffin blocks from which slides were cut for *in situ* hybridization.

### In Situ Hybridization

*In situ* hybridization was performed according to Prosser et al.<sup>32</sup> with slight modifications. Briefly, sections were deparaffinized and pretreated with 1  $\mu$ g/mL proteinase K (Sigma, St. Louis, MO) for 20 minutes at 37°C and then acetylated, washed in 2  $\times$  SSC (0.3 mol/L NaCl in 0.03 mol/L sodium citrate), dehydrated in graded ethanol, and air dried. Hybridization was performed overnight at 55°C with 25  $\mu$ L of hybridization solution containing 300,000 cpm of  $^{35}$ S-labeled antisense or sense RNA probe. After several washes in SSC containing 25 mmol/L  $\beta$ -mercaptoethanol to minimize background, sections were briefly treated with ribonuclease A and, after further washes, dehydrated in graded ethanol and air dried. Slides were then processed for autoradiography using standard procedures, counterstained with H&E, and examined by light microscopy under a Leitz microscope equipped with bright and dark field. All sections used for analysis were hybridized with the same batches of probes. Comparison of slides hybridized with different preparations of the same probe showed a substantial agreement for all probes tested.

### Complementary RNA Probes for In Situ Hybridization

$\lambda$ BCI, a 1050 base pair complementary DNA (cDNA) corresponding to the precursor region of human TGF- $\beta$ 1<sup>33</sup>

subcloned in the *Eco*RI site of pSP65 (Promega, Madison, WI), was linearized with *Kpn*I so that only 325 base pairs were transcribed. A sense probe was transcribed from the same sequence subcloned in opposite orientation in Bluescript KS+ (Stratagene, La Jolla, CA). A 1314-base pair fragment of human PDGF-A cDNA,<sup>34</sup> subcloned in the *Eco*RI site of Bluescript KS+, was linearized with *Bam*H, allowing transcription of a 470-base pair fragment using T7 polymerase (antisense) and an 844-base pair fragment using T3 polymerase (sense). The pSP-sis 2 plasmid,<sup>35</sup> containing a 2144-base pair fragment of human PDGF-B (c-sis) cDNA, was linearized with *Bst*EII, which generated a 391-base pair fragment contained within exon 7. A 1072-base pair fragment containing the same sequence was subcloned in pSP64 (*Pst*I-*Bam*H) and linearized with *Bam*H to generate a sense probe that was used after controlled base hydrolysis. Finally, Hf677, a 1500-base pair fragment of human procollagen  $\alpha_1$ (I) cDNA,<sup>36</sup> subcloned into the *Eco*RI site of pSP65, was linearized with *Ava*I, allowing transcription of a 700-base pair fragment corresponding to the C-propeptide region. The same 700-base pair fragment was subcloned in pSP64 and linearized with *Eco*RI to generate the sense probe.

Controls for specificity consisted of pretreatment of slides with ribonuclease (20 mg/mL for 30 minutes) before hybridizing, hybridization of companion slides with sense RNA probes generated from the above cDNAs, and hybridization with anti-sense and sense probes transcribed with the appropriate polymerase from a 599-base pair cDNA for human fibronectin cDNA subcloned from the plasmid pFH-6<sup>37</sup> into Bluescript KS+.

### Immunohistochemistry and Identification of Cell Phenotype

Cell types were identified on the basis of morphology and immunohistochemistry. The analysis was performed on sections cut sequentially to those used for *in situ* hybridization to identify mRNA expressing cell types. We used the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, MA) with the following antibodies (all obtained from Dako): monoclonal anti-desmin, anti-vimentin, and anti-Factor VIII to detect endothelial cells. Macrophages were detected using Dako PGM1 anti-CD68, and bile ductular cells were detected with Dako anti-cytokeratin 19. A rabbit polyclonal antibody, designated CC, raised to a synthetic peptide representing the N-terminal 30 amino acids of the mature form of human TGF- $\beta$ 1 (Collagen Corporation, Palo Alto, CA), was used to localize extracellular TGF- $\beta$ 1 protein.<sup>38</sup> Preincubation of the CC antibody with the peptide antigen eliminated staining.

Proliferating ductules were characterized as typical or atypical on the basis of morphology and location.<sup>39</sup> Typical ductules were characterized by the presence of a well-defined lumen lined by biliary cells and by their location within the connective tissue of portal tracts or fibrous septa. Atypical ductules were identified on the basis of a poorly defined or absent lumen and by their characteristic position in the region between the connective tissue and the periportal parenchyma or at the periphery of regenerating nodules. In addition to typical biliary

**Table 1.** Clinical and Histological Characteristics and mRNA Scores of Cases Studied

| Case | Clinical diagnosis                      | Histology                                     | HAI | DPI | Pro-I<br>(G/D) | TGF- $\beta$<br>(G/D) | PDGF-A<br>(G/D) | PDGF-B<br>(G/D) |
|------|---|---|-----|-----|----------------|-----------------------|-----------------|-----------------|
| 1    | Biliary atresia                         | Cirrhosis, minimal activity                   | 4   | 4   | 3/4            | 1/3                   | 1/3             | 1/2             |
| 2    | Biliary atresia                         | Cirrhosis, minimal activity                   | 4   | 4   | 2/3            | 1/2                   | 1/3             | 0/0             |
| 3    | Biliary atresia                         | Cirrhosis, mild activity                      | 6   | 4   | 3/4            | 3/4                   | 3/4             | 1/2             |
| 4    | Biliary atresia                         | Cirrhosis, mild activity                      | 6   | 2   | 3/3            | 3/3                   | 3/3             | ND              |
| 5    | Biliary atresia                         | Cirrhosis, mild activity                      | 6   | 3   | 3/4            | 1/2                   | 2/4             | 1/2             |
| 6    | Primary biliary cirrhosis               | Stage IV, mild activity                       | 5   | 3   | 3/3            | 1/2                   | 2/4             | 1/2             |
| 7    | Primary biliary cirrhosis               | Stage I-II, moderate activity                 | 9   | 0   | 0/0            | 1/1                   | 1/1             | 0/0             |
| 8    | Primary biliary cirrhosis               | Stage IV, moderate activity                   | 9   | 3   | 3/3            | 2/3                   | 1/2             | 0/0             |
| 9    | Primary biliary cirrhosis               | Stage III, moderate activity                  | 14  | 2   | 1/1            | 1/3                   | 0/0             | 0/0             |
| 10   | Secondary biliary cirrhosis             | Cirrhosis, moderate activity                  | 13  | 3   | 2/2            | 1/1                   | 1/2             | 0/0             |
| 11   | Primary sclerosing cholangitis          | Cirrhosis, moderate activity                  | 9   | 2   | 3/3            | 1/2                   | 1/2             | 0/0             |
| 12   | Autoimmune CLD                          | Cirrhosis, mild activity                      | 6   | 2   | 2/1            | 1/1                   | 1/1             | 0/0             |
| 13   | Autoimmune CLD                          | Cirrhosis, mild activity                      | 6   | 2   | 0/0            | 1/1                   | 2/1             | 0/0             |
| 14   | Alcohol-related CLD                     | Cirrhosis, moderate activity                  | 13  | 2   | 1/1            | 1/0                   | 1/2             | 0/0             |
| 15   | Alcohol-related CLD                     | Cirrhosis, moderate activity                  | 9   | 3   | 0/0            | 2/3                   | 2/3             | 0/0             |
| 16   | Alcohol-related CLD                     | Cirrhosis, moderate activity                  | 11  | 4   | 2/1            | 2/3                   | 1/2             | ND              |
| 17   | HBV-related CLD                         | Cirrhosis, moderate activity                  | 9   | 2   | 2/1            | 1/1                   | 1/1             | 0/0             |
| 18   | HCV-related CLD                         | Cirrhosis, mild activity                      | 6   | 1   | 1/0            | 1/0                   | 1/1             | 0/0             |
| 19   | HCV-related CLD                         | Cirrhosis, mild activity                      | 7   | 2   | 2/1            | 2/2                   | 1/1             | 0/0             |
| 20   | HCV-related CLD                         | Cirrhosis, moderate activity                  | 13  | 3   | 1/1            | 2/4                   | 2/2             | 0/0             |
| 21   | HCV-related CLD                         | Cirrhosis, moderate activity                  | 13  | 2   | 2/1            | 1/1                   | 1/1             | 0/0             |
| 22   | HCV-related CLD                         | Cirrhosis, mild activity                      | 9   | 3   | 2/3            | 1/1                   | 1/1             | 0/0             |
| 23   | HCV-related CLD                         | Cirrhosis, severe activity                    | 14  | 2   | 1/1            | 1/0                   | 1/1             | 0/0             |
| 24   | Cryptogenic CLD                         | Cirrhosis, moderate activity                  | 10  | 2   | 2/1            | 3/4                   | 1/2             | 1/1             |
| 25   | Cryptogenic CLD                         | Cirrhosis, moderate activity                  | 11  | 4   | 2/3            | 1/3                   | 1/3             | 1/1             |
| 26   | Cryptogenic CLD                         | Cirrhosis, mild activity                      | 8   | 3   | 1/1            | 1/2                   | 2/3             | 1/2             |
| 27   | Granulomatous CLD                       | Cirrhosis, moderate activity                  | 9   | 4   | 2/3            | 2/4                   | 2/4             | 0/0             |
| 28   | Polycystic liver disease                | Cirrhosis, minimal activity                   | 4   | 1   | 1/1            | 1/1                   | 1/1             | 0/0             |
| 29   | Budd-Chiari syndrome                    | Fibrous portal expansion,<br>minimal activity | 3   | 1   | 1/0            | 1/1                   | 1/2             | 0/0             |
| 30   | HAV-related acute liver failure         | SMN, bridging fibrosis                        | 20  | 2   | 2/3            | 2/4                   | 1/3             | 0/0             |
| 31   | HCV-related acute liver failure,<br>CLD | SMN, cirrhosis                                | 18  | 4   | 2/3            | 1/4                   | 2/4             | 0/0             |
| 32   | HCV-related acute liver failure         | SMN, fibrous portal expansion                 | 18  | 4   | 2/4            | 2/4                   | 3/4             | ND              |
| 33   | HCV-related acute liver failure         | SMN, fibrous portal expansion                 | 16  | 4   | 3/4            | 3/4                   | 2/4             | 1/4             |
| 34   | HCV-related acute liver failure         | SMN, fibrous portal expansion                 | 18  | 3   | 3/4            | 1/4                   | 1/4             | 0/0             |
| 35   | HBV-related acute liver failure         | SMN, bridging fibrosis                        | 20  | 3   | 2/4            | 2/4                   | 2/4             | 0/0             |
| 36   | Undetermined acute liver failure        | SMN, fibrous portal expansion                 | 19  | 3   | 2/3            | 3/4                   | 1/4             | 2/3             |
| 37   | Undetermined acute liver failure        | SMN, bridging fibrosis                        | 20  | 4   | 2/3            | 1/4                   | 1/1             | 0/0             |
| 38   | Liver donor                             | Steatosis, no inflammation                    | 0   | 1   | 1/0            | 0/0                   | 0/0             | 0/0             |
| 39   | Liver donor                             | Normal  | 0   | 0   | 1/0            | 0/0                   | 0/0             | 0/0             |

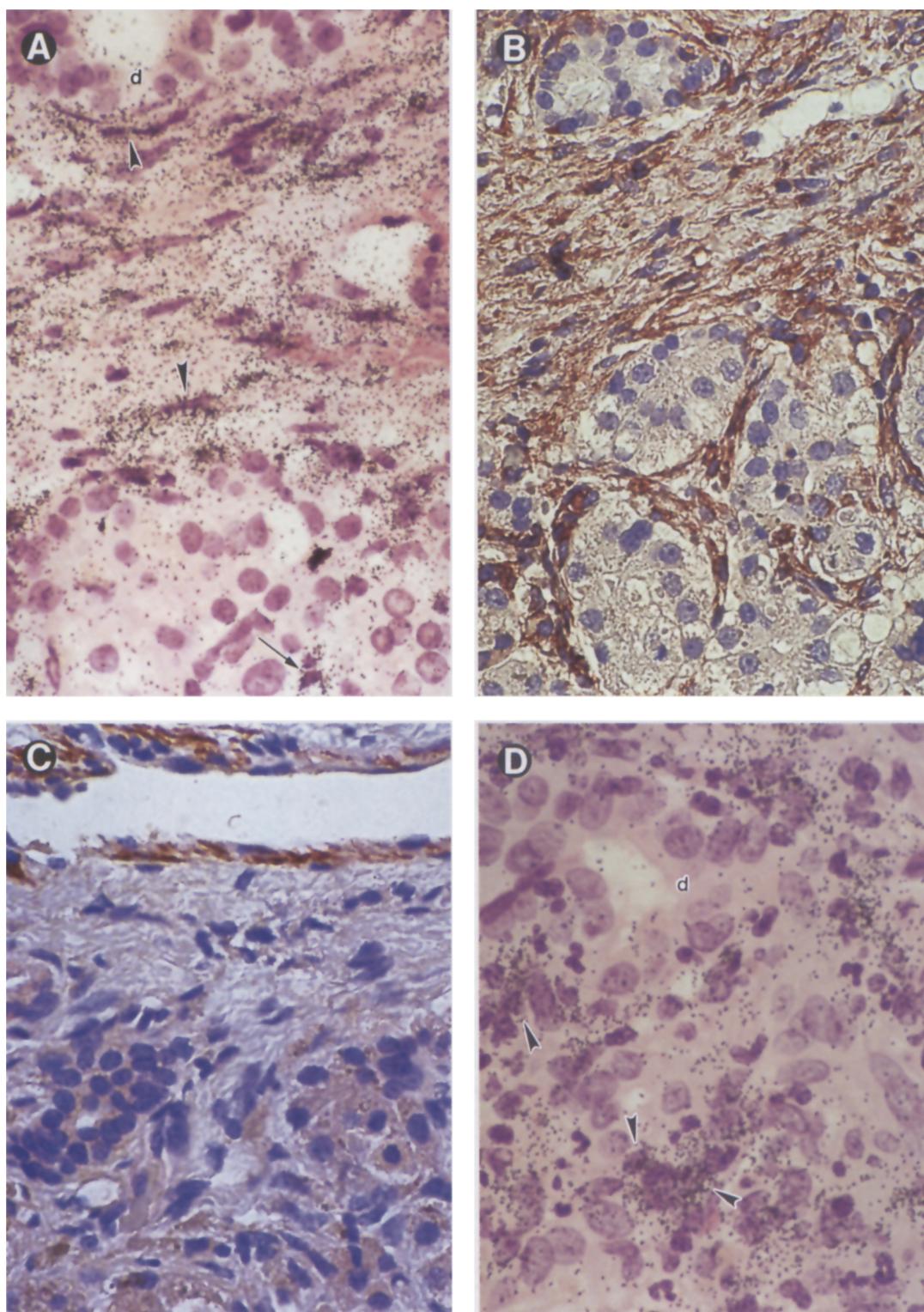
CLD, chronic liver disease; D, score of the mean tissue density of mRNA expressing cells; G, score of the mean number of silver grains per cell; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; SMN, submassive necrosis.

cells, the atypical ductules also have cells with cytological features intermediate between bile duct cells and true hepatocytes. These cells show strong positivity for cytokeratin 19 and were termed "ductular hepatocytes" by Gerber et al.<sup>40</sup> and Vandersteenoven et al.<sup>41</sup> In this report, we collectively refer to atypical ductules.

#### Semiquantitative Assessment of mRNA Abundance by In Situ Analysis

Standard exposure times for all cases included for analysis were 2 weeks for sections hybridized with probes for pro-I and 8 weeks for those hybridized with TGF- $\beta$ 1, PDGF-A, and PDGF-B probes. The same exposure times were used for the respective sense probes. Assessment of relative mRNA

abundance was performed by 2 observers on at least 20 high-power (40 $\times$  objective) fields (HPFs) randomly chosen from each section to avoid selection bias. Cells were considered specifically labeled when they showed more than 5 silver grains above background. The relative abundance of silver grains for each probe was scored on a scale from 0 to 3 using the average number of grains per cell (0, 0-5; 1, 6-15; 2, 16-25; 3,  $\geq 25$ ) counted on at least 20 random HPFs. The mean tissue density of positive cells was assessed on the same 20 HPFs and scored using a range from 0 to 4 (0,  $\leq 2$  cells per HPF; 1, 2-15 cells; 2, 16-30 cells; 3, 31-45 cells; 4,  $\geq 45$  cells). Sections were considered negative when no clearly detectable signal above background was found in the parenchyma or when the mean tissue density of positive cells was below 2 cells per



**Figure 1.** Biliary atresia and cirrhosis with minimal activity. (A) In situ hybridization with antisense probe to pro-*I* shows high level message in spindle-shaped fibroblastlike cells (arrowheads) in the fibrous tissue around a regenerative hepatocyte nodule and some proliferated bile ductules (d). Perisinusoidal cells are also strongly positive (arrow). The proliferated bile ductular cells and the hepatocytes show only some low background signal. (B) Vimentin-positive and (C) desmin-negative. Smooth muscle cells in the vessel wall are desmin-positive, as expected. (D) Hepatitis C virus-related submassive necrosis. In situ hybridization with antisense probe to pro-*I* showing high level message in medium to large, irregular rounded cells (arrowheads) around a bile duct (d). There is considerable inflammatory infiltrate present as well (original magnification 500 $\times$ ).

HPF. These 2 scores were used for analysis and comparison between different mRNAs or with HAI and DPI.

### Interobserver and Intraobserver Variation

All numeric scoring was performed by the same two observers (G.M. and A.R.). Interobserver and intraobserver variation were assessed for both histological indexes (HAI and DPI) and for mRNA abundance (mean tissue density of positive cells and mean number of grains per cell) readings. For all features tested, the interobserver and intraobserver agreement was  $\geq 90\%$ .

### Statistical Analysis

The results were analyzed by the Spearman's rank correlation test.

## Results

### Normal Liver

In normal liver, pro-I mRNA was found in some fibroblasts in the hepatic capsule, in occasional endothelial and spindle-shaped cells (presumably fibroblasts) around terminal hepatic venules, and, rarely, in perisinusoidal cells. The abundance of pro-I mRNA did not exceed an average of 15 silver grains per cell, and the mean density of positive cells was below 2 per 20 HPFs (see Table 1). TGF- $\beta$ 1 mRNA was virtually absent from both normal samples examined with the exception of a low level in rare subcapsular cells in one specimen. There was no signal above background for PDGF-A and PDGF-B under standard exposure conditions of 8 weeks.

### End-Stage Liver Disease

**Pro-I mRNA expression.** Pro-I mRNA was mainly expressed by vimentin-positive mesenchymal cells that varied in morphology from spindle-shaped to rounded. In cirrhosis, pro-I mRNA was expressed primarily by spindle-shaped mesenchymal cells resembling fibroblasts located in fibrous septa and in areas of periductular fibrosis (Figure 1). The distribution of pro-I mRNA expressing cells in cirrhotic septa ranged from diffuse throughout fibrous scars to restricted to marginal cells adjacent to regenerating nodules. Pro-I mRNA was also expressed by perisinusoidal cells that seemed to be mostly vimentin-positive and desmin-negative (Figure 1A-C). In a smaller number of cases characterized by high levels and diffuse expression of pro-I mRNA, there was also expression in the endothelial and smooth muscle cells of the vascular structures found in the scar tissue (Table 2). Very high levels of pro-I mRNA were observed in submassive necrosis, where pro-I mRNA transcripts were found in medium- to large-sized cells with an irregular to rounded shape that were distributed throughout the

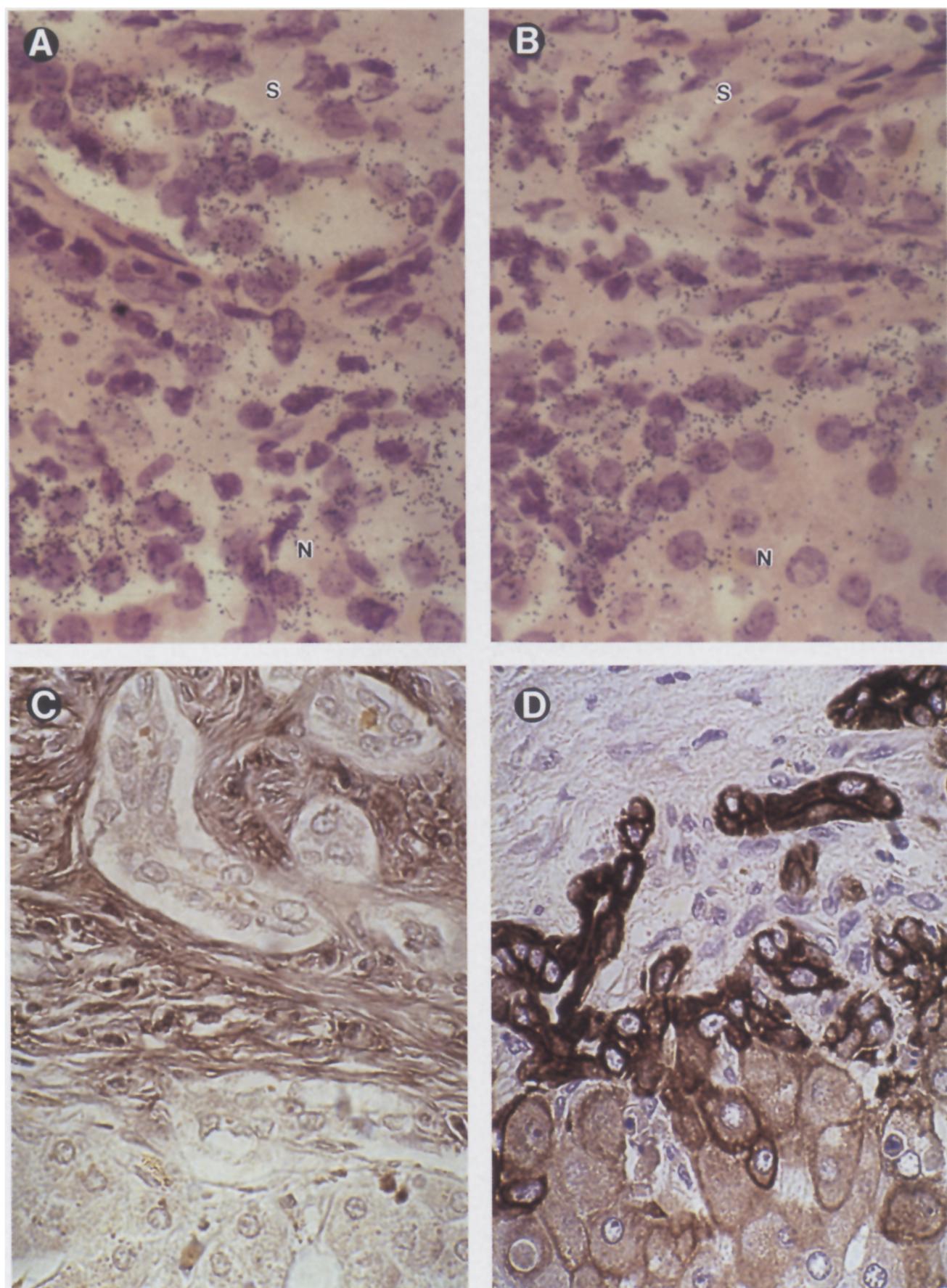
**Table 2.** Liver Cell Types Expressing Pro-I and Growth Factor mRNAs With All Patients Combined

| Cell type            | Probes <sup>a</sup> |                |        |        |
|----------------------|---------------------|----------------|--------|--------|
|                      | Pro-I               | TGF- $\beta$ 1 | PDGF-A | PDGF-B |
| Hepatocytes          | 0/39                | 0/39           | 0/39   | 0/36   |
| Atypical ductules    | 0/39                | 24/39          | 30/39  | 0/36   |
| Typical ductules     | 0/39                | 13/39          | 22/39  | 5/36   |
| Bile ducts           | 0/39                | 1/39           | 5/39   | 0/36   |
| Mononuclear          | 0/39                | 27/39          | 19/39  | 1/36   |
| Smooth muscle        | 4/39                | 1/39           | 10/39  | 1/36   |
| Vascular endothelial | 7/39                | 5/39           | 20/39  | 6/36   |
| Perisinusoidal       | 16/39               | 12/39          | 0/39   | 0/36   |
| Fibroblastlike       | 33/39               | 0/39           | 2/39   | 0/36   |

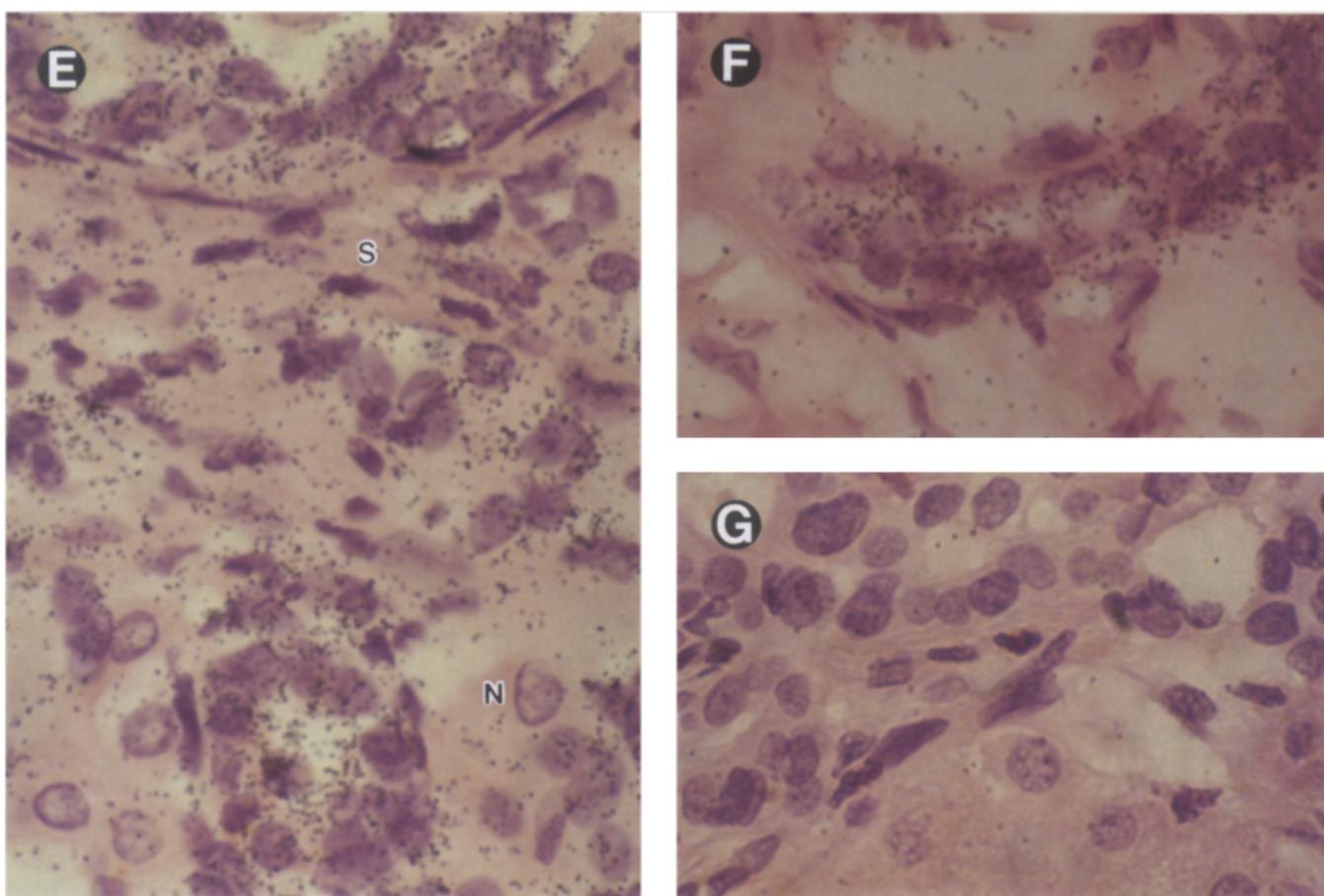
<sup>a</sup>Number of specimens showing at least 2 cells per HPF with at least 6–15 grains per cell/total number of specimens examined.

parenchyma and in areas where severe inflammation was present (Figure 1D). No signal above background was found in hepatocytes, bile duct epithelial cells, ductular hepatocytes, or inflammatory cells (Table 2).

**TGF- $\beta$ 1 mRNA expression.** TGF- $\beta$ 1 mRNA was mainly present in areas of ductular reaction and at sites of inflammatory infiltration and fibrous scarring. Expression of TGF- $\beta$ 1 mRNA was particularly frequent in cytokeratin-19-positive typical (13 of 37 cases) and atypical proliferated ductules (25 of 37 cases), whereas interlobular and septal bile ducts were mostly negative. The levels of TGF- $\beta$ 1 mRNA expression by ductular structures was especially high in cases of biliary atresia in which ductular reaction was prominent and the inflammatory infiltration very mild (Figure 2). Otherwise, mononuclear cells were the most important source of TGF- $\beta$ 1 mRNA both in cirrhosis and submassive necrosis. Although TGF- $\beta$ 1 message was found in some lymphocytes of the portal and periportal infiltrate of active cirrhosis, the main "inflammatory" cells expressing TGF- $\beta$ 1 mRNA were mononuclear phagocytes that appeared to be PGM1 CD68-positive in sequential sections and were particularly numerous in cases of submassive necrosis (Figure 3). TGF- $\beta$ 1 mRNA was also unequivocally expressed by perisinusoidal cells in about one third of cases (12 of 39). Moreover, in submassive necrosis, some of the irregular, rounded, large, vimentin-positive mesenchymal cells with a high level of pro-I mRNA expression may also express TGF- $\beta$ 1 mRNA. However, TGF- $\beta$ 1 mRNA was never found in the typical spindle-shaped fibroblastlike cells present in the cirrhotic fibrous tissue or in true hepatocytes. Finally, TGF- $\beta$ 1 mRNA was rarely found in vascular endothelial and smooth muscle cells. Cells expressing TGF- $\beta$ 1 mRNA were often closely associated with pro-I expressing mesenchymal cells both in cirrhosis and submassive necrosis. This was particu-



**Figure 2.** See legend on next page.



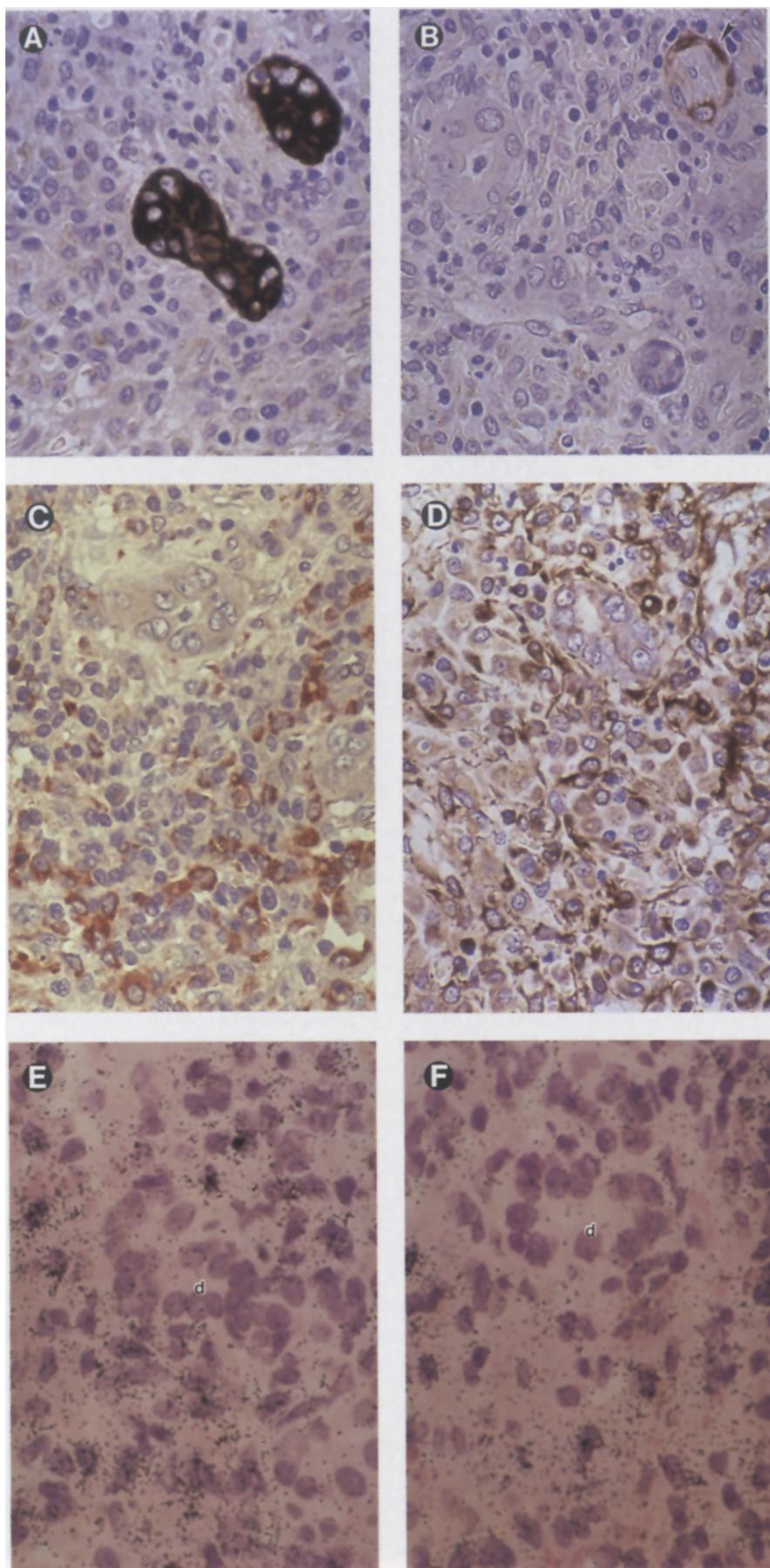
**Figure 2.** Biliary atresia and cirrhosis with minimal activity. *In situ* hybridization with antisense probe to TGF- $\beta$ 1 shows high levels of mRNA expression in the scar tissue (S) surrounding the regenerative nodule (N). (A and B) Signal is mainly found in typical and atypical proliferated bile ductules at the interface of the regenerative nodule and the scar. Immunohistochemistry for TGF- $\beta$ 1 with the CC antibody shows that TGF- $\beta$ 1 protein is localized in the extracellular matrix in the scar tissue. (C) The epithelial cells involved in the ductular reaction and the parenchymal hepatocytes do not stain for TGF- $\beta$ 1. Anti-cytokeratin 19 immunostaining in an area of ductular reaction shows typical bile ductules in the fibrous septum and atypical ductular cells at the periphery of a regenerating nodule. (D) Some marginal hepatocytes are strongly expressing cytokeratin 19. (E) PDGF-A mRNA is found in the epithelial cells involved in the ductular reaction at the periphery of the regenerative nodule (N) and in the fibrous scar (S). (F) PDGF-B mRNA, although not as intense as PDGF-A, is also found in the epithelial cells of a typical proliferated bile ductule within the scar tissue surrounding a regenerative nodule. (G) *In situ* hybridization with a sense probe to TGF- $\beta$ 1 shows no signal above background on a sequential section of the same sample (original magnification 500 $\times$ ).

larly evident in areas of ductular proliferation, where adjacent mesenchymal cells contained abundant pro-I mRNA.

**PDGF-A and PDGF-B mRNA expression.** The highest signal for PDGF-A mRNA was found in both typical and atypical proliferated ductules present at the periphery of cirrhotic nodules and in areas of extensive ductular reaction (Figure 2E). PDGF-A mRNA was also highly expressed in inflammatory cells. This was particularly evident in submassive necrosis, where the signal seemed to be mainly localized in cells that in sequential sections could be identified as PGM1 CD68-positive mononuclear phagocytes (Figure 3F). As observed for TGF- $\beta$ 1, there were some irregularly shaped, vimentin-positive mesenchymal cells that seemed to express PDGF-A mRNA. PDGF-A mRNA was expressed by

vascular endothelial and smooth muscle cells and occasionally by biliary cells in interlobular and septal bile ducts. PDGF-A transcripts were also occasionally found in spindle-shaped fibroblastlike cells in the fibrous scars but were never found in perisinusoidal cells (Table 2). PDGF-A mRNA expressing cells were present in specimens with all degrees of inflammation and often in the same areas in which pro-I and TGF- $\beta$ 1 positive cells were present (Table 1). PDGF-B mRNA was detected in only 9 of 36 cases examined (Tables 1 and 2). PDGF-B mRNA was restricted to atypical and typical proliferated bile ductules where expression was particularly high (Figure 2F) and occasionally in vascular endothelial cells.

**Semiquantitative analysis of mRNA expression.** The mean tissue density of pro-I mRNA expressing cells correlated with both the HAI of histological



**Table 3.** Spearman's Rank Correlation of Mean Tissue Density of mRNA Expressing Cells With Histological Activity and Ductular Proliferation

| Probe              | HAI   |        | DPI   |         |
|--------------------|-------|--------|-------|---------|
|                    | $r_s$ | P      | $r_s$ | P       |
| Pro- $\alpha_1(I)$ | 0.35  | <0.003 | 0.71  | <0.0001 |
| TGF- $\beta 1$     | 0.55  | <0.001 | 0.67  | <0.0001 |
| PDGF-A             | 0.27  | NS     | 0.68  | <0.0001 |
| PDGF-B             | -0.05 | NS     | 0.42  | <0.05   |

activity and the DPI score of ductular proliferation (Table 3) and with the tissue density of TGF- $\beta 1$  and PDGF-A mRNA expressing cells (Table 4). Moreover, there was a correlation between pro-I mRNA expression and the subindex of periportal inflammation ( $r_s = 0.33$ ;  $P < 0.05$ ; Spearman's rank correlation test). On the other hand, TGF- $\beta 1$  mRNA was correlated with both HAI (and with the subindexes of periportal inflammation and lobular necrosis) and DPI, whereas PDGF-A mRNA expressing cells seemed to be related only with DPI (Table 3). Mean tissue density of TGF- $\beta 1$  mRNA expressing cells was also related to that of PDGF-A mRNA (Table 4). Finally, the tissue density of PDGF-B mRNA expressing cells was correlated with the DPI score of ductular proliferation (Table 3) and with the tissue density of pro-I, TGF- $\beta 1$ , and PDGF-A mRNA (Table 4). When the mean number of grains per cells was considered, pro-I, PDGF-A, and PDGF-B mRNA scores correlated only with DPI ( $r_s = 0.51$ , 0.48, and 0.42;  $P < 0.005$ ,  $P < 0.005$ , and  $P < 0.02$ , respectively), whereas TGF- $\beta 1$  mRNA correlated with both HAI and DPI ( $r_s = 0.36$ ;  $P < 0.02$ ).

### Immunohistochemistry

Immunoreactivity with the anti-desmin monoclonal antibody was almost exclusively found in the smooth muscle cells of the vessel walls. Perisinusoidal lining cells and fibroblastlike mesenchymal cells, that likely include fat-storing cells, were desmin-negative using this monoclonal antibody and vimentin-positive. However, the fibrous septa of some cases of biliary atresia occasionally showed fibrillar staining for desmin. Staining for TGF- $\beta 1$  was associated with the extracellular matrix in fibrous septa and in areas of periductular fibro-

**Table 4.** Spearman's Rank Correlation Between the Mean Tissue Density mRNA Expressing Cells for the Four mRNAs Studied

|                    | TGF- $\beta 1$                 | PDGF-A                         | PDGF-B                       |
|--------------------|--------------------------------|--------------------------------|------------------------------|
| Pro- $\alpha_1(I)$ | $r_s = 0.65$ ;<br>$P < 0.0001$ | $r_s = 0.74$ ;<br>$P < 0.0001$ | $r_s = 0.46$ ;<br>$P < 0.01$ |
| TGF- $\beta 1$     | —                              | $r_s = 0.68$ ;<br>$P < 0.0001$ | $r_s = 0.36$ ;<br>$P < 0.05$ |
| PDGF-A             | —                              | —                              | $r_s = 0.5$ ;<br>$P < 0.001$ |

sis in cases with high expression of TGF- $\beta 1$  and pro-I mRNA (Figure 2). There was no TGF- $\beta 1$  protein detected with the CC antibody in normal liver tissue.

### Discussion

Our results show that the expression of TGF- $\beta 1$  mRNA and protein and PDGF-A mRNA are increased in human end-stage liver disease. There was a highly significant correlation between the tissue density of TGF- $\beta 1$  mRNA expressing cells, inflammation, and ductular proliferation, whereas PDGF-A was more clearly related only to ductular proliferation. These data confirm and extend previous data on the correlation between TGF- $\beta 1$ , fibrogenesis, and inflammatory activity in chronic liver disease.<sup>14</sup> The expression of TGF- $\beta 1$  mRNA in the liver correlated significantly with that of pro-I, a definitive indicator of fibrogenic activity.<sup>14</sup> Indeed, there were only two cases (no. 14 and 23; Table 1) with pro-I expression, albeit low, that showed minimal TGF- $\beta 1$  expression. On the other hand, there were four cases (no. 7, 13, 15, and 29; Table 1) with hepatic TGF- $\beta 1$  expression without increased pro-I mRNA expression. Two of these cases (no. 7 and 29) had essentially no fibrosis apart from some expansion of the portal tracts. The presence of TGF- $\beta 1$  mRNA without active pro-I mRNA expression in rare cases is not surprising given the complex posttranscriptional regulation of TGF- $\beta$  processing and biological activity. TGF- $\beta 1$  mRNA was highly expressed by mononuclear cells and ductular cells in cases with marked inflammation as found in some cases of active cirrhosis or submassive necrosis. However, there was a dramatic increase in expression of TGF- $\beta 1$  mRNA in some cases with only minimal inflammation. This was particularly evident in biliary atresia in which TGF- $\beta 1$  mRNA ex-

**Figure 3.** Hepatitis C virus-related submassive necrosis. (A) The remnant of a portal tract shows the presence of some bile ducts that are strongly positive for the anti-cytokeratin 19 monoclonal antibody. (B) Desmin immunoreactivity is found only in some subendothelial smooth muscle cells (arrowhead) in the wall of a small portal artery. There are numerous mononuclear phagocytes strongly positive for the (C) PGM1 anti-CD68 monoclonal antibody, (D) some of which are vimentin-positive. Many of these cells seem to express mRNA for (E) TGF- $\beta 1$  and (F) PDGF-A, whereas bile ducts (d) show no signal above background (original magnification: A, B, C, and D, 500 $\times$ ; E and F, 625 $\times$ ).

pression was virtually restricted to typical and atypical proliferated ductules. Milani et al.<sup>5</sup> showed that biliary cells mainly express TGF- $\beta$ 2. However, they also reported low TGF- $\beta$ 1 expression in hepatocytes near regenerating nodules and in bile duct epithelial cells. It was not specified whether the bile duct epithelial cells were present in proliferated ductules or in interlobular bile ducts. In our study, the very high expression of TGF- $\beta$ 1 mRNA by the proliferated ductular cells, including the so-called ductular hepatocytes, but not by the interlobular bile ducts seemed to be directly correlated to the degree of ductular reaction. On the other hand, the expression of TGF- $\beta$ 1 and pro-I mRNA in the perisinusoidal cells in about one third of the cases studied further supports the evidence for a role of fat-storing cells in liver fibrogenesis. Interestingly, the cells with the highest expression of pro-I mRNA (i.e., mesenchymal fibroblastlike cells and perisinusoidal cells) did not express desmin, at least as identified by the monoclonal anti-desmin antibody, but only vimentin intermediate filaments. However, it should be noted that specific staining of human fat-storing cells has been reported using a polyclonal anti-desmin antibody.<sup>42</sup> Moreover, the morphology of fibroblastlike cells varied from spindle-shaped to irregularly rounded with the latter morphological type being much more frequent in cases of submassive necrosis.

The apparent absence of TGF- $\beta$ 1 mRNA in clearly identifiable fibroblasts differs from the results found in carbon tetrachloride-induced hepatic fibrosis in the rat.<sup>43</sup> This may result from the very advanced stage of disease in the human cases in our study in contrast to the acute animal model study. However, in human idiopathic pulmonary fibrosis, there was abundant TGF- $\beta$ 1 mRNA in the macrophages adjacent to fibrotic foci with less abundant TGF- $\beta$ 1 mRNA in the fibroblastic foci. In the fibrotic lesions, immunohistochemical staining for TGF- $\beta$ 1 protein using the CC antibody was very intense and showed a distinctive fibrillar pattern.<sup>38</sup> Similarly, in our study, the localization of TGF- $\beta$ 1 protein in the extracellular matrix in fibrous septa and in areas of periductular fibrosis found in cases with high TGF- $\beta$ 1 and pro-I mRNA levels suggests that TGF- $\beta$ 1 might be involved in the synthesis and accumulation of ECM proteins. The hypothesis that TGF- $\beta$ 1 may play a role in regulating connective tissue gene expression in liver fibrosis is further supported by the increased expression of fibronectin as well as  $\alpha$ 5 and  $\beta$ 1 integrin genes (unpublished data, Malizia et al., 1994) whose expression is stimulated by TGF- $\beta$ 1 in several cell lines.<sup>8,44,45</sup> The lower sensitivity of the in situ hybridization technique on paraffin sections may account for the low expression of TGF- $\beta$ 1 mRNA

we found in normal liver, differing from other studies in which Northern blot analysis<sup>14,15,22</sup> or in situ hybridization on frozen sections<sup>5,38</sup> were used. The specificity of the in situ hybridization technique we used seems high because none of the sense probes yielded signal greater than background.

Our data also provide convincing evidence for PDGF gene activation in human liver disease. In many cases, cells expressing PDGF were closely associated with cells expressing pro-I and TGF- $\beta$ 1 mRNA (Table 1). The main cellular sources of PDGF mRNA in human liver disease were the proliferated ductular cells, vascular endothelial cells, and mononuclear cells. One model commonly proposed in liver fibrosis implicates the release of PDGF by activated macrophages, including Kupffer's cells. The PDGF binds to its receptors and stimulates fat-storing cell proliferation and consequently ECM protein deposition.<sup>3,28</sup> This effect may be potentiated by TGF- $\beta$ 1, which increases the transcriptional rate of both A and B chains of PDGF (at least in endothelial cells)<sup>46</sup> and regulates the expression of PDGF receptor A and B subunits.<sup>47</sup> All possible dimeric combinations of PDGF chains (PDGF-AB, PDGF-BB, and PDGF-AA) have been identified in normal and transformed cells (reviewed in Raines et al.<sup>48</sup>). However, in our cases, there was an apparent higher frequency of PDGF-A transcripts as compared with PDGF-B. This might suggest that the PDGF secreted in these livers might be mainly the A-A homodimeric form. These findings, in the absence of PDGF-AA protein immunostaining, must be interpreted with caution because a number of studies have implicated the B chain of PDGF in fibroproliferative disorders.<sup>19,49</sup>

If future studies verify increased expression of PDGF A-A homodimers, there may be pathophysiological consequences. PDGF A-A seems to be less mitogenic than B-B dimers for certain cell lines<sup>50</sup> and for cultured rat fat-storing cells.<sup>51</sup> However, the mitogenic effect of PDGF isoforms will vary among cell types depending on their ability to bind the different isoforms rather than on intrinsically different mitogenic potential.<sup>48,52</sup> Studies on aortic smooth muscle cells have shown that PDGF-AA expression is induced by TGF- $\beta$ 1. The PDGF-AA stimulates mitogenesis through this pathway of autocrine stimulation.<sup>53</sup> Interestingly, recent evidence suggests that TGF- $\beta$ 1 increases PDGF-A mRNA expression and PDGF-AA protein secretion by human fat-storing cells, which also seem to express the cognate A-type receptor for PDGF.<sup>54</sup> In this context, it might be inferred that the release of PDGF A-A homodimers induced by TGF- $\beta$ 1 may play an important role in the processes of mesenchymal cell proliferation and extracellular matrix protein deposition leading to liver cirrhosis. In situ analysis of

the distribution and mechanisms of regulation of PDGF receptors are required to further clarify the role of different PDGF isoforms in human liver fibrogenesis.

Finally, the observation that atypical proliferated ductular cells, including the so-called ductular hepatocytes and, to a lesser extent, typical proliferated ductular cells, express high levels of both TGF- $\beta$ 1 and PDGF mRNA suggests that ductular proliferation may also have a role in organizing and promoting inflammatory and fibrotic reactions as previously postulated by Popper.<sup>55</sup> In light of our findings, ductular cells, by virtue of their ability to express growth factors, might be directly implicated in neutrophil infiltration and periductular fibrosis generally associated with ductular proliferation. Moreover, it is tempting to speculate that the presence of growth factors in these cells may also sustain ductular proliferation through an autocrine pathway when hepatocyte replication is inhibited, as suggested for oval cell proliferation.<sup>40,56,57</sup>

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