Transforming growth factor betas and their receptors in human liver cirrhosis

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\textbf{Background} Transforming growth factor betas (TGF-\(\beta\))s are a group of homologous polypeptides that exert pleiotropic effects on various cell types and stimulate the formation of extracellular matrix and fibrosis. To evaluate whether TGF-\(\beta\) isoforms (TGF-\(\beta\)1, TGF-\(\beta\)2 and TGF-\(\beta\)3) and their receptors (types I–III) are also of importance in the pathophysiology of liver cirrhosis, we analysed their concomitant expression and localization in human liver cirrhosis.

Patients Cirrhotic liver tissue samples were obtained from 17 patients (four women, 13 men) with a median age of 41 years (range 22–67). Normal liver tissues from ten patients (seven women, three men) with a median age of 55 years (range 45–75) served as controls.

Methods The tissues were fixed in Bouin's solution and paraffin-embedded for histological analysis. For RNA analysis, freshly obtained tissue samples were snap-frozen in liquid nitrogen and stored at –80°C until analysed. Northern blot analysis was used to examine the expression of TGF-\(\beta\)1, \(\beta\)2 and \(\beta\)3 and their receptors, type I (\(\beta\)R-I), type II (\(\beta\)R-II) and type III (\(\beta\)R-III). Immunohistochemistry was performed to determine the localization of the corresponding proteins in the normal and the cirrhotic liver.

Results Northern blot analysis revealed enhanced expression (\(P<0.05\)) of TGF-\(\beta\)1 (twofold increase), TGF-\(\beta\)2 (threefold increase) and TGF-\(\beta\)3 (8.5-fold increase) and of \(\beta\)R-II (threefold increase) mRNA in liver cirrhosis in comparison with normal controls. In contrast, \(\beta\)R-I (ALK-5) and \(\beta\)R-III mRNA expression showed no significant changes. No TGF-\(\beta\) isoform immunoreactivity was present in hepatocytes in either normal livers or in liver cirrhosis. However, in liver cirrhosis, intense TGF-\(\beta\)1 immunoreactivity was present in bile duct and ductular epithelial cells (including ductular proliferations) and in inflammatory cells. In a few sinusoidal lining cells, faint TGF-\(\beta\)1 and moderate TGF-\(\beta\)2 immunoreactivity was present. TGF-\(\beta\)3 immunostaining was present in bile duct and ductular epithelial cells, in inflammatory cells and in fibroblast-like spindle cells in liver cirrhosis. For \(\beta\)R-I and \(\beta\)R-II, the immunoreactivity was localized in hepatocytes and biliary cells in normal and cirrhotic liver tissues, with higher intensity for \(\beta\)R-II in the cirrhotic liver.

Conclusion Enhanced expression of all three TGF-\(\beta\) isoforms and of \(\beta\)R-II in liver cirrhosis suggests their involvement in this fibrotic disorder. The higher immunoreactivity of the three TGF-\(\beta\) isoforms in the bile duct epithelial cells in cirrhotic tissues suggests a possible role of these cells in the pathogenesis of liver cirrhosis.

Keywords: fibrogenesis, immunohistochemistry, liver cirrhosis, Northern blot analysis, transforming growth factor-beta, transforming growth factor-beta receptors

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Introduction

Transforming growth factor betas (TGF-\(\beta\))s belong to the TGF-\(\beta\) gene superfamily of regulatory polypeptides, which act as signalling molecules by binding to specific receptors located on the surface of the cell membrane [1]. TGF-\(\beta\)s are produced as an inactive molecule that must be activated before it can bind to its specific cell membrane receptors. In mammalian cells, three isoforms of TGF-\(\beta\) have been characterized: TGF-\(\beta\)1, TGF-\(\beta\)2 and TGF-\(\beta\)3. TGF-\(\beta\)s play an important role in the regulation of cell growth [2,3], cell differentiation, angiogenesis, immune reactions, formation of the extracellular matrix and fibrosis [4–6]. In the liver they stimulate activation of perisinusoidal cells [7,8] and induce growth of normal fibroblasts [9] in general. However, they are also recognized as inhibitors of the proliferation of hepatocytes.
TGF-βs have been implicated in a variety of fibrotic disorders, including liver fibrosis, due to their stimulation of procollagen and fibronectin synthesis [10,11], induction of collagenase inhibitors, stimulation of monocyte nitrogens, chemotaxis of monocytes and fibroblasts [12] and their role in the increase of incorporation of fibronectin and collagen into the extracellular matrix. In liver cirrhosis, TGF-β1 has been analysed and found in the extracellular matrix between liver nodules and fibrous septa where fibrogenesis occurs [13].

TGF-βs act after binding to specific transmembrane receptors. Three major TGF-β receptors have been characterized: TGF-β receptor type I (TβR-I), TGF-β receptor type II (TβR-II) and TGF-β receptor type III (TβR-III) [14–18]. TβR-I and TβR-II are involved in signal transduction [16,17], whereas TβR-III participates in storage of TGF-βs and in the presentation of the isoforms to their signalling receptors. Although only one subtype of TβR-II has been identified, several subtypes of TβR-I have been found [17,19,20]. One of these, ALK-5, participates mainly in mediation of TGF-β signalling. The exact functions of the other TβR-I subtypes in TGF-β signalling are not known [19,20]. The presence of both TβR-I (ALK-5) and TβR-II is required for signal transduction by a serine/threonine kinase [15].

Due to the importance of TGF-βs in fibrogenesis, we analysed in the present report on liver cirrhosis the concomitant expression and cellular localization of all three mammalian TGF-β isoforms. Because TGF-βs can only function in the presence of their signalling receptors, we also analysed them simultaneously. We found enhanced expression of all three TGF-β isoforms and of TβR-II in liver cirrhosis, which implies that they play an important role in this disorder. To our knowledge, this is the first report that has investigated concomitant expression of TGF-β isoforms and their three major receptors in liver cirrhosis.

Patients and methods
Normal human liver tissue samples were obtained from ten individuals (seven women, three men; median age 55 years, range 45–75). Four normal liver tissues were obtained through an organ donor programme in which there were no recipients available for liver transplantation and six specimens were obtained from patients undergoing liver resection due to a single colorectal metastasis. In the case of liver resection of metastasis, normal liver tissue was taken at the greatest distance from the localization of the metastasis. The tissue samples used as controls were histologically analysed and clearly shown to have no relevant changes, in particular no inflammation or fibrosis.

Hepatic cirrhosis tissues were obtained from 17 patients (four women, 13 men). The median age of the hepatic cirrhosis patients was 41 years (range 22–67). The aetiology of liver cirrhosis was hepatitis B in three cases, hepatitis C in three, primary biliary cirrhosis in three, primary sclerosing cholangitis in two, alcoholic liver disease in two, sarcoidosis in one and cryptogenic in three cases.

Freshly removed tissue samples were fixed in Bouin’s solution for 12–24 h and paraffin-embedded for histological analysis. In addition, a part of the tissue samples destined for RNA extraction was snap-frozen in liquid nitrogen immediately upon surgical removal and maintained at −80°C until use.

The studies were approved by the Human Subject Committee of the University of Bern.

Immunohistochemistry
Paraffin-embedded tissue sections (2–4 μm thick) were subjected to immunostaining using streptavidin–peroxidase for TGF-β1–3 and TβR-I (ALK-5) and TβR-II (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). Tissue sections were submerged for 15 min in Tris-buffered saline (TBS) (10 mM Tris–HCl, 0.85% NaCl, pH 7.4) containing 0.1% (v/v) Triton X-100, and were washed for 5 min in TBS solution, as previously reported [21,22]. Endogenous peroxidase activity was blocked by incubating the slides in methanol and in methanol/0.6% hydrogen peroxide, followed by washings in methanol and TBS containing 0.1% bovine serum albumin (BSA) [21,22]. Following treatment with hyaluronidase (1 mg/ml in 100 mM sodium acetate, 0.85% NaCl), the sections were incubated for 30 min at 23°C with 10% normal goat serum, and then incubated overnight at 4°C with isofrom-specific polyclonal antibodies detecting TGF-β1, TGF-β2 and TGF-β3 (2.5 μg/ml) diluted in 5% normal goat serum [21,22]. Bound antibody was detected with a biotinylated goat anti-rabbit IgG secondary antibody and a streptavidin–peroxidase complex (Kirkegaard & Perry Laboratories, Inc.), followed by incubation with diaminobenzidine tetrahydrochloride (DAB, 0.05%) as the substrate and counter-staining with Mayer’s haematoxylin.

To ensure antibody specificity, control slides were incubated either in the absence of primary antibody or with an irrelevant IgG antibody. In both cases no immunostaining was detected. The isofrom-specific antibodies that detected mature TGF-β1, TGF-β2 and TGF-β3 were produced in the laboratory of Leslie Gold [21,22]. Briefly, the antibodies were raised in rabbits to synthetic peptides corresponding to amino acid residues 4–19 of TGF-β1 and TGF-β2 and 8–29 of TGF-β3. Each antiserum was purified by ammonium sulphate precipitation followed by peptide affinity chromatography using the respective peptide as immunogen. The antisera were shown to be isofrom-specific by Western blot analysis using mature
recombinant TGF-β1 and TGF-β3 and porcine native TGF-β2 [23]. No cross-reactivity among the antisera was observed. Antibody activity was previously shown to be absorbed with 33 molar excess of each peptide used as immunogen. Each antiserum absorbed with the peptide showed no immunoreactivity, proving antibody specificity. The antibodies against TBR-I (ALK-5) and TBR-II were purchased from Santa Cruz Biotechnology. All tissue sections compared were treated at the same time using identical conditions. Negative controls included samples incubated without primary antibodies and samples incubated with non-specific IgG (rabbit) antibodies.

Northern blot analysis
Total RNA was extracted by the guanidine isothiocyanate method, size-fractionated on 1.2% agarose/1.8 M formaldehyde gels, and stained with ethidium bromide for verification of RNA integrity and loading equivalency [24,25]. The RNA was electrophoresed onto nylon membranes (Gene Screen, Du Pont, Boston, MA, USA) and cross-linked by UV irradiation [25]. The filters were then prehybridized, hybridized, and washed under conditions appropriate for specific antisense cRNA riboprobes (TGF-β2, TGF-β3, TBR-I, TBR-II, TBR-III) or cDNA probes (TGF-β1, 7S) [21,26,27].

In the case of the antisense riboprobes, the blots were prehybridized overnight at 65°C in 50% formamide, 0.5% sodium dodecyl sulphate (SDS), 5x SSC, 5x Denhardt’s solution (1x Denhardt’s = 0.02% ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA), 250 μg/ml salmon sperm DNA and 50 mM Na2PO4, pH 6.5. The blots were then hybridized for 18 h at 65°C in the presence of 1 x 10⁶ cpm/ml of the [α-32P]-labelled antisense riboprobe, washed twice at 65°C in a solution containing 1x SSPE (150 mM NaCl, 10 mM Na2HPO4 and 1 mM EDTA) and 0.5% SDS, and washed twice at 65°C in a solution containing 0.1x SSPE and 0.5% SDS [25].

In the case of cDNA probes, blots were prehybridized overnight at 42°C in a prehybridization buffer that contained 50% formamide, 1% SDS, 0.75 M NaCl, 5 mM EDTA, 5x Denhardt’s solution, 100 μg/ml salmon sperm DNA, 10% dextran sulphate and 50 mM Na2PO4, pH 7.4. The hybridization was carried out at 42°C for 18 h with the [α-32P]-labelled cDNA probe (1 x 10⁶ cpm/ml for TGF-β1, 1 x 10⁵ cpm/ml for 7S) and washed twice at 37°C in 0.2x SSC and three times at 55°C in 0.2x SSC and 2% SDS [25].

Blots were then exposed at -80°C to Fuji X-ray film using intensifying screens, and the intensity of the radiographic bands was quantified by laser densitometry (BioRad 620, New York, USA), as previously reported [25].

Probes and probe synthesis
The TGF-β1 cDNA probe consisted of a 280 bp EcoRI/XhoI fragment of human TGF-β1 cDNA [26,27].

The TGF-β2 cDNA probe consisted of a 600 bp HindIII/PstI fragment of human TGF-β2 cDNA [22,26]. The TGF-β3 cDNA probe consisted of a 125 bp XhoI/BglII fragment of human TGF-β3 cDNA. The TBR-I cRNA probe specifically detecting ALK-5 consisted of a 377 bp BamHI fragment of human TBR-I cDNA [28,29]. The TBR-II cRNA probe consisted of a 476 bp BamHI fragment of human TBR-II cDNA. The TBR-III cRNA probe consisted of a 723 bp BamHI fragment of human TBR-III cDNA [27].

Antisense cRNA probes were radiolabelled with [α-32P] CTP (Du Pont), and the cDNA probes were labelled with [α-32P] dCTP (Du Pont) [26,27]. Northern blots were also hybridized with a 190 bp BamHI fragment of the mouse 7S cytoplasmic cDNA that cross-hybridizes with human 7S RNA, in order to assess equivalent RNA loading [25,30]. [α-32P] dCTP was incorporated into cDNA probes using a random primer labelling system (Boehringer, Mannheim, Germany) [31].

Statistical analysis
Results are expressed as median and range or as mean ± SD (standard deviation). For statistical analysis, the Mann–Whitney U test was used [32]. Significance was defined as P < 0.05.

Results
Immunohistochemistry of TGF-βs and receptors
Immunostaining of TGF-β isoforms and receptors in the normal liver
Hepatocytes, bile duct epithelial cells and extracellular matrix were devoid of any immunostaining for TGF-β1, TGF-β2 and TGF-β3 (Fig. 1A,C,E). For TBR-I and TBR-II, there was moderate to strong immunoreactivity in the hepatocytes and bile duct epithelial cells (Fig. 2A,C).

Immunostaining of TGF-β isoforms and receptors in hepatic cirrhosis
There was no immunostaining in hepatocytes for any of the TGF-β isoforms, except for some hepatocytes located at the border of the fibrous tracts. TGF-β1–3 immunoreactivity was intense in bile duct and ductular epithelial cells (including ductular proliferations), however, and mild to moderate in a few sinusoidal cells and in inflammatory cells (Fig. 1B,D,F). With respect to TGF-β receptors, there was marked immunostaining of hepatocytes and of bile duct and ductular epithelial cells for TBR-II (Fig. 2D). The same pattern was found for TBR-I, but with lower intensity (Fig. 2B).

No difference in the expression of TGF-βs and their receptors was found among the different aetiologic subgroups of liver cirrhosis, except higher immunoreactivity for cases that showed marked ductular proliferation.
Northern blot analysis
To determine mRNA expression of TGF-βs and TGF-β receptors, Northern blot analysis of total liver RNA was performed (Fig. 3). Low levels of TGF-β1, TGF-β2 and TGF-β3 mRNA were observed in normal human liver specimens. In some of these samples, the transcripts corresponding to TGF-β1, TGF-β2 and TGF-β3 mRNA were visible only on the original autoradiograph and could not be readily photographed.

In the cirrhotic hepatic tissue samples, there was a marked increase in all three TGF-β mRNA moieties and in TβR-II
Immunohistochemical analysis of TGF-β receptors. (A,B) TβR-I (ALK-5) and (C,D) TβR-II immunostaining in the normal liver (A,C) and in hepatic cirrhosis (B,D). There is moderate to strong TβR-I (ALK-5) and TβR-II immunostaining of hepatocytes and of bile duct and ductular epithelial cells in the normal liver. The same immunohistochemical staining pattern was found in cirrhotic liver samples, with higher intensity for TβR-II, magnification x400.

mRNA. In contrast, TβR-I and TβR-III mRNA levels were unchanged in liver cirrhosis samples compared to normal controls. There was some heterogeneity among cirrhotic samples. This is because cirrhotic liver tissues with higher degrees of fibrosis and bile duct proliferation exhibited higher TGF-βs mRNA expression. However, the underlying aetiology of liver cirrhosis had no influence on the mRNA expression levels of TGF-βs and their receptors.

Densitometric analysis of the Northern blots indicated that, in comparison with the normal liver, in hepatic cirrhosis there were two-, three- and 8.5-fold increases in the mRNA encoding TGF-β1 (P < 0.05), TGF-β2 (P < 0.01) and TGF-β3 (P < 0.01), respectively. Analysis of the same samples showed a threefold increase only in TβR-II mRNA levels (P < 0.02), whereas for TβR-I and TβR-III the changes were not significant (Fig. 4).

**Discussion**

An increased synthesis and deposition of extracellular matrix proteins and proteoglycans characterizing hepatic...
TGF-β1  
TGF-β2  
TGF-β3  

TBR-I  
TBR-II  
TBR-III

Denitometric analysis of the Northern blots. The ratios of optical density between TGF-β1, TGF-β2, TGF-β3, TBR-I, TBR-II and TBR-III mRNA expression levels and the corresponding 7S signals were calculated for each sample. Bars represent the mean values (± SD).

Fibrosis is initiated both in the perisinusoidal space of Disse and in portal tracts. Typically, chronic injury to the liver results in high-density extracellular matrix in the perisinusoidal space [33] and activation of portal tract connective tissue, which is followed by the formation of fibrous septa. The primary sources of perisinusoidal extracellular matrix components are Ito cells and their myofibroblast-like derivatives [34,35], whereas the critical cells
involved in portal tract and septal fibrosis are still not well characterized. Initiation of Ito cell activation appears to be mediated by soluble factors released by other cells, followed by perpetuation of activation via autocrine and paracrine pathways [36].

The isoforms of the TGF-β family are the molecules currently considered responsible for the induction and modulation of fibrogenesis in various disorders [4]. In addition, in the liver, TGF-β1 plays an important role in epithelial cell turnover, regulating both hepatocyte proliferation [37] and apoptosis [38] and contributing to hepatocarcinogenesis [39]. TGF-β1 is abundantly expressed during regenerative growth, immune and inflammatory responses, and hepatic fibrosis [40,41]. Furthermore, TGF-β stimulates human Ito cell proliferation [42]; however, the source of this cytokine in the liver is still a matter of debate.

In the present study, TGF-β1 mRNA expression was found to be significantly higher in cirrhotic versus normal human liver tissues. Immunohistochemistry disclosed marked immunoreactivity for TGF-β1 in the cirrhotic liver, chiefly detectable in epithelial cells of bile ducts and ductules, including ductular proliferations. A less marked signal was found in sinusoidal and inflammatory cells. However, TGF-β immunostaining was absent in hepatocytes. These findings are in accordance with previous studies in liver fibrosis, which described the expression of this cytokine in bile duct cells, macrophages and mesenchymal cells but not in hepatocytes [43,44]. Although former studies demonstrated TGF-β1 expression in perisinusoidal cells [45], we did not detect TGF-β immunoreactivity there, except for weak immunoreactivity in sinusoidal cells, most probably Kupffer cells.

In the light of a stimulatory action of TGF-β1 on Ito cells, its presence in bile duct and ductular epithelial cells is of particular interest, both with respect to the anatomical relationship between these cells and possible or potential signal pathways. It is not clear how TGF-β, apparently produced by bile duct cells, interacts with Ito cells, which are located in different tissue compartments. Several possible mechanisms can be considered. First, the perisinusoidal space of Disse, which harbours Ito cells and myofibroblast-like cells, is in direct contact with portal tract lymphatics, which closely follow portal tract vessels, bile ducts and ductules [46,47]. Recently it has been proposed that lymphatic function plays a pivotal role in macromolecular transport in cases of deteriorated sinusoidal function, such as occurs in cirrhosis [48]. Second, in addition to occupying their well-known anatomical site, Ito cells and myofibroblasts appear to form an extralittoral compartment involving perivascular, periductal and periductular tissue in portal tracts and septa [49-51]. These particular features may represent the microenvironmental substrate for a direct interaction between TGF-β-producing portal tract cells and cells critically involved in fibrogenesis. Third, Ito cells could be a target for downstream effectors of TGF-β.

In the present study, TGF-β2 was increased twofold in the cirrhotic liver, while TGF-β3 showed an 8.5-fold increase in comparison with normal liver tissue. TGF-β3 exhibited the highest mRNA level among the three TGF-β isoforms tested, and was immunohistochemically detectable in bile duct and ductular cells (including ductular proliferation), in sinusoidal cells, and in non-specifically spindled cells of portal tracts. In contrast to a report on TGF-β3 expression in human liver allografts [52], we did not detect TGF-β3 immunoreactivity primarily in Ito cells of cirrhotic liver samples. Our immunohistochemical findings indicate that the three TGF-β isoforms exhibit a common expression pattern with respect to bile duct and ductular cells, but they differ regarding several non-epithelial cell systems in liver cirrhosis, suggesting that TGF-β3s are differentially expressed in human liver cirrhosis. Furthermore, the observation that TGF-β3 exhibited the highest level of mRNA expression may mean that this isoform plays a key role in hepatic fibrogenesis. However, marked up-regulation of TGF-β3 mRNA, and, to a lesser degree, the other two TGF-β isoforms may also be related to pathways other than fibrogenesis in cirrhosis, e.g. modulation of liver cell proliferation taking place in cirrhosis. With respect to the expression of TGF-β2 and TGF-β3 in bilocytes, we hypothetically favour the same mechanisms proposed for TGF-β1 (see above).

It is not clear whether portal tract spindle cells immunoreactive for TGF-β3 represent putative extralittoral myofibroblast-like cells involved in an autocrine pathway stimulating extracellular matrix. Clarification of this question requires a more precise identification of these cells, which is histologically difficult.

In cirrhotic liver tissue, a marked increase in TBR-II mRNA levels, but not of receptors I and III, was detectable. Immunohistochemically, a similar staining pattern was observed for receptors I and II. Immunoreactivity for both receptors was present in hepatocytes and epithelial cells of bile ducts and ductules, with higher intensity for TBR-II in cirrhotic liver.

In summary, the concomitant overexpression of all three TGF-β isoforms and TBR-II in liver cirrhosis, specifically in bile ducts and ductular cells, supports the importance of TGF-β signalling pathways in the pathogenesis of this disease, and suggests that TGF-β3 and TBR-II may be especially important in this regard.

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References


