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Rapid Communication

Transforming Growth Factors β1 and β2 Are Differentially Expressed in Fibrotic Liver Disease

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Transforming growth factor (TGF) β1 has been implicated in the control of hepatocyte growth and stimulation of extracellular matrix synthesis in acute and chronic liver disease. The cellular localization of transforming growth factor (TGF) β1 and β2 RNA transcripts was determined in normal and fibrotic liver by* in situ *hybridization with [^{35}S]-labeled RNA probes in combination with immunostaining for cell type characteristic markers. Fibrotic specimens were from patients with hepatitis B virus infection or alcohol abuse and rats with fibrosis secondary to bile duct ligation and scission. In normal liver, low levels of TGFβ1 transcripts were found in some portal tract stromal cells, and TGFβ2 RNA was not detectable. In fibrotic liver, high TGFβ1 RNA levels were present in most mesenchymal liver cells, in most inflammatory cells, and in few bile duct epithelial cells. Hepatocytes did not express this cytokine with the exception of few limiting plate hepatocytes in cases of human cirrhosis with high activity. TGFβ2 transcripts were detected at high levels in proliferating bile ducts of fibrotic livers, but were absent in all other cell types. TGFβ1 expression in the liver is thus a function predominantly of mononuclear and mesenchymal cells as well as of some hepatocytes, whereas TGFβ2 expression is a specific property of bile duct epithelial cells that may be related to the formation of specialized periductular connective tissue during bile duct proliferation. (Am J Pathol 1991, 139:1221–1229)

Transforming growth factors (TGF) β1 and β2 are multifunctional cytokines with positive or negative influences on cell growth and differentiation in a broad spectrum of tissues.[1,2] TGFβ1 is a strong inhibitor of proliferation of, among others, bronchial epithelial cells, keratinocytes, hepatocytes, endothelial cells, and megakaryocytes.[1–6] TGFβ1 promotes differentiation of keratinocytes and is upregulated during differentiation of stem cells.[2,7] Stimulating influences are seen on the mesenchyme that result in extracellular matrix synthesis and deposition.[8,9] TGFβ1 upregulates the expression of laminin, procollagen type I, fibronectin, tissue inhibitor of metalloproteases (TIMP) and integrins on the one side[8–11] and downregulates the expression of transin/stromelysin and collagenase on the other.[12–14] The activation of procollagen type I gene transcription by TGFβ1 is mediated by a nuclear factor 1 binding site in the procollagen promoter region.[15] *In vivo*, the injection of TGFβ1 results in a local fibrotic response and acclerated wound healing.[16]

In the liver, TGFβ1 has been implicated in the induction of synthesis and accumulation of extracellular matrix, which may eventually result in hepatic fibrosis.[17–20] Stimulation of rat hepatic lipocytes with TGFβ1 *in vitro* increases mRNA levels of various connective tissue proteins and the synthesis of proteoglycans by these cells.[21–24] These cells are considered to represent the principal source of collagen in the liver.[25–30] Lipocytes are not only sensitive to the stimulating effects of TGFβ1, but may additionally synthesize this cytokine, thus pointing to stimulation of fibrogenesis by means of paracrine and autocrine regulatory loops.[22,31] *In vivo*, elevated levels of TGFβ1 mRNA were found in liver extracts from CCl_4-intoxicated rats, in mice with schistosomal liver fibrosis, and in fibrotic human livers.[32–34] Analysis of

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RNA from isolated liver cells and *in situ* hybridization on rat livers with CCl_4-induced fibrosis indicated that TGFβ1 expression is predominantly a function of mesenchymal cells rather than of hepatocytes.[17,29,32] However, TGFβ1 immunoreactivity has been described in hepatocytes and bile duct epithelial cells of normal mice[7] and of rats with chronic CCl_4 intoxication,[35] raising the question as to whether this observation might be related to cellular uptake of TGFβ1 rather than to *de novo* synthesis in these cell types. The cellular distribution of TGFβ1 RNA transcripts in human fibrotic liver disease and in animal models of biliary fibrosis is still not characterized. Also, no data are available yet regarding the expression of other members of the TGFβ gene family in the liver. Here we report on the cellular localization of TGFβ1 and TGFβ2 RNA transcripts in human liver with fibrosis secondary to hepatitis B virus (HBV) infection and toxic damage due to alcohol abuse as well as in rat liver after common bile duct ligation and scission.

Materials and Methods

Animals

Female Wistar rats (200–250 g, Zentralinstitut für Versuchstiere, Hannover, FRG) were treated by ligation and subsequent scission of the common bile duct.[27,36] Animals were sacrificed 3 and 5 weeks after surgery. Untreated rats served as controls. Animal experiments were conducted in accordance with the state laws governing the use of experimental animals.

Human Liver Biopsies

Wedge liver biopsies were submitted for diagnostic procedures and informed consent was obtained before surgery. Results from two biopsies with normal histologic appearance were from patients undergoing noncomplicated cholecystectomy. One cirrhotic section with minimal inflammatory activity was obtained during portocaval shunt surgery from a 65-year-old female with a history of alcohol abuse. Nine specimens were the explanted livers from nine male patients (median age, 62 years) with hepatitis B viral liver disease obtained during liver transplantation. Histology revealed cirrhosis with minimal-to-moderate inflammation (5 cases) or severe activity (4 cases). Tissue aliquots were snapfrozen and stored in liquid nitrogen. Cryostat sections (4 μm) were airdried on a hot plate at 80°C for a few minutes, and fixed in 4% paraformaldehyde/PBS (phosphate-buffered saline) for 20 minutes.

Immunohistology

Monoclonal antibodies specific for vimentin (V9, DAKOPATTS, Glostrup, Denmark) and cytokeratin 7 (Boehringer Mannheim, Mannheim, FRG) as a marker for bile duct epithelial cells[37,38] were diluted to working concentration with dilution buffer (see below) and applied to sections for up to 30 minutes at 20°C. Optimal antibody concentrations and minimal incubation periods had been determined in preceding experiments. The dilution buffer for all immunohistologic reagents (pH 7.5 at 20°C), freshly made up with diethylpyrocarbonate (DEPC)-treated water before use, consisted of 10× RPMI 1640 concentrate (Gibco-BRL, Karlsruhe, FRG), 10 mg/ml bovine serum albumin (fraction V; Sigma, Deisenhofen, FRG), 5000 IU/ml heparin sodium salt (Sigma), 0.6 mg/ml yeast tRNA (Gibco-BRL).[39] After incubation with the primary antibody, slides were rinsed in TRIS-buffered saline (TBS), pH 7.5, and incubated further 30 minutes with affinity-purified rabbit anti-mouse immunoglobulin antibodies (DAKOPATTS), diluted 1:20 in the aforementioned buffer containing 10% normal rat or human serum for rat or human tissues, respectively. Sections were then rinsed again in TBS and incubated with APAAP-complex (DAKOPATTS) in 1:20 dilution for 30 minutes before development with new fuchsin substrate.[40] After a final washing step in TBS, a control slide was counterstained and examined before subjecting the batch to the *in situ* hybridization procedure.

Probes

For the preparation of single-stranded RNA probes, the 0.55 kb SmaI fragment of a human TGFβ1 cDNA,[17] provided by Dr. N. Fausto, Brown University, Rhode Island, USA, and the 0.75 kb HindIII-EcoRI fragment of the human TGFβ2 cDNA as present in the plasmid pBS2/sup40-1,[41] donated by Dr. de Martin, Sandoz Ltd., Basel, Switzerland, were subcloned into the vector pGEM1 (Promega Biotec, Madison, WI, USA) at the appropriate restriction sites. The crosshybridization of the human TGFβ cDNA clones to the corresponding rat gene sequences has been established.[2,17,41,42] After linearization of the plasmids with EcoRI or HindIII restriction endonucleases singe-stranded RNA probes complementary (anti-sense probe) or anti-complementary (sense probe, negative control) to cellular RNA transcripts were obtained by run-off transcription using SP6 or T7 RNA polymerase (Gibco-BRL, Karlsruhe FRG).[26] [^{35}S]-labeled nucleotides were used for generating *in situ* hybridization probes with a specific activity of 1.2–1.4 × 10^9 cpm/μg.

Hybridization and Autoradiography

Prehybridization, hybridization, washing procedures, and RNase digestion of mismatched sequences as well as autoradiography were performed as described.[26] In brief, sections were treated with 0.2 M HCl, digested with pronase, and fixed in 4% paraformaldehyde/PBS. Slides were then acetylated, rinsed again in PBS, dehydrated in graded ethanols and airdried before hybridization. Hybridization was performed for 18 hours at 50°C using 5×10^5 cpm of [^{35}S]-labeled RNA probe. Slides were washed for 5 hours at 50°C in modified hybridization buffer and subjected to a brief RNase A digestion. After some further washing steps, the slides were dehydrated in graded ethanols, airdried, and dipped into Ilford G5 photographic emulsion (Ilford, Mobberley Cheshire, UK). After exposure for 2 to 10 days at 4°C, slides were developed in Kodak D19 developer (Kodak, Hemel Hampstead, UK) for 2.5 minutes, rinsed in 1% acetic acid and fixed in Kodak Fixer for 3 minutes. After extensive washing, the slides were finally counterstained in hematoxylin-eosin and mounted. All sections from normal and fibrotic livers were processed in parallel using the same batches of probes and reagents.

Results

Normal Liver

Applying the TGFβ1 probe, a weak autoradiographic signal was found over few cells of the portal tract stroma in normal human and rat liver tissues. Hepatocytes, lobular mesenchymal cells, and bile-duct epithelial cells displayed only occasional silver grains, which were interpreted as being related to nonspecific signal, because a similar number of grains was also observed after hybridization with the sense control probes (Figure 1). TGFβ2 expression was not detectable.

Fibrotic Liver

The *in situ* hybridization results on normal and fibrotic rat and human liver are summarized in Table 1. In human fibrotic specimens, *in situ* hybridization revealed an intense signal with the TGFβ1 anti-sense RNA probe over many cells of portal tracts, fibrous septa, and sinusoids infiltrated by inflammatory cells, particularly at the periphery of regenerating nodules (Figure 2). Morphology and expression of vimentin as assessed by a combination of immunohistology and *in situ* hybridization allowed for their identification as mesenchymal cells. In both rat and human livers, high TGFβ1 gene transcript levels were found in nonepithelial cells surrounding proliferating bile ducts, whereas bile duct epithelial cells showed only weak labeling of occasional cells. Leukocytes of the portal and periportal inflammatory infiltrate represented another quantitatively relevant source of TGFβ1 in human specimens. Hepatocytes showed no evidence for specific TGFβ1 transcript levels above background with the exception of some intensely labeled hepatocytes in the periphery of regenerating nodules in 2 of 10 cirrhotic human liver biopsies with severe inflammatory activity. These particular hepatocytes were found close to fibrotic septa and were partially surrounded by vimentin- and TGFβ1-positive mesenchymal cells (Figure 4).

In comparison to TGFβ1, TGFβ2 showed a strikingly different expression pattern in both human and rat liver. Autoradiographic signals for TGFβ2 transcripts were

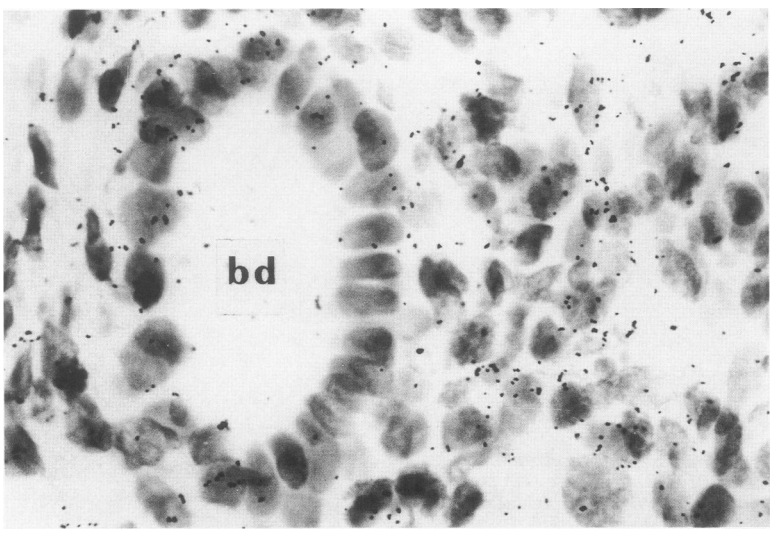


Figure 1. In situ *hybridization with [^{35}S]-labeled TGF-β1 antisense RNA probe on normal human liver. Grains are localized over mesenchymal cells of the portal tracts and over some cells distributed along sinusoids. bd, bile duct. Exposure time 10 days, magnification ×400.*

Table 1. *Summary of* In Situ *Hybridization Results*

		Portal tract			Liver lobule						
					Zone 1			Zone II		Zone III	
Tissue	Probe	Stromal cells	Leukocytes	BDC	HC	BDC	NEC	HC	NEC	HC	NEC
Fibrotic human liver	TGF-β1	+ +	+ +	±	−*	±	+ +	−	+ +	−	+ +
	TGF-β2	−	−	+ + +	−	+ + +	−	−	−	−	−
Fibrotic rat liver	TGF-β1	+ +	+ +	±	−	±	+ +	−	+ +	−	+ +
	TGF-β2	−	−	+ + +	−	+ + +	−	−	−	−	−

* Weak labeling of few hepatocytes of the limiting plate in two cases.
HC, hepatocytes; BDC, bile duct epithelial cells; NEC, nonepithelial cells.
−, ±, + +, + + +; intensity of the autoradiographic signal: background levels, weak, moderate, and intense labeling, respectively.

found exclusively over epithelial cells of proliferating bile ducts (Figure 3), as confirmed by double labeling with a cytokeratin 7-specific antibody (Figure 5). Occasional groups of small TGFβ2-positive cells at the periphery of some lobules could also be identified as bile duct epithelial cells as they expressed cytokeratin 7. TGFβ2 transcripts were homogeneously distributed among bile duct epithelial cells, with enhanced signal intensity in small ductules at the periphery of fibrotic portal tracts as compared to larger bile ducts. The intensity of cytokeratin 7-specific staining appeared to be inversely related to the TGFβ2-specific signal, with bile duct epithelial cells of

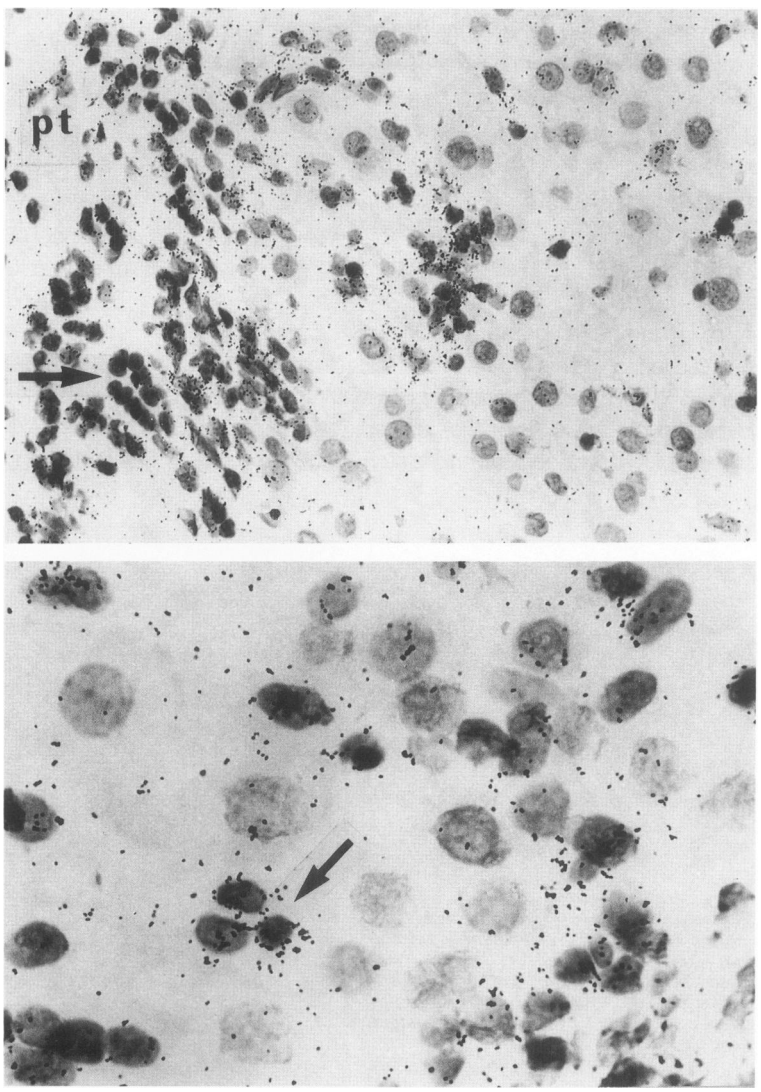


Figure 2. In situ *hybridization with [^{35}S]-labeled TGF-β1 antisense RNA probe on cirrhotic human liver with high activity. Autoradiographic signal mainly localized over perisinusoidal cells (arrow). Exposure time 7 days, magnification ×180* **(A)**, *×400* **(B)**.

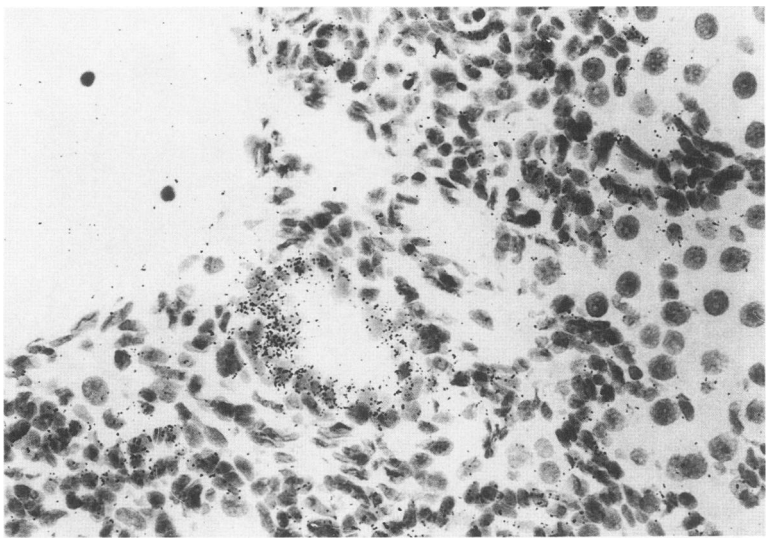


Figure 3. In situ *hybridization with [^{35}S]-labeled TGF-β2 antisense RNA probe on rat liver 3 weeks after bile duct interruption. Autoradiographic signal present over epithelial cells of newly formed bile ductules. Exposure time 5 days, magnification ×180.*

large ducts showing the most intense staining. In comparison with hybridizations employing sense-probes, no significant TGFβ2 gene transcript steady-state levels were detectable in nonepithelial cells and hepatocytes, some of which expressed cytokeratin 7. This weak background signal was of similar intensity over all parenchymal cells regardless of their localization within the lobule.

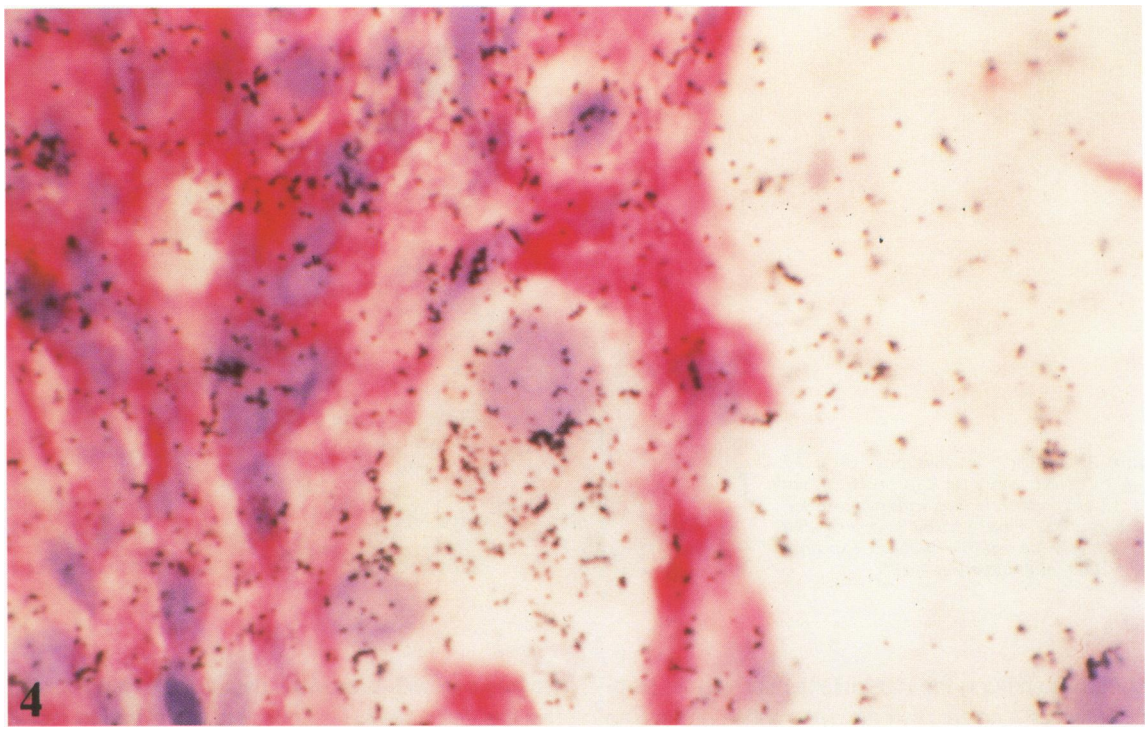
Discussion

It has previously been shown that TGFβ polypeptides stimulate the production of ECM components and inhibit degradation of these proteins in many tissues.[1,2] Inhibition or modulation of TGFβ polypeptide effects may thus represent potential approaches to fibroprevention or antifibrotic therapy. In chronic liver disease, TGFβ1 expression correlated closely with the histologic activity index and other parameters such as procollagen type I mRNA and procollagen type III serum peptide levels.[34] We were thus interested in characterizing the celluar sources of TGFβ transcripts in liver tissues with fibrosis of different etiology. In this study we demonstrate that TGFβ1 and TGFβ2 are differentially expressed in fibrotic rat and human liver, and that enhanced cellular RNA transcript levels of these cytokines apparently occur in parallel with the activity of liver fibrosis and bile duct proliferations, respectively.

In human liver, TGFβ1 transcripts were present in various mesenchymal cell types, positively identified by vimentin staining in double labeling experiments. Their phenotype, as judged by morphology and tissue distribution, was compatible with inflammatory cells, fibroblasts, myofibroblasts, and lipocytes. The labeling of some bile duct epithelial cells by the TGFβ1 probe is in agreement with its previous detection by immunostaining in this cell type and is unlikely to be related to crosshybridization to TGFβ2, as the pattern of TGFβ1 expression differed considerably from that of TGFβ2. We also found that parenchymal cells did not express TGFβ1 RNA in rat liver. This is in agreement with previously published *in situ* hybridization data on rat liver with acute and chronic carbon tetrachloride intoxication.[29,30] The finding of occasional groups of TGFβ1 RNA-expressing hepatocytes along fibrotic septa in a proportion of human liver biopsies was therefore unexpected.

In vivo, expression of TGFβ1 RNA has never been documented in hepatocytes. TGFβ1-immunoreactivity previously shown in murine hepatocytes was considered to be exogenous in origin since the liver plays a role in the clearance of circulating TGFβ.[7] Our data support the view that hepatocytes may well be capable of expressing the TGFβ1 gene under special circumstances. This observation raises the possibility that hepatocytes may directly contribute to the induction of fibrogenesis by stimulating the ECM synthesis in neighboring mesenchymal cells. In addition, since TGFβ1 is an inhibitor of hepatocyte growth *in vitro,*[17,18] it is also conceivable that TGFβ1 may also influence parenchymal cell growth in distinct areas of the lobule by negative autocrine regulatory loops.

To date, few functional differences between TGFβ1 and TGFβ2 have been described. TGFβ2, also termed glioblastoma-derived T-cell suppressor factor by virtue of its suppressive effects on interleukin-2-dependent T-cell growth, is the equivalent of bovine cartilage-inducing factor B.[41,42] The human TGFβ1 and TGFβ2 genes share approximately 70% of common nucleotide sequence and their peptides bind to specific types of receptors exhibiting different affinities.[43] Administration of exoge-



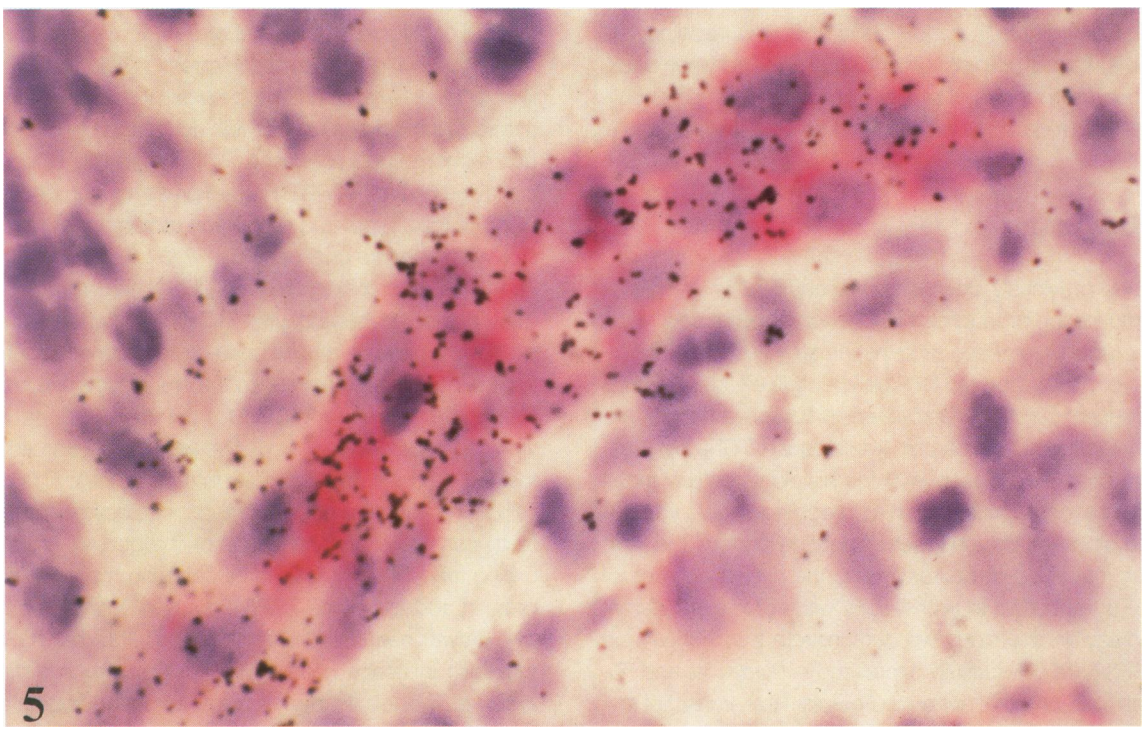


Figure 4. In situ *hybridization with [^{35}S]-labeled TGF-β1 antisense RNA probe and vimentin-specific immunostaining on cirrhotic human liver with high activity. The autoradiographic signal is present over vimentin-positive nonparenchymal cells and a vimentin-negative hepatocyte in the periphery of a renerating nodule. Exposure time 7 days, APAAP method, magnification ×480.*
Figure 5. In situ *hybridization with [^{35}S]-labeled TGF-β2 antisense RNA probe and cytokeratin 7-specific immunostaining on human liver with HBV-related chronic active hepatitis. The* in situ *hybridization signal is present over cytokeratin 7-positive bile duct epithelial cells. Exposure time 7 days, APAAP method, magnification ×480.*

nous TGFβ1 and TGFβ2 to cultured human colon carcinoma cells induces the expression of different, although largely overlapping sets of genes, implying that these proteins have distinct functions.[44] Immunolocalization studies revealed that TGFβ2 is developmentally regulated, e.g., during the pre- and peri-implantation stages of in murine embryos. This lead to the suggestion that TGFβ2 may be involved directly in the regulation of

growth or the specific patterns of extracellular matrix production.[45]

In rat and human livers, TGFβ2 RNA transcripts were restricted to bile duct epithelial cells as confirmed by double labeling with a cytokeratin 7-antibody. Accumulation of desmin-positive mesenchymal cells and synthesis of interstitial and basement membrane extracellular matrix components by these cells around newly formed bile ducts is characteristic of the fibroductular reaction.[37] The expression of TGFβ2 in epithelial cells of proliferating bile ducts is therefore likely to reflect the induction of a specific periductular environment by paracrine stimulation of ECM synthesis in adjacent mesenchymal cells. This interpretation is in keeping with the expression of the basement membrane components collagen type IV and laminin B1 by proliferating bile duct epithelial cells which indicates that these cells also contribute to the synthesis of their basement membranes.[27,28,46] Contrary to hepatocytes expressing TGFβ1, the production of TGFβ2 is unlikely to have negative autocrine growth effects on the bile duct epithelial cells synthesizing this factor, as these cells display proliferative activity.[37] It will therefore prove interesting to characterize the differences among those sets of genes activated by TGFβ1 and TGFβ2 polypeptides in the liver. The expression of TGFβ1 in addition to TGFβ2 in some of the bile duct epithelial cells also points to the possibility of TGFβ1.2 heterodimer secretion by these cells.

Whereas TGFβ2 expression is restricted to bile duct epithelial cells and may be characteristic of liver disease associated with bile duct proliferation, TGFβ1 expression involves several cell types of fibrotic liver. This suggests that TGFβ1 expression may represent a general response to acute and chronic liver injury. The cellular distribution of TGFβ1 RNA expression and its association with fibrosis and inflammation in human and rat liver further supports the view that this cytokine plays an important role in the development and progression of hepatic fibrosis. However, cytokine RNA expression may not always be followed by the synthesis and the release of the corresponding protein, and TGFβ polypeptides are secreted in inactive form requiring activation by transient acidification or proteolysis in the extracellular space prior to binding to receptor structures. The present RNA expression studies thus have to be extended to the immunohistology of activated TGFβ polypeptides and TGFβ receptors in the liver to obtain a more detailed understanding of the pathophysiologic functions of TGFβ1 and TGFβ2 in hepatic fibrosis.

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