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Precision-cut liver slices as a model for the early onset of liver fibrosis to test antifibrotic drugs

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ABSTRACT

Induction of fibrosis during prolonged culture of precision-cut liver slices (PCLS) was reported. In this study, the use of rat PCLS was investigated to further characterize the mechanism of early onset of fibrosis in this model and the effects of antifibrotic compounds. Rat PCLS were incubated for 48 h, viability was assessed by ATP and gene expression of *PDGF-B* and *TGF-β1* and the fibrosis markers *Hsp47*, *αSma* and *Pcol1A1* and collagen1 protein expressions were determined. The effects of the antifibrotic drugs imatinib, sorafenib and sunitinib, PDGF-pathway inhibitors, and perindopril, valproic acid, rosmarinic acid, tetrandrine and pirfenidone, TGFβ-pathway inhibitors, were determined. After 48 h of incubation, viability of the PCLS was maintained and gene expression of *PDGF-B* was increased while *TGF-β1* was not changed. *Hsp47*, *αSma* and *Pcol1A1* gene expressions were significantly elevated in PCLS after 48 h, which was further increased by PDGF-BB and TGF-β1. The increased gene expression of fibrosis markers was inhibited by all three PDGF-inhibitors, while TGFβ-inhibitors showed marginal effects. The protein expression of collagen 1 was inhibited by imatinib, perindopril, tetrandrine and pirfenidone. In conclusion, the increased gene expression of *PDGF-B* and the down-regulation of fibrosis markers by PDGF-pathway inhibitors, together with the absence of elevated *TGF-β1* gene expression and the limited effect of the TGFβ-pathway inhibitors, indicated the predominance of the PDGF pathway in the early onset of fibrosis in PCLS. PCLS appear a useful model for research of the early onset of fibrosis and for testing of antifibrotic drugs acting on the PDGF pathway.

Introduction

Liver fibrosis is the progressive accumulation of connective tissue that affects the normal function of the liver and eventually leads to liver cirrhosis (Bataller and Brenner, 2005). Hundreds of millions of patients are affected by cirrhosis worldwide (Friedman, 2003). As antifibrotic drugs are currently not available, patients can only be treated by organ transplantation. To improve and accelerate the drug discovery and development process, there is an urgent need for reliable in vitro methods to test the efficacy of potential antifibrotic compounds, as in vivo experiments use a large number of animals with considerable discomfort, and are expensive and time consuming.

During fibrosis, different signaling pathways are activated. The TGFβ/Smad signaling pathway is stimulated by transforming growth factor β (TGFβ), which is secreted by hepatic stellate cells (HSC), Kupffer cells, hepatocytes and platelets (Dooley and Ten Dijke, 2012; Friedman, 2008; Liu et al., 2009; Tsukada et al., 2006). In addition, upon activation, macrophages and HSC also produce platelet derived growth factor (PDGF), triggering the PDGF signaling pathway (Deng et al., 2009; Friedman, 2008; Liu et al., 2009; Tsukada et al., 2006). These pathways are activated upon chronic liver injury, which causes damage of endothelial cells and apoptosis of hepatocytes. In addition to the activation of the TGFβ and PDGF secretion, these injured cells recruit inflammatory cells to the injured liver (Cong et al., 2012). HSC play an essential role in the onset and progression of liver fibrosis as activation by TGFβ causes them to proliferate and to produce extracellular matrix components (Borkham-Kamphorst et al., 2004). In addition, PDGF is a stimulator of HSC growth which is further stimulated by an increased expression of the PDGF receptor β in activated HSC (Borkham-Kamphorst et al., 2004). Thus, there is a continuous interaction between HSC, hepatocytes and Kupffer cells during the onset and progression of liver fibrosis. To study the process of fibrosis in vitro in a physiologic milieu and to test antifibrotic drugs, it is important to use an in vitro system, which closely reflects the in vivo situation. In precision-cut liver slices (PCLS) all cell types of the liver are present in their original context and remain viable up to 48 h (van de Bovenkamp et al., 2005). As each slice contains ca 70–100 lobules, the acinar heterogeneity of the liver or inhomogeneous distribution of portal spaces

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plays only a minor role in the variability of the results in this ex vivo model. Furthermore, the possibility to make PCLS of human liver tissue makes this model very promising to investigate fibrosis in the human liver. Previously, we and others (van de Bovenkamp et al., 2008; Vickers et al., 2004) showed that a fibrotic process is initiated during prolonged culture of PCLS. Moreover, van de Bovenkamp et al. showed the antifibrotic effect of imatinib, pentoxifyllin and dexamethasone in fibrotic rat PCLS, prepared from bile duct ligated rats, and these compounds decreased the gene expression of specific fibrosis markers heat shock protein 47 (*Hsp47*), α-smooth muscle actin (*αSma*) and pro-collagen 1A1 (*Pcol1A1*) (van de Bovenkamp et al., 2006).

In the current study, prolonged incubation of the rat PCLS was used to induce the onset of fibrosis (van de Bovenkamp et al., 2008). Fibrogenesis was measured by determining the expression of genes and proteins that indicate the activation of HSC and (myo) fibroblasts, using gene and protein expression of specific markers of fibrosis as previously described (van de Bovenkamp et al., 2006). To investigate the involvement of the two main fibrosis pathways, the gene expressions of *TGF-β1* and *PDGF-B*, in addition to *Ctgf* were measured, as well as the effects of exposure of the PCLS to exogenous TGF-β1 and PDGF-BB.

The aim of the present study was to investigate the mechanism of the early onset of fibrosis in prolonged culture of PCLS, and to determine if this in vitro model can be used to test antifibrotic compounds. The antifibrotic compounds used in this study were all selected based on the published effects in vivo in animals and/or in vitro in cell lines and primary cells. The concentrations used in this study correspond with the concentrations utilized in experiments with cell cultures. The compounds were selected to represent different modes of action, reflecting the main pathways involved in liver fibrosis, the PDGF and TGFβ signaling pathways. The compounds mainly inhibiting the PDGF pathway (PDGF-inhibitors) were imatinib, sorafenib and sunitinib. Imatinib is a competitive inhibitor of the tyrosine kinases PDGF receptor, Bcr-Abl and c-Kit (Buchdunger et al., 2000; Neef et al., 2006). Sorafenib is a receptor tyrosine kinase inhibitor that targets the PDGF receptor and the Raf/ERK signaling pathway (Wang et al., 2010). Sunitinib, the third tyrosine kinase inhibitor used in this study, targets amongst others the PDGF receptor, c-Kit and the VEGF receptor (Minkin et al., 2008). In this study, rosmarinic acid, perindopril, valproic acid, tetrandrine and pirfenidone were investigated as the inhibitors known to be mainly acting on the TGFβ signaling pathway (TGF-inhibitors). Rosmarinic acid naturally occurs in many medicinal species of the plant Lamiaceae (mint family) (Li et al., 2010; Mannaerts et al., 2010; Osakabe et al., 2004) and it has been shown that rosmarinic acid has antifibrotic effects that were achieved through the inhibitory effect on TGFβ (Domitrovic et al., 2012; Zhang et al., 2011). In addition, perindopril, an angiotensin converting enzyme (ACE) inhibitor and valproic acid, a histone deacetylase inhibitor, has been shown to decrease the TGFβ expression (Helmy et al., 2000; Watanabe et al., 2011). Tetrandrine is an alkaloid that is isolated from the Chinese medicinal herb *Stephania tetrandra* (Chen et al., 2005; Hsu et al., 2007). Tetrandrine's antifibrotic effect is at least partially caused by an up-regulation of Smad 7, which in turn blocks the TGFβ expression and its downstream signaling (Chen et al., 2005). Pirfenidone acts as an antifibrotic agent, by down-regulating the gene expression of *TGFβ* (Schaefer et al., 2011). To investigate the effect of direct inhibition of collagen disposition, the drug colchicine, known to have a direct effect on collagen disposition by causing disruption of microtubule formation and inhibition of collagen transport and synthesis, was investigated (Chung and Kang, 1999; Lee et al., 2004).

The expression of *PDGF-B* and *TGF-β1* and the specific properties of the compounds under investigation, enabled us to assess the involvement of the PDGF and TGFβ pathways in the onset of fibrosis during prolonged incubation of PCLS.

Materials and methods

Slice experiments. Livers of adult male Wistar rats (Harlan PBC, Zeist, The Netherlands) anaesthetized with isoflurane/O_2 (Nicholas Piramal, London, UK) were freshly isolated and used for preparing liver slices in ice-cold Krebs–Henseleit buffer supplemented with 25 mM D-glucose (Merck, Darmstadt, Germany), 25 mM $NaHCO_3$ (Merck), 10 mM HEPES (MP Biomedicals, Aurora, OH, US0041) and saturated with carbogen (95% O_2/5% CO_2) using a Krumdieck tissue slicer as described before in detail (de Graaf et al., 2010). PCLS with a diameter of 5 mm and a thickness of 250 μm were incubated individually in 1.3 ml of Williams Medium E (with L-glutamine, Invitrogen, Paisly, Scotland) supplemented with 25 mM glucose and 50 μg/ml gentamycin (Invitrogen) at 37 °C and under continuous supply of 95% O_2/5% CO_2 in 12-well plates while gently shaken. After 1 h of preincubation the slices were transferred to fresh medium and further incubated for 24, 48 and 72 h and for 48 h with antifibrotic compounds. After 24 and 48 h the slices were transferred to new 12 well plates with fresh medium containing the drug. The slices were incubated with the antifibrotic compounds imatinib (1–10 μM) (Novartis, Basel, Switzerland), valproic acid (0.1–1 mM) (Sigma Aldrich, Zwijndrecht, Netherlands), perindopril (10–100 μM) (Sigma Aldrich), pirfenidone (0.5–2.5 mM) (Sigma Aldrich), rosmarinic acid (120–270 μM) (Sigma Aldrich), colchicine (30–200 nM) (Sigma Aldrich), tetrandrine (1–10 μM) (Sigma Aldrich), sunitinib (0.5–5 μM) (LC laboratories, Woburn, USA) and sorafenib (0.5–2 μM) (LC laboratories). Stock solutions of the compounds were prepared in water or DMSO and diluted in the culture medium with a final concentration of the solvent of ≤1%. Before the incubations of PCLS with TGF-β1 (1–5 ng/ml) (hTGF-β1, Roche Applied Science, Mannheim, Germany) the 12 well plates were pretreated with 10% BSA in Milli Q water solution for 20 min, whereafter the solution was removed and plates were air dried, in order to prevent non-specific binding of the TGF-β1 to the walls of the plates. Incubations with PDGF-BB (10 and 50 ng/ml) (Recombinant Human PDGF-BB, Peprotech, Bioconnect) were performed as described above for the antifibrotic compounds. All incubations were performed in triplicate (using 3 slices incubated individually in separate wells) and were repeated with livers from 3-x different rats. The rats were housed on a 12 hour light/dark cycle in a temperature-and-humidity-controlled room with food (Harlan chow no 2018, Horst, The Netherlands) and water ad libitum. The animals were allowed to acclimatise for at least seven days before the start of the experiment and all animals received human care. The experiments were approved by the Animal Ethical Committee of the University of Groningen.

Viability. After incubation, slices were transferred to a 1 ml sonication solution, containing 70% ethanol and 2 mM EDTA, and snap frozen in liquid nitrogen and stored at −80 °C. To determine the cell viability, ATP levels were measured in the supernatant of samples sonicated for 45 s and centrifuged for 2 min at 13,000 rpm, using the ATP bioluminescence kit (Roche diagnostics, Mannheim, Germany). ATP values (pmol) were normalized to the total protein content (μg) of the slice estimated by Lowry (BIO-rad RC DC Protein Assay) (Bio Rad, Veenendaal, The Netherlands) (Lowry et al., 1951). Values displayed are relative values compared to the related controls.

Gene expression. To determine the antifibrotic effect of the drugs, gene expressions of fibrosis markers were determined using Real-Time PCR. The triplicate slices were pooled and snap frozen and total RNA was isolated with the use of the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). The amount of isolated RNA was measured with the ND-1000 spectrophotometer (Fisher Scientific, Landsmeer, The Netherlands).

Reverse transcriptase was performed with 2 μg RNA using Reverse Transcription System (Promega, Leiden, The Netherlands). The RT-PCR reaction was performed in the Eppendorf mastercycler gradient at 25 °C for 10 min, 45 °C for 60 min and 95 °C for 5 min.