BILIARY DISEASE

Pentoxifylline downregulates profibrogenic cytokines and procollagen I expression in rat secondary biliary fibrosis

C Raetsch, J D Jia, G Boigk, M Bauer, E G Hahn, E-O Riecken, D Schuppan

Background: The trisubstituted methylxanthine derivative pentoxifylline inhibits hepatic stellate cell proliferation and collagen synthesis in vitro. The antifibrotic effect of pentoxifylline in a suitable in vivo model of chronic liver fibrogenesis remains to be tested.

Methods: Groups of adult rats (n=20–23) received oral pentoxifylline at a dose of 8 mg/kg/day from week 1 to week 6, and 16 mg/kg/day from week 1 to week 6 or week 4 to week 6 after complete bile duct occlusion. Animals who underwent sham operation that received 16 mg/kg/day pentoxifylline and untreated rats with bile duct occlusion alone served as controls. After six weeks, animals were sacrificed and parameters of fibrogenesis determined.

Results: Bile duct occlusion caused portal cirrhosis with a 10-fold increased hepatic collagen content in the absence of inflammation or necrosis. This was accompanied by an 11-fold elevated serum amino-terminal procollagen III peptide (PIIINP). The drug induced a dramatic eightfold downregulation of procollagen I mRNA, and suppression of the fibrogenic factors transforming growth factor β1 and connective tissue growth factor by 60–70%. However, profibrogenic tissue inhibitor of metalloproteinase 1 (TIMP-1) mRNA was increased twofold, resulting in only a moderate decrease in liver collagen, fibrosis score, and PIIINP.

Conclusions: We conclude that targeting pentoxifylline to the fibrogenic cells, thereby avoiding upregulation of TIMP-1, could become a potent antifibrogenic tool in chronic liver disease.
METHODS
Rat model of secondary biliary cirrhosis

Adult female Wistar rats (Schoenwalde, Germany) weighing 206 ±19 g were maintained in 12 hour light/dark cycles at 23 ±2°C, with 60 ±10% humidity. Complete bile duct occlusion (BDO) was performed as reported previously. Briefly, after midline abdominal incision and proper isolation, the common bile duct was occluded by injection of sodium amidotriazole (Ethibloc 0.2 ml/kg body weight; Ethicon, Germany) in a retrograde direction using a Teflon catheter (Abbocath-T 26 G; Abbott, Chicago, Illinois, USA), followed by double ligation and siccision in-between. Sham operation was a midline abdominal incision, isolation of the common bile duct, and wound closure. PTX (Trental; Aventis, Germany) was added to the drinking water and did not alter fluid consumption by the animals. The following therapeutic groups were formed: (1) BDO and PTX at 16 mg/kg/day for six weeks (n=20); (2) BDO and 8 mg/kg/day PTX for six weeks (n=23); (3) BDO and PTX at 16 mg/kg/day from week 4 to week 6 (n=20); (4) BDO and PTX at 16 mg/kg/day from week 1 to week 6, or with PTX at 16 mg/kg/day from week 4 to week 6, respectively. Animals without treatment (n=21) served as controls (fig 1).

Sham operation and daily treatment with PTX at 16 mg/kg/day from week 1 to week 6, or with PTX at 16 mg/kg/day from week 4 to week 6, respectively. Animals who underwent sham operation and daily treatment with PTX at 16 mg/kg/day from week 1 to week 6 (sham PTX) served as controls.

Histological scoring

For each liver, 1 µm paraffin sections of the right and left lobe were stained with haematoxylin/eosin, trichrome (Masson-Goldner), and silver impregnation (Gomori), and scores (see below) of both lobes averaged. Inflammation and necrosis were graded according to the histological activity index of Knodell and colleagues. Staging of fibrosis followed the slightly modified method of Ruvart and colleagues which, contrary to conventional scores, is particularly suited for detecting slight changes in portal fibrosis: normal liver (0 points); increased collagen with some periportal stellate bile duct proliferations (1 point); increased collagen with incomplete septa that do not form portal-portal or portal-central connections (2 points); strongly increased collagen, more incomplete than complete septa (2.25 points); an equal number of incomplete and complete septa (2.5 points); more complete than incomplete septa (2.75 points); only complete septa (3); complete septa and less than 25% diffuse parenchymal fibrosis (3.25 points); complete septa and 25–50% diffuse parenchymal fibrosis (3.5 points); complete septa and 50–75% diffuse parenchymal fibrosis (3.75 points); and complete septa and more than 75% diffuse parenchymal fibrosis (4 points). Point scores from each animal were derived from the means of the right and left liver lobes.

Determination of hepatic hydroxyproline and serum aminoterminal procollagen III peptide

Hepatic HYP content was determined in duplicate from approximately 0.2 g hydrolysed tissue from the right and left liver lobes using the method of Jamall and colleagues with minor modifications. Liver collagen content was calculated from mean HYP concentration of both lobes (both lobes differed by less than 8%). Serum aminoterminal procollagen III peptide (PIIINP) was measured by a radioimmunoassay based on rat PIIINP, a monospecific rabbit antiserum to rat PIIINP and a goat antiserum to rabbit IgG, as detailed previously.

Table 1: Clinical, chemical, and fibrosis parameters in treated and untreated groups of rats

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<tr>
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<th>Sham 16 w 1–6</th>
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<th>BDO 16 w 4–6</th>
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<tr>
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<td>127* (108/162)</td>
<td>135* (106/161)</td>
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<td>26.0 (22/33)</td>
<td>26.5 (23/33)</td>
<td>26.0 (21/31)</td>
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<tr>
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<td>16.5 (16/23)</td>
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<td>262 (243/305)</td>
<td>312 (263/370)</td>
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<td>Bili (µmol/l)</td>
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<td>171 (162/192)</td>
<td>165 (145/180)</td>
<td>153 (128/178)</td>
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<tr>
<td>Crea (µmol/l)</td>
<td>42.0 (41/47)</td>
<td>32.0 (32/36)</td>
<td>34.0 (32/38)</td>
<td>32.0 (31/35)</td>
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<td>27.1 (6.8)</td>
<td>27.3 (11.5)</td>
<td>22.6 (8.9)</td>
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<td>25.1 (2.6)</td>
<td>23.3 (3.1)</td>
<td>21.4** (3.1)</td>
<td>24.9 (3.4)</td>
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<tr>
<td>Spleen weight (g)</td>
<td>0.63 (0.06)</td>
<td>1.83 (0.36)</td>
<td>1.60* (0.48)</td>
<td>1.38** (0.28)</td>
<td>1.81 (0.48)</td>
</tr>
</tbody>
</table>

Data are median [25th/75th percentiles].

* p<0.05, ** p<0.01 versus untreated group (BDO alone).
Antifibrotic effect of pentoxifylline in liver fibrosis

Figure 2 Effect of pentoxifylline (PTX) on parameters of liver fibrosis of normal and bile duct occluded (BDO) rats. (A) Total liver collagen expressed as total hydroxyproline. (B) Relative content of collagen, as hydroxyproline per gram of wet liver weight. (C) Modified histo score. (D) Serum aminoterminal procollagen type III peptide (PIIINP). Sham 16 w 1–6, sham operation and PTX at 16 mg/kg/day for six weeks; BDO w 1–6, bile duct occlusion for six weeks; BDO 16 w 1–6, bile duct occlusion and PTX at 16 mg/kg/day for six weeks; BDO 8 w 1–6, bile duct occlusion and PTX at 8 mg/kg/day for six weeks; BDO 4 w 1–6, PTX at 16 mg/kg/day from week 4 to week 6. Significant differences were calculated using the Mann-Whitney rank sum test, and data are presented as box plots, with medians and boxes representing the 25th and 75th percentiles. *p<0.05, **p<0.01 versus the untreated group (BDO alone).

cDNA probes and multiprobe RNase protection assay

A 1.3 kb PstI/HindIII fragment of plasmid α1R1 containing cDNA encoding rat procollagen α1(I), kindly provided by Dr D Rowe (Department of Pediatrics, University of Connecticut Health Center, Farmington, Connecticut 06032, USA), was subcloned into pGEM1, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TGF-β1 plasmids were gifts from Dr XL Tian (Max Delbrueck Centre for Molecular Medicine, Berlin-Buch, Germany). For generation of all other probes, rat liver total RNA was reverse transcribed with SuperscriptII Reverse Transcriptase (Gibco Life Technologies, Freiburg, Germany). For generation of all other probes, rat liver total RNA was reverse transcribed with SuperscriptII Reverse Transcriptase (Gibco Life Technologies, Freiburg, Germany) and oligo-dT primer according to the manufacturer’s instructions. The cDNA for rat TIMP-1 was generated by polymerase chain reaction (PCR) using a mixture of Taq and Pfu DNA polymerases (Gibco and Stratagene, Amsterdam, the Netherlands) and primers binding to positions 109–133 and 415–439 of the published sequence. Similarly, 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 398–417 and 806–827 of the mouse CTGF sequence (GenBank accession No M70642). PCR products were cloned into the EcoRI site of pZErO-1 and sequenced on both strands. The rat specific sequence of the CTGF amplicon has been submitted to GenBank/EMBL Data Libraries with accession No AJ236872. The rat protein displays 95% homology to the mouse, human, and porcine protein.

Liver total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform method, and its integrity documented by visualisation of 18S and 28S ribosomal bands after electrophoresis. Radiolabelled RNA probes were produced by in vitro transcription with T7 polymerase (Ambion, Austin, Texas, USA) using [α-32P]UTP (800 Ci/mmol, 10 mCi/ml; NEN, Boston, Massachusetts, USA). Specific activity of all radiolabelled transcripts was usually about 10×106 cpm/µl. RNA was incubated with 2.0–4.0×105 cpm of ·32P labelled RNA probes, denatured at 90°C, and hybridised overnight at a temperature optimised by preliminary experiments (range 43–46°C). After hybridisation, RNase T1 (Ambion) was added to digest unbound label and unprotected mRNA. The protected RNA-RNA hybrids were denatured and separated by electrophoresis through a 5% polyacrylamide/urea sequencing gel. Gels were exposed to x-ray films for 16–24 hours, and autoradiographs analysed with the public domain NIH Image program. Signals for procollagen α1(I) and TIMP-1 mRNA were normalised to the GAPDH mRNA signal and expressed as relative abundance (arbitrary units). Expected RNAase protected transcript sizes were as follows: procollagen α1(I) 230 bp (from BamHI to RsaI site, M11432, M12198, and M12199); TIMP-1 264 bp (from position 176 to 439 bp, L29512); CTGF approximately 430 bp (the rat CTGF sequence homologous to positions 398–827 of mouse CTGF); TGFB-1 253 bp (from position 1197 to 1449 in X52498); and GAPDH 102 bp (from position 335 to 436 bp, M17701).

Statistics

Data are presented as mean (SD) and median (25th/75th percentiles). Statistical analysis was performed using the Mann-Whitney rank sum test and ANOVA. Differences in relative abundance of mRNAs were analysed using the Kruskal-Wallis test.

RESULTS

A total of 84 of 93 rats survived beyond day 4 after BDO (early mortality 9%, with no mortality thereafter). Groups of 20–23 surviving rats received either no PTX (untreated BDO controls), PTX at a dose of 8 mg/kg/day (low), or 16 mg/kg/day (high) for six weeks, or PTX (high) from week 4 to week 6 of BDO. Ten sham operated rats given PTX (high) served as normal controls (fig 1). All rats with BDO became icteric after a few days and displayed severe secondary biliary fibrosis at sacrifice. Body weights increased in all groups over the six weeks but were significantly lower only in rats with BDO that received PTX (high) (table 1). Liver and spleen weights were elevated 2–3-fold in all rats with BDO compared with

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DISCUSSION

We previously showed that experimental liver fibrosis due to complete obstruction of the biliary tract is a suitable model for testing drugs that inhibit hepatic fibrogenesis in vivo. This model is characterised by a progressive portal fibrosis accompanying the proliferating bile ducts. As inflammation and necrosis are virtually absent, a significant reduction in liver collagen in this model should allow better selection of true antifibrotic and not merely anti-inflammatory or radical scavenging agents that could be useful in human liver fibrosis.

Figure 3  Modulation of hepatic procollagen α1(I) [pro α1 (I)], tissue inhibitor of metalloproteinase 1 (TIMP-1), transforming growth factor β (TGF-β1), and connective tissue growth factor (CTGF) mRNA expression by pentoxifylline (PTX) treatment. RNA from livers of five rats in each experimental group was extracted and analysed for pro α1 (I), TIMP-1, TGF-β1, CTGF and GAPDH mRNA content by multiprobe ribonuclease protection assays. The protected bands showed the expected sizes and there were no equivalent bands in the negative controls. After six weeks of BDO, hepatic procollagen α1(I), TIMP-1 and TGF-β1 mRNA, normalised to GAPDH mRNA, were highly increased relative to the sham operated controls (all p<0.001) (fig 3). PTX downregulated the 20-fold increased procollagen α1(I) mRNA by a factor of eight (p<0.001) whereas it upregulated TIMP-1 mRNA twofold (p<0.05). TGF-β1 and CTGF transcript levels were decreased by approximately 60–70% (fig 3).

Alkaline phosphatase (ALP), gamma glutamyl transpeptidase (γGT), and bilirubin were increased 3.5, 9, and 27-fold, respectively, in the BDO compared with the sham operated animals. These cholestasis parameters were not affected by treatment with PTX (table 1). Similarly, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), reflecting inflammation and necrosis, did not differ among groups except for a moderate elevation in AST in rats that received both doses of PTX for six weeks (p<0.05).

To investigate if one of the serum parameters reflected the decrease in hepatic collagen accumulation in animals treated with PTX, correlations were calculated using the Spearman rank order test (table 2). Liver hydroxyproline correlated well with PTX, correlations were calculated using the Spearman rank order test (table 2). Liver hydroxyproline correlated well with PTX (table 1). Similarly, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), reflecting inflammation and necrosis, did not differ among groups except for a moderate elevation in AST in rats that received both doses of PTX for six weeks (p<0.05).

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Chymal cell proliferation and collagen synthesis in vitro. PTX, an inhibitor of phosphodiesterases, can inhibit mesen-
ligation for only three days. PTX (50 mg/kg body weight) in rats 24 hours prior to bile duct ligation for only three days.

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ligation for only three days. PTX (50 mg/kg body weight) in rats 24 hours prior to bile duct ligation for only three days.

Table 2 Correlations between the investigated parameters. Coefficients were calculated using the Spearman rank order correlation, and highly significant correlations (p<0.01) are printed in bold.

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<th>BW</th>
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<th>Spleen</th>
<th>Kidneys</th>
<th>Heart</th>
<th>AST</th>
<th>ALT</th>
<th>γ-GT</th>
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BW, per cent increase in body weight over the six weeks of the experiment; Hyp, hepatic hydroxyproline (mg); liver/spleen/kidneys/heart (organ weights in g); AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GT, gamma glutamyltransferase; ALP, alkaline phosphatase; Bili, total bilirubin; Crea, creatinine; ΔBW, difference in body weight; Histo, histo score (points); Hyp, hydroxyproline; PIIINP, serum aminoterminal propeptide of procollagen type III.

Figure 4 Correlations between total liver collagen, aminoterminal propeptide of procollagen type III (PIIINP), and the histo score. Correlations were calculated using the Spearman rank order correlation, as described in the methods.

Antifibrotic effect of pentoxifylline in liver fibrosis

Several studies showed that the methylxanthine derivative PTX, an inhibitor of phosphodiesterases, can inhibit mesenchymal cell proliferation and collagen synthesis in vitro. Most of these investigations had been performed with dermal fibroblasts but recently several groups confirmed these effects of PTX in rat and human hepatic stellate and myofibroblast cell cultures. Previous in vivo studies used porcine liver fibrosis subsequent to feeding of yellow phosphorus which bears little similarity to human disease, or rat carbon tetrachloride induced fibrosis, a model that involves free radicals and severe necroinflammation of the pericentral zone. Importantly, the numbers of animals in these studies were low. A recent report investigated the effect of a single intraperitoneal injection of PTX (50 mg/kg body weight) in rats 24 hours prior to bile duct ligation for only three days. The authors demonstrated an antiproliferative effect of PTX on hepatic myofibroblasts in vivo but did not investigate fibrosis. A single study suggested no significant antifibrotic effect of PTX (30 mg/kg/day for four weeks) in rats with bile duct ligation. However, lower numbers of animals were used, fibrosis and cholestasis were less advanced, and apart from morphometry, no biochemical collagen determinations or investigations of matrix metabolism were carried out. Therefore, we studied the antifibrotic efficacy of PTX and parameters of matrix turnover in our model of chronic BDO.

We showed that PTX, when given orally at a dose of 16 mg/kg/day, reduced total hepatic collagen accumulation moderately but significantly whereas the effect of half the dose did not reach statistical significance. In contrast with its moderate antifibrotic effect, PTX potently downregulated hepatic steady state levels for procollagen α1(I) mRNA, which encodes the major collagen deposited during fibrogenesis, from 20-fold to 2.5-fold above baseline in bile duct obstructed rats. This eightfold reduction in procollagen α1(I) mRNA is the most dramatic effect of a drug on procollagen I expression in this progressive model of fibrosis and, to our knowledge, unmatched in any other in vivo model of hepatic fibrosis. It was shown before that apart from being an antiproliferative drug for mesenchymal cells, PTX also potently suppresses interstitial procollagen expression in vitro. In rat biliary fibrosis, only silymarin, a mixture of well defined flavonoids, and LU135252, a specific endothelin A receptor antagonist, inhibited collagen accumulation and procollagen α1(I) mRNA expression by up to 60%. However, in contrast with these two drugs, PTX upregulated expression of TIMP-1, the most important endogenous inhibitor of most matrix metalloproteinases, more than twofold in rats with BDO, whereas silymarin and LU135252 suppressed TIMP-1 three and twofold, respectively. These findings strongly suggest that in vivo, upregulation of TIMP-1 expression by PTX counteracts its powerful suppressive effect on procollagen I expression, resulting in the observed modest net reduction in fibrosis. While PTX downregulates TIMP-1 in fibroblasts and culture activated hepatic stellate cells, it could upregulate TIMP-1 mRNA expression in hepatocytes, bile duct epithelia, and Kupffer cells which do not express procollagen I but could upregulate TIMP-1. However, in contrast with these two drugs, PTX upregulated expression of TIMP-1, the most important endogenous inhibitor of most matrix metalloproteinases, more than twofold in rats with BDO, whereas silymarin and LU135252 suppressed TIMP-1 three and twofold, respectively. These findings strongly suggest that in vivo, upregulation of TIMP-1 expression by PTX counteracts its powerful suppressive effect on procollagen I expression, resulting in the observed modest net reduction in fibrosis. Thus our findings caution against extrapolation of in vitro data using activated stellate cells or myofibroblasts alone for the far more complex in vivo situation of fibrogenesis.

The effect of PTX on procollagen I mRNA expression is even more remarkable when we consider that the higher dose of the drug did not exceed the upper dose that is usually prescribed for humans to improve the macro and microcirculation. The published plasma levels at this oral dose range from 2 to 5 µg/ml and are thus below concentrations that are required to suppress collagen synthesis of activated hepatic stellate cells and myofibroblasts in vitro, reaching 27% (at 100 µg/ml) or

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67% (at 500 μg/ml). In other in vivo experimental settings such as thrombin induced platelet activation, this was explained by an as yet undetermined in vivo accumulation or activation of the drug. At the applied dosage, PTX did not cause hepatic inflammation or necrosis while AST was slightly elevated in the groups that received PTX over six weeks (table 1). Similarly, PTX did not change the laboratory parameters of cholestasis, suggesting that its antifibrotic properties are unrelated to an effect on hepatobiliary function but rather due to a direct impact on the effector cells of hepatic fibrogenesis—that is, activated stellate cells and (portal tract derived) myofibroblasts. This is supported by Desmouliere and colleagues who found significantly reduced myofibroblast proliferation in rats with bile duct occlusion, two key markers of fibrogenesis that act in concert (13, 16–18, 1999) (Gastroenterology 1999;118:A1227).

ACKNOWLEDGEMENT

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Part of this work was presented in abstract form at the meeting of the American Gastroenterological Association, Orlando, Florida, May 16–19, 1999 (Gastroenterology 1999;118:A1227).

REFERENCES

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