Short Communication

Proliferating Bile Duct Epithelial Cells Are a Major Source of Connective Tissue Growth Factor in Rat Biliary Fibrosis

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Connective tissue growth factor (CTGF) is a downstream mediator of transforming growth factor-β1 (TGF-β1) and thus a potential target for antifibrotic treatment strategies. CTGF is up-regulated in disorders such as atherosclerosis, scleroderma, and fibrosis of kidneys and lungs. We investigated the temporospatial expression patterns of CTGF and TGF-β1 mRNA in rat livers with acute fibrogenesis (after a single dose of CCl_4) and with advanced fibrosis (6 weeks after complete bile duct occlusion). Multiprobe ribonuclease protection assay revealed increasing TGF-β1 and CTGF mRNA levels 6 hours after injection of CCl_4, with peak levels after 72 hours. In biliary fibrosis TGF-β1 and CTGF mRNA levels increased fourfold and sevenfold, respectively ($P <$ 0.001). *In situ* hybridization combined with cell-specific markers revealed CTGF transcripts in desmin-positive cells after a single dose of carbon tetrachloride, whereas no transcripts were found in normal livers. In biliary fibrosis, however, proliferating bile duct epithelial cells were the predominant source of CTGF mRNA. We conclude that in rat liver fibrogenesis CTGF is up-regulated in close association with TGF-β1 and that, contrary to a previous report, not solely hepatic stellate cells but activated bile duct epithelial cells are the main source of this profibrogenic factor. (Am J Pathol 2001, 158:1239–1244)

Connective tissue growth factor (CTGF) is a cysteine-rich polypeptide identified originally in human umbilical vein endothelial cells by its chemotactic and mitogenic effects on fibroblasts.[1] *In vitro* and *in vivo* experiments have shown that expression of CTGF in endothelial cells and fibroblasts is induced by transforming growth factor-β1 (TGF-β1), but not by other mediators such as platelet-derived growth factor, epidermal growth factor, or basic fibroblast growth factor.[2–5] CTGF shares biological properties with TGF-β1 such as triggering rat kidney (NRK) fibroblast proliferation and up-regulating mRNA expression of procollagen α1(I), fibronectin, and the fibronectin receptor, integrin α5β1. Unlike TGF-β1, however, CTGF does not stimulate anchorage-independent growth of NRK fibroblasts or inhibit growth of mink lung epithelial cells, indicating that CTGF does not share all biological activities with TGF-β1.[2,6] In this line CTGF seems to be a downstream mediator for only some actions of TGF-β1, notably promotion of fibroblast proliferation and extracellular matrix production.[7,8] Co-injection of CTGF in subcutaneous tissue of mice enhances and perpetuates the fibrotic response after injection of TGF-β1.[9] Because of its potentiating effect in organ fibrosis and because of its specific expression by and action on mesenchymal cells, CTGF is considered an attractive target for antifibrotic therapies.

Several studies demonstrated that CTGF mRNA is coordinately expressed with TGF-β1 in fibrogenic lesions such as atherosclerosis,[10] scleroderma,[11–14] and fibrosis of kidneys,[15] lungs,[16] and intestine.[17] Recently, hepatic stellate cells (HSCs) were identified as major cellular source of CTGF in liver fibrogenesis,[18] in which TGF-β1 is a prominent profibrogenic cytokine.[19] However, CTGF transcripts in activated HSC were only shown for a single case of (human) liver cirrhosis. By studying the temporospatial expression of CTGF in rats with acute and chronic hepatic fibrogenesis, we identified bile duct epithelia as a

dominant source of CTGF mRNA in liver subsequent to bile duct occlusion (BDO).

Materials and Methods

Cloning of Partial Rat CTGF cDNA by Reverse Transcription-Polymerase Chain Reaction

Total liver RNA was reverse transcribed with an oligo-dT primer and Superscript II reverse transcriptase (Life Technologies, Karlsruhe, Germany) according to the manufacturer's instructions. Rat CTGF cDNA was amplified with primers CAA CCG CAA GAT TGG AGT GT and CTC CAG TCT GCA GAA GGT ATT G according to positions 398 to 417 and 806 to 827 of the mouse CTGF sequence (fisp-12, GenBank accession number: M70642).[7,20] Polymerase chain reaction products were cloned into the *Eco*RV site of pZErO-1 and sequenced on both strands. The rat sequence of the CTGF amplicon has been submitted to GenBank/EMBL (accession number AJ236872). The rat protein displays 95% homology to mouse, human, and porcine CTGF.

Animal Models

Animal experiments were performed in accordance with local institutional and governmental regulations on the use of experimental animals. Wistar rats were maintained in 12-hour light/dark cycles at 23°C with a humidity of $60 \pm 10\%$. Fifteen male rats (~125 g; Animal Production Facilities, Schoenwalde, Germany) received a single intraperitoneal injection of CCl_4 (1.25 ml/kg body weight), mixed with an equal volume of vegetable oil. Groups of three animals were sacrificed 6, 12, 24, 48, and 72 hours after the injection, and three untreated rats served as controls. In eight female rats (~250 g) the common bile duct was injected in a retrograde manner with the sclerosant sodium amidotriazoate (0.2 ml/kg body weight, Ethibloc; Ethicon, Norderstedt, Germany) as reported previously.[21,22] Another eight rats, in whom the abdominal cavity was opened and resealed, were used as sham-operation controls. After 6 weeks, rats were sacrificed and aliquots of right and left liver lobes were snap-frozen in liquid nitrogen.

In Situ *Hybridization Combined with Immunohistochemistry*

Plasmids containing rat CTGF (430 bp, see above), TGF-β1 (a 570-bp fragment from position 987 to 1550 in X52498), kindly provided by Dr. X. L. Tian, Max Delbrueck Center for Molecular Medicine, Berlin, Germany)[23] and rat procollagen α1(I) (a 1.3-kb *Pst*I/*Hin*dIII-fragment of plasmid α1R1, provided by Dr. D. Rowe, Department of Pediatrics, University of Connecticut Health Center, Farmington, CT, subcloned in pGEM (Promega, Mannheim, Germany)[24] were linearized prior to transcription. Antisense and sense (negative control) RNA probes were prepared by *in vitro* transcription with [^{35}S]-UTP (NEN, Cologne, Germany) and used for *in situ*

hybridization combined with immunohistochemistry on a single section as described before.[25-27] Briefly, 5-μm frozen sections were mounted onto 3-aminopropyl triethoxysilane-coated slides and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 20 minutes, followed by washes in PBS, dehydration in graded ethanol, and air-drying. After prehybridization, slides were hybridized with antisense or sense probes at 52°C for 16 hours, followed by high stringency washing, and RNase A digestion to remove unhybridized RNA. For double labeling, sections were stained with monoclonal antibodies to desmin (DAKO, Hamburg, Germany) by the alkaline phosphatase anti-phosphatase (APAAP) method before prehybridization under RNase-free conditions. Sections were dehydrated and dipped into photo emulsion (Amersham, Braunschweig, Germany), followed by autoradiographical exposure at 4°C for 1 to 2 weeks, development, fixation, and counterstaining with hematoxylin and eosin.

RNA Isolation and Multiprobe Ribonuclease Protection Assay

RNA was extracted from snap-frozen liver tissues by the single-step method of Chomczynski and Sacchi.[28] Concentration and purity of RNA were determined by spectrophotometry at 260/280 nm and integrity of RNA was verified by visualization of the 18S and 28S rRNA bands after agarose (1.4%) electrophoresis and ethidium bromide staining.

[α-^{32}P]-UTP (NEN)-labeled antisense riboprobes for rat CTGF (the full length of our cloned sequence), glyceraldehyde-3- phosphate dehydrogenase (GAPDH)[29] and TGF-β1 were prepared by *in vitro* transcription and purified by polyacrylamide gel electrophoresis. The ribonuclease protection assay (RPA) was performed as described[22] using the RPA II kit (Ambion, Austin, TX) according to the manufacturer's instructions. In brief, 20 μg of total RNA and 30,000 cpm of each probe (20,000 cpm for the GAPDH mRNA) were hybridized in 20 μl of hybridization buffer containing 80% formamide at 45°C for 16 hours and digested with RNase T1 at 37°C for 1 hour. Protected probes were separated on a 5% polyacrylamide/8 mol/L urea gel followed by autoradiography with X-ray films (Kodak, Rochester, NY) at −70°C for 16 hours. Sizes of the protected sequences for CTGF, TGF-β1, and GAPDH were 430, 253, and 102 nucleotides, respectively.

Signal Quantification and Statistical Analysis

Autoradiographies were analyzed with the public domain program NIH Image (developed at the National Institutes of Health, Bethesda, MD) on a Power Macintosh 7500/100 computer. Target mRNA signals of CTGF and TGF-β1 were normalized to the GAPDH signal and expressed as relative abundance (arbitrary units). The differences among the various time points after injection of CCl_4 were analyzed by one-way analysis of variance. The differences between sham-operated and BDO rats were compared by the unpaired *t*-test. $P < 0.05$ was regarded as statistically significant.
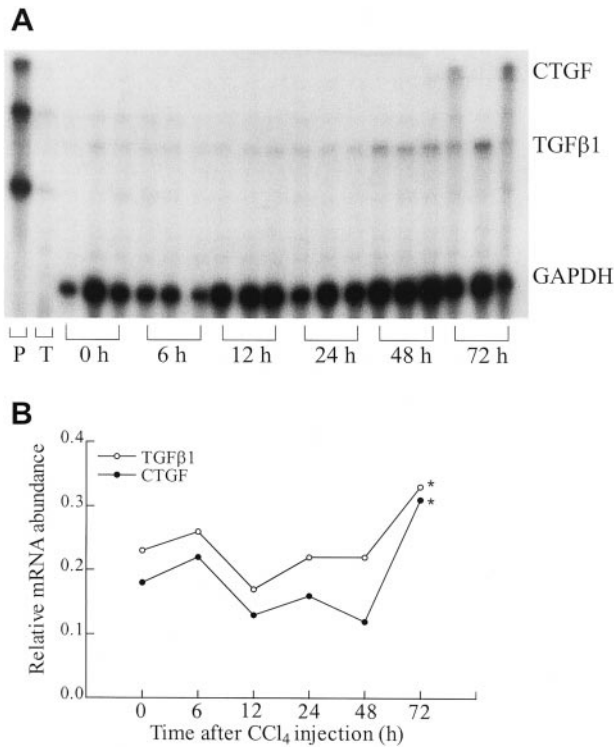
Figure 1. Coordinate expression of CTGF and TGF-β1 in acute liver fibrogenesis. **A:** Multiprobe ribonuclease protection assay for CTGF and TGF-β1 mRNA in rats after acute CCl_4-intoxication. Twenty μg of liver RNA were hybridized with [α-^{32}P]-labeled CTGF and TGF-β1 riboprobes. After RNase T1 digestion the protected probes were resolved through denaturing polyacrylamide gel electrophoresis and subjected to autoradiography at −70°C for 16 hours. **B:** P, full-length probes; T, tRNA control. Autoradiographic signals were normalized to that of GAPDH and expressed as arbitrary units. Each time point represents the mean value of three rats. *, $P < 0.05$ compared with all other time points.

Results

Rat CTGF Is Highly Homologous to CTGF of Other Species

Using primers derived from the mouse CTGF mRNA sequence, we amplified a 430-bp fragment from rat liver cDNA. After excluding the 42-bp mouse primer regions, this rat sequence shared 95 and 97% identity with mouse CTGF at the mRNA and the deduced amino acid level, respectively. Amino acid identity was also 95% with human and pig, and 82% with bovine and frog CTGF.

CTGF mRNA Levels in Rats with Acute and Chronic Liver Fibrogenesis

CTGF mRNA levels in normal rat livers were low. After a single injection of CCl_4, mRNAs for TGF-β1 and CTGF rose slowly, but only increased significantly (~50% greater than baseline) 72 hours after injection (Figure 1). However, at 72 hours one out of three rats showed a low CTGF and a high TGF-β1 mRNA steady state level, indicating that at later time points expression of these two cytokines may dissociate (Figure 1A). Furthermore, this finding underlines the well-known individual heterogeneity of the CCL_4 model of fibrogenesis. In contrast, nor-
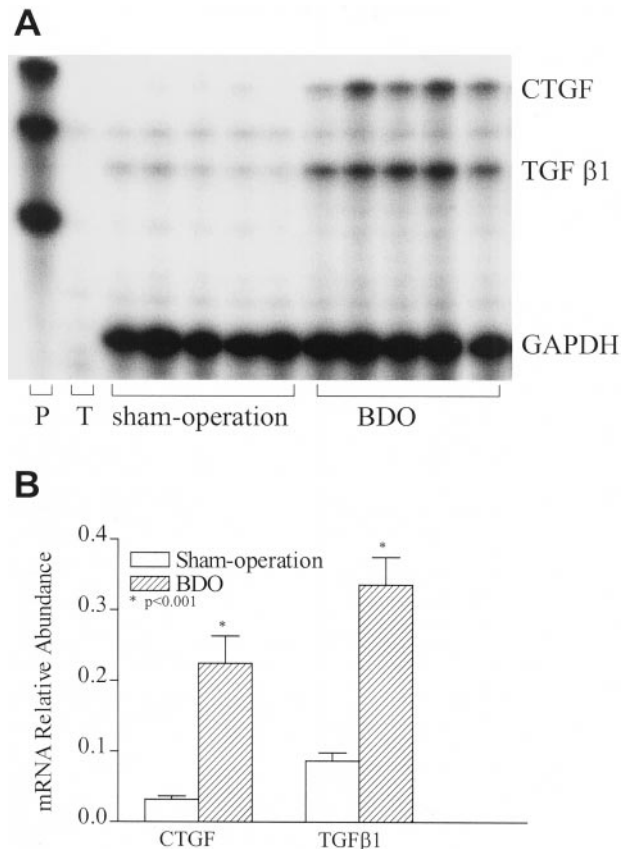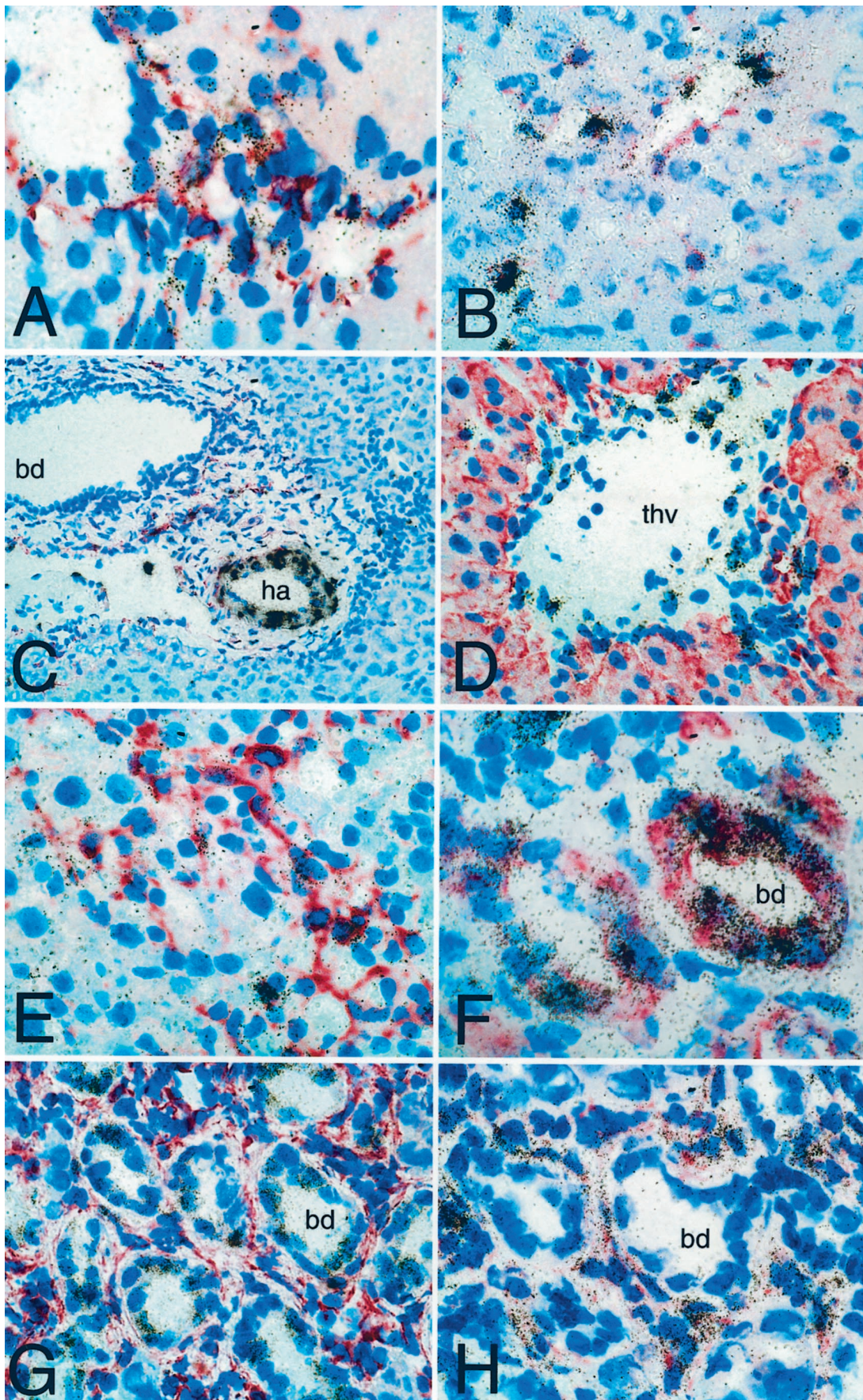

Figure 2. Coordinate expression of CTGF and TGF-β1 in chronic liver fibrogenesis. Multiprobe ribonuclease protection assay for CTGF and TGF-β1 mRNA from rats after BDO for 6 weeks. Twenty μg of liver RNA were hybridized with [α-^{32}P]-labeled CTGF and TGF-β1 riboprobes and processed as described in Figure 1. P, full-length probes; T, tRNA control. Error bars represent standard errors derived from five rats. *, $P < 0.001$ compared with sham-operated controls.

malized mRNA levels of TGF-β1 and CTGF in livers of rats with secondary biliary cirrhosis (BDO) were fourfold and sevenfold higher than those of the sham-operated controls ($P < 0.001$, Figure 2).

Cellular Sources of CTGF in Rat Liver

By combining *in situ* hybridization with immunostaining for cell-specific markers CTGF mRNA was barely detectable in liver sections of normal rats, except for a local up-regulation in portal and central vein endothelia and in myocytes of portal arteries (Figure 3, A and B). After acute CCl_4 intoxication signals for CTGF mRNA were increased predominantly in activated HSCs/myofibroblasts, identified by positivity for desmin (Figure 3C). Surprisingly, CTGF mRNA was highly up-regulated and almost exclusively found over proliferating, pan-cytokeratin-positive and desmin-negative bile duct epithelia in rats after 6 weeks of BDO (Figure 3; D, E, F, and G). Here, signal intensity varied among cells of a single bile duct. These CTGF-expressing cells were surrounded by a rim of procollagen α1(I) expressing, desmin-positive HSCs/myofibroblasts (Figure 3H).

Coordinate Expression of TGF-β1 and CTGF mRNA

Although in acute fibrogenesis the time course and quantitative expression of TGF-β1 and CTGF mRNA were virtually superimposable (Figure 1), in BDO-induced fibrosis proliferating bile duct epithelia expressed TGF-β1 mRNA at minimal levels whereas it was highly up-regulated in the surrounding HSCs/myofibroblasts (data not shown).

Discussion

CTGF is a member of the emerging CCN (connective tissue growth factor/*fisp 12*, *Cyr61/cef10* and *nov*) family that comprises several immediate early gene products induced by growth factors or oncogenes. Recent data indicate that CTGF is up-regulated by and acts in concert with TGF-β1. Because mesenchymal cells have been held to be both the major source and target of CTGF, inhibition of expression or blockade of this growth factor and its yet unidentified cellular receptor are attractive targets for an antifibrogenic therapy.

By combining *in situ* hybridization and immunohistochemistry we identified activated HSCs as the main cellular source of CTGF in later time points of acute fibrogenesis triggered by CCl₄, confirming a recent report.[18] In normal as well as CCl₄-injured livers, however, CTGF transcripts were most prominent over venous endothelial cells and arterial myocytes. This is consistent with the results obtained in rats and other species, where CTGF transcripts were predominantly found in fibroblasts and endothelia of the skin,[13] gingiva,[30] kidney,[15] pancreas,[31] and lung,[16] as well as in smooth muscle cells of atherosclerotic blood vessels[10] and in hypertrophic chondrocytes of costal cartilage.[32] The basal expression of CTGF in these cell types was low, but highly up-regulated during wound healing or fibrogenesis. A single *in vivo* study showed some CTGF expression by renal tubular epithelial cells.[33] Our observation of a prominent expression of CTGF by proliferating bile duct epithelial cells *in vivo* is unexpected. However, it explains the prominent fibroductular reaction around proliferating bile duct epithelia in biliary fibrosis, as exemplified by an up-regulated procollagen I expression by periductular myofibroblast-like cells.[34] It is also in line with the TGF-β1 and TGF-β2 mRNA expression patterns of these ductular epithelial cells and periductular myofibroblasts that we found in an earlier study.[35] Furthermore, the deposition of CTGF immune-reactive material in the periductular extracellular matrix observed by Paradis and colleagues[18] mostly

likely reflects the affinity of this growth factor to abundant matrix proteins such as collagens and fibronectin.[36]

The observed patchy distribution of CTGF mRNA in proliferating bile duct cells does not seem to be related to proliferation, because we showed previously that the proliferation marker Ki-67 is distributed homogeneously in larger as well as proliferating biliary epithelia.[34,35]

Our findings may have therapeutic implications. TGF-β1 has been regarded as a key profibrogenic cytokine that is capable of promoting mesenchymal proliferation and extracellular matrix production and of suppressing extracellular matrix degradation.[37] Thus blocking the expression or activity of TGF-β1 can effectively prevent organ fibrosis in various experimental models.[38] However, TGF-β1 is a pleiotropic, multifunctional, and context-dependent cytokine, including a strong immunosuppressive and proapoptotic effect for lymphocytes, macrophages, and hepatocytes.[39] TGF-β1 knockout mice die shortly after birth of an excessive inflammatory response, because of massive mononuclear infiltration into organs such as intestine and liver. Furthermore, depending on the context the role of TGF-β1 in carcinogenesis can be divergent, varying from inhibition to promotion of tumor formation or metastasis.[40] Therefore, complete blockade of its expression or activity is accompanied by unfavorable if not lethal side effects.

On the other hand, the effect of CTGF, which acts in conjunction with TGF-β1, seems to be limited to the mesenchymal compartment.[2] Therefore, inhibition of CTGF bioactivity is expected to be a better target for antifibrotic therapies.[41] However, as we showed in our study, activated epithelial cells have to be considered as a major source of CTGF *in vivo*.

In conclusion, we found that in rat biliary fibrosis bile-duct epithelial cells are the dominant cellular source of CTGF and that the expression of CTGF mRNA is coordinately up-regulated with that of TGF-β1, supporting the suggested role of CTGF as an associated if not downstream mediator of TGF-β actions.

Acknowledgment

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Figure 3. Expression patterns of CTGF and procollagen α1(I) mRNA in acute and chronic hepatic fibrogenesis. Radioactive *in situ* hybridization, in part in combination with immunostaining for desmin (**A-D**, **G**, and **H**) or pan-cytokeratin (**E**) on rat livers. CTGF antisense: **A:** Normal liver portal tract; labeling of HSCs/portal fibroblasts (original magnification, ×600). **B:** Six hours after CCl₄, labeling of desmin-positive HSC and endothelial cells (original magnification, ×400). **C:** Six hours after CCl₄, prominent signals in hepatic arterial (ha) myocytes in a larger portal tract, no expression over a larger bile duct (bd) (original magnification, ×200). **D:** Seventy-two hours after CCl₄, strong labeling of single centrilobular HSC and endothelial cells of a terminal hepatic vein (thv) (original magnification, ×600). **E:** Seventy-two hours after CCl₄, enhanced CTGF mRNA expression in most portal tract fibroblasts (original magnification, ×400). **F** and **G:** CTGF mRNA is almost exclusively expressed in desmin-negative (**F:** original magnification, ×900) and pan-cytokeratin-positive (**G:** original magnification, ×600) bile duct epithelia (bd in biliary fibrosis); note the patchy ductular CTGF mRNA expression pattern. **H:** Up-regulation of procollagen α1(I) mRNA in desmin-positive myofibroblasts surrounding proliferating bile ducts (bd) after 6 weeks of BDO (original magnification, ×700).

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