Human precision-cut liver slices as a model to test antifibrotic drugs in the early onset of liver fibrosis

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ABSTRACT

Liver fibrosis is the progressive accumulation of connective tissue ultimately resulting in loss of organ function. Currently, no effective antifibrotics are available due to a lack of reliable human models. Here we investigated the fibrotic process in human precision-cut liver slices (PCLS) and studied the efficacy of multiple putative antifibrotic compounds.

Our results demonstrated that human PCLS remained viable for 48 h and the early onset of fibrosis was observed during culture, as demonstrated by an increased gene expression of Heat Shock Protein 47 (HSP47) and Pro-Collagen 1A1 (PCOL1A1) as well as increased collagen 1 protein levels. SB203580, a specific inhibitor of p38 mitogen-activated protein kinase (MAPK) showed a marked decrease in HSP47 and PCOL1A1 gene expression, whereas specific inhibitors of Smad 3 and Rac-1 showed no or only minor effects. Regarding the studied antifibrotics, gene levels of HSP47 and PCOL1A1 could be down-regulated with sunitinib and valproic acid, while PCOL1A1 expression was reduced following treatment with rosmarinic acid, tetrandrine and pirfenidone. These results are in contrast with prior data obtained in rat PCLS, indicating that antifibrotic drug efficacy is clearly species-specific.

Thus, human PCLS is a promising model for liver fibrosis. Moreover, MAPK signaling plays an important role in the onset of fibrosis in this model and transforming growth factor beta pathway inhibitors appear to be more effective than platelet-derived growth factor pathway inhibitors in halting fibrogenesis in PCLS.

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1. Introduction

Fibrosis is an integral part of the pathophysiological mechanism of a diverse range of chronic diseases, such as Crohn's disease, chronic kidney disease and viral hepatitis. The fibrotic process is characterized by augmented production and excessive deposition of extracellular matrix proteins resulting in scar formation and the progressive loss of organ function. Liver cirrhosis, the end stage of liver fibrosis, is possibly the most clinically relevant form of tissue fibrosis in the world due to the high prevalence of viral hepatitis (Zeisberg and Kalluri, 2013). Consequently, liver fibrosis is widely studied and there is extensive knowledge regarding the process of fibrogenesis (Pellicoro et al., 2014). And throughout the years a plethora of potential therapeutic targets have been described (Schuppan and Kim, 2013). Nevertheless, an effective therapy for liver fibrosis remains elusive, and transplantation remains the sole successful treatment modality. Moreover, antifibrotic drug discovery is hampered by the lack of reliable and reproducible (human) in vitro models. Recently, precision-cut tissue slices have been used as a model for incipient and established fibrosis (Westra et al., 2013; Stribos et al., 2015). Of note, this model replicates most of the multicellular characteristics of organs and the different cells are retained in their original environment. Previously, rat and murine precision-cut liver slices (PCLS) have been successfully used to test the antifibrotic efficacy of several putative antifibrotic drugs (van de Bovenkamp et al., 2006a; Westra et al., 2014a, 2014b; Iswandana et al., 2016). Here we report the use of human PCLS to test antifibrotic compounds.

There are several well-known common signaling pathways involved in the fibrotic process in all organs, including the archetypical transforming growth factor beta (TGF-β) and platelet-derived growth factor (PDGF) pathways as well as the p38 mitogen-activated protein kinase (MAPK) pathway (Zeisberg and Kalluri, 2013; Bonner, 2004; Parsons et al., 2007). Even though these pathways are generally involved in fibrogenesis, there remain tissue-, species- and strain-specific
differences (Zeisberg and Kalluri, 2013; Liu et al., 2013; Inoue et al., 2015). In general, TGF-β signaling is associated with an increased deposition of collagen, whereas PDGF is a potent mitogen affecting cells of mesenchymal origin, such as myofibroblasts (Bonner, 2004). Both growth factors activate a myriad of transcription factors by binding to their respective receptors (e.g. type 1 TGF-β receptor or receptor tyrosine kinase; (Liu, 2011)). TGF-β acts mainly via Smad signaling, which can be targeted by the specific inhibitor of Smad3 (Sis3), known to inhibit TGF-β-induced Smad3 phosphorylation (Jinnin et al., 2006). PDGF stimulates cell proliferation via a host of downstream intracellular signaling cascades involving for instance glycogen synthase kinase 3β or Rho GTPases (e.g. Rac1). The latter can be specifically inhibited by NSC23766 (Gao et al., 2004). MAPK is involved in the regulation of collagen 1α1 gene expression and mRNA stability, and can be activated by both PDGF and TGF-β (Parsons et al., 2007). SB203580 is a known inhibitor of MAPK (Cuenda et al., 1995). Thus, next to studying the efficacy of antifibrotic compounds, the current study was also designed to identify the pathways underlying the (early) fibrotic response in healthy human PCLS using these pathway specific inhibitors.

2. Methods

2.1. Ethics statement

This study was approved by the Medical Ethical Committee of the University Medical Centre Groningen (UMCG), according to Dutch legislation and the Code of Conduct for dealing responsibly with human tissue in the context of health research (www.federa.org), refraining the need of written consent for ‘further use’ of coded-anonymous human tissue. The procedures were carried out in accordance with the experimental protocols approved by the Medical Ethical Committee of the UMCG.

2.2. Chemicals

All chemicals were obtained from Sigma Aldrich ( Zwijndrecht, The Netherlands) unless stated otherwise. Stock solutions were prepared in either milli-Q or dimethyl sulfoxide (DMSO) and stored at −20 °C. During experiments, stocks were diluted in culture medium with a final solvent concentration of ≤1%.

2.3. Human liver tissue

Healthy human liver tissue was obtained either from patients following partial hepatectomy due to metastatic colorectal cancer (PH-livers) or from donors, remaining as surgical surplus after reduced-size liver transplantation (TX), as described previously (Ellerink et al., 2011). Clinical characteristics of study subjects who provided liver tissue are listed in Table 1. Of note, donor variability has limited impact on the fibrotic response in our model (van de Bovenkamp et al., 2006b).

2.4. Precision-cut liver slice preparation and experimental treatment

All liver tissue was perfused with cold University of Wisconsin (UW) organ preservation solution (DuPont Critical Care, Waukegab, IL, USA) at the time of collection and stored in ice-cold UW solution until use (de Graaf et al., 2010). Liver slices were prepared in ice-cold Krebs–Henseleit buffer supplemented with 25 mM D-glucose (Merck, Darmstadt, Germany), 25 mM NaHCO3 (Merck), 10 mM HEPES (MP Biomedicals, Aurora, OH, USA) and saturated with carbogen (95% O2, 5% CO2) using a Krumdieck tissue slicer as previously described (de Graaf et al., 2010). In addition, slices were kept on ice-cold UW solution before culture, during which time viability and metabolic activity was maintained as described before (Olinga et al., 1998). PCLS—diameter: 5 mm, thickness: 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 under continuous supply of 95% O2, 5% CO2 in 12- well plates while gently shaken. After 1 h of preincubation the slices were transferred to new plates with fresh medium and subsequently incubated for 24 or 48 h in the presence or absence of antifibrotic compounds. Medium was refreshed every 24 h. PCLS were treated with antifibrotics demonstrated to be effective in previous studies utilizing animal models, primary human cells and/or cell lines (Westra et al., 2014a; Jinnin et al., 2006; Tsukada et al., 2005; Xu et al., 2009) i.e. imatinib (Novartis, Basel, Switzerland), sorafenib (LC laboratories, Woburn, USA), sunitinib (LC laboratories), perindopril, valproic acid, rosmarinic acid, tetrandrine, pirfenidone and the specific MAPK inhibitor SB203580 (Biococonnect, Huisken, The Netherlands), the Smad 3 inhibitor Sis3 (Biococonnect) and the Rac1 inhibitor NSC23766 (Tocris Bioscience, Bristol, UK). For the tested concentrations see Table 2 and to illustrate clinically relevant levels, the maximum serum concentration (Cmax) of compounds tested in humans is also provided. Furthermore, the PCLS were incubated with the growth factors PDGF-BB (10 and 50 ng/ml; Recombinant Human PDGF-BB, Peprotech, Biococonnect) and TGF-β1 (1 – 5 ng/ml; hTGF-β1, Roche Applied Science, Mannheim, Germany). Non-specific binding of TGF-β1 was prevented by preincubating the culture plates with 10% BSA in milli-Q for 20 min, whereafter the solution was removed and plates were dried at room temperature. All experiments were performed in triplicate (technical replicates) using liver tissue from 3 to 5 different subjects.

2.5. Histomorphological examination

Integrity of the slices was assessed by immunohistochemistry as previously described (de Graaf et al., 2000). In short, PCLS were fixated with 70% ethanol at 4 °C for 24 h, and subsequently rehydrated in successive baths of xylene and graded alcohols using a Krumdieck tissue slicer as previously described (de Graaf et al., 2000). PCLS were dried at room temperature. All experiments were performed in triplicate (technical replicates) using liver tissue from 3 to 5 different subjects.

<p>| Table 2 Experimental treatment. |
|-------------------------------|----------------|--------------|</p>
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Compound</th>
<th>Concentration (μM)</th>
<th>Cmax (μM), reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF</td>
<td>Imatinib</td>
<td>1 – 10</td>
<td>5, (Leveque and Maloisel, 2005)</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>0.3 – 2</td>
<td>9, (Strumbreg et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>Sunitinib</td>
<td>0.3 – 5</td>
<td>14, (Minkin et al., 2008)</td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Perindopril</td>
<td>10 – 100</td>
<td>0.04, (Devisaguet et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>Valproic acid</td>
<td>100 – 1000</td>
<td>1000, (Chavez-Blanco et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Rosmarinic acid</td>
<td>120 – 270</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Tetrindrine</td>
<td>1 – 10</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Pirfenidone</td>
<td>500 – 2500</td>
<td>85, (Rubino et al., 2009)</td>
</tr>
<tr>
<td>Smad 3</td>
<td>Sis3</td>
<td>0.3 – 3</td>
<td>–</td>
</tr>
<tr>
<td>MAPK</td>
<td>SB203580</td>
<td>5 – 10</td>
<td>–</td>
</tr>
<tr>
<td>Rac1</td>
<td>NSC23766</td>
<td>5 – 50</td>
<td>–</td>
</tr>
</tbody>
</table>

*After oral administration of 8 mg perindopril. Needs to be metabolized in vivo into the active form perindoprilat (Devisaguet et al., 1990).
2.6. ATP determination

The viability of the slices was determined by measuring ATP levels, as reported previously (Hadi et al., 2013). In short, following treatment, PCLS were transferred to 1 ml sonication solution, containing 70% ethanol and 2 mM EDTA, snap frozen in liquid nitrogen and stored at −80 °C until use. Subsequently, samples were thawed on ice, homogenized for 45 s using a Mini-BeadBeater-8 (Biospec, Bartlesville, OK, USA) and centrifuged for 2 min at 16,000 × g. ATP levels were measured in the supernatant with the ATP bioluminescence kit (Roche diagnostics, Mannheim, Germany), and corrected for the total protein content of the sample estimated via the Lowry assay (Bio-rad RC DC Protein Assay; Bio Rad, Veenendaal, The Netherlands; (Lowry et al., 1951)).

2.7. Gene expression of fibrosis markers

To assess gene expression, total RNA was isolated, from pooled (n = 3) and snap frozen slices, using the RNeasy Mini kit (Qiagen, Venlo, the Netherlands) according to the manufacturer’s recommendations. The amount of isolated RNA was measured with the ND-1000 spectrophotometer (Fisher Scientific, Landsmeer, The Netherlands), and 2 μg RNA was reversed transcribed using the 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. The mRNA expression levels of heat shock protein 47 (HSP47), alpha smooth muscle actin (αSMA) and pro-collagen 1A1 (PCOL1A1) were detected using specific primer-probe sets (Table 3) and the qPCR master mix plus (Eurogentec, Maastricht, The Netherlands). The PCR reaction was performed using a 7900HT Real Time PCR (Applied Biosystems, Bleswijck, The Netherlands) with 1 cycle of 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Gene expression levels of PDGF-BB and TGF-β1 were determined using the SYBR Green master mix (Qiagen, Alphen aan de Rijn, The Netherlands) and specific primers (Table 3). The PCR reaction was performed using the same PCR system (see above) with 1 cycle of 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 25 s at 60 °C followed by a dissociation curve (95 °C, 15 s: 60 °C, 15 s: 95 °C, 15 s). GAPDH was used as reference gene and relative expression levels were calculated as fold change using the $2^{-\Delta\Delta CT}$ method.

2.8. Western blot

Collagen 1 protein expression was determined by Western blot. Stored PCLS (see above) were lysed for 1 h on ice in 250 μl RIPA buffer containing 1 Protease inhibitor cocktail tablet (Boehringer, Alkmaar, The Netherlands), in 10 ml 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 0.5% sodiumdeoxycholaat and 0.1% SDS. The tissue was homogenized on ice by a Potter homogenizer and centrifuged for 2 min at 16,000 × g. ATP levels were measured in the supernatant using a Biorad DC protein assay according to the sample estimated via the Lowry assay (Bio-rad RC DC Protein Assay; Bio Rad, Veenendaal, The Netherlands; (Lowry et al., 1951)).

Table 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5′−3′)</th>
<th>Reverse (5′−3′)</th>
<th>Probe (5′−3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP47</td>
<td>GCCAGGCGCTCGTGGCCGCA</td>
<td>GCCAGGCGCTCGTGGCCGCA</td>
<td>CTCCCTCTCTGCTTCTGAGG</td>
</tr>
<tr>
<td>αSMA</td>
<td>AGGCGGGATGTTGGCAGAAA</td>
<td>AGGCGGGATGTTGGCAGAAA</td>
<td>GGGCTACCAAAGAACAGCA</td>
</tr>
<tr>
<td>PCOL1A1</td>
<td>CAATCACCGTCTGAGCAGG</td>
<td>CAATCACCGTCTGAGCAGG</td>
<td>GGGCTACCAAAGAACAGCA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACCAGGGGCTCTTCTTTACTCT</td>
<td>GGGCTACCAAAGAACAGCA</td>
<td>CTCCCTCTCTGCTTCTGAGG</td>
</tr>
<tr>
<td>PDGF-β1</td>
<td>CTGGCAGTGGACAGTGGACAG</td>
<td>CTGGCAGTGGACAGTGGACAG</td>
<td>GAGTCACCAAGAACAGCA</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>GGCACGGTGGCACCTCTTCTA</td>
<td>GGCAGAAGATGAGGTAGGCTT</td>
<td>CTCCCTCTCTGCTTCTGAGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCTGCTGCTGCTGATCTGCG</td>
<td>CTGCTGCTGCTGATCTGCG</td>
<td>GGGCTACCAAAGAACAGCA</td>
</tr>
</tbody>
</table>

3. Results

3.1. Viability and expression profile of fibrosis markers in PCLS during culturing

As shown in Fig. 1A, ATP levels of the untreated PCLS increase during the 1 h preincubation as compared to the ATP values directly following slicing (0 h). Moreover, the levels remain stable for at least 24 h with an average ATP content of 7.3 ± 0.6 pmol/μg protein. After 48 h of culturing, there is a small 28% decrease of the ATP levels in untreated slices compared to 24 h. Since there is a clear correlation between ATP content of the slices and viability as determined by morphological scoring (Fig S1, r = 0.78, p = 0.021), ATP levels can be used as a reliable viability marker. These results indicate that the viability of healthy PCLS could be sustained for 48 h. Next, we studied the gene expression of several fibrosis markers (e.g. HSP47, αSMA and PCOL1A1) during culturing. Fig. 1B demonstrates that the expression of HSP47 increases at all studied time points in healthy PCLS, PCOL1A1 levels were only elevated after 48 h and αSMA expression was lower at all time points as compared to the gene level at 0 h. Moreover, collagen 1 protein expression time-dependently increased 3 fold (24 h) and 10 fold (48 h) compared to 0 h (Fig 1C), in line with the observed PCOL1A1 gene levels. Quantitative PCR further revealed that after 48 h of culture, PCOL1A1 expression in PCLS is similar to the level observed in slices prepared from cirrhotic tissue (Fig 1D). These findings illustrate the spontaneous induction of fibrogenesis in healthy PCLS, with HSP47 and PCOL1A1 serving as clear markers for this process. Therefore, these two genes were used as markers in the remainder of the study when evaluating the potency of antifibrotics.

3.2. Pathways associated with the fibrogenic response in PCLS

Next, we investigated which pathway underlies the fibrotic response in human PCLS. After 48 h of culturing, expression levels of both PDGF and TGF-β3 were significantly up-regulated compared to control (0 h;
After incubation, PCLS were collected and total mRNA was isolated. Afterwards, cDNA was synthesized and HSP47 (black bars; n = 21), αSMA (white bars; n = 7) and PCOL1A1 (red bars; n = 21) gene expression was studied using qPCR. GAPDH was used as reference gene and relative expression levels were calculated using the 2−ΔΔCT method. (C) Collagen 1 protein expression was studied by Western blotting. Proteins were separated via SDS/PAGE and blotted onto polyvinylidene difluoride membranes. Collagen was detected at 130 kD and GAPDH at 37 kD. Band intensities were corrected for GAPDH (n = 3). (D) PCOL1A1 gene expression was studied in PCLS prepared from healthy and cirrhotic liver tissue using qPCR. GAPDH was used as housekeeping gene and relative expression levels were calculated using the 2−ΔΔCT method. Results are presented as mean ± SEM of minimally three experiments performed in triplicate. * indicates p < 0.05 compared to 0 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 1. Viability of PCLS and expression of fibrosis markers. Human PCLS were cultured for 48 h. (A) The viability of the slices was assessed by determining the ATP content (n = 23). (B) After incubation, PCLS were collected and total mRNA was isolated. Afterwards, cDNA was synthesized and HSP47 (black bars; n = 21), αSMA (white bars; n = 7) and PCOL1A1 (red bars; n = 21) gene expression was studied using qPCR. GAPDH was used as reference gene and relative expression levels were calculated using the 2−ΔΔCT method. (C) Collagen 1 protein expression was studied by Western blotting. Proteins were separated via SDS/PAGE and blotted onto polyvinylidene difluoride membranes. Collagen was detected at 130 kD and GAPDH at 37 kD. Band intensities were corrected for GAPDH (n = 3). (D) PCOL1A1 gene expression was studied in PCLS prepared from healthy and cirrhotic liver tissue using qPCR. GAPDH was used as housekeeping gene and relative expression levels were calculated using the 2−ΔΔCT method (n = 3). Results are presented as mean ± SEM of minimally three experiments performed in triplicate. * indicates p < 0.05 compared to 0 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2A), 6.2-fold and 2.7-fold, respectively. As shown in Fig. 2B and C, addition of either growth factor could not significantly augment the fibrotic response in PCLS. Nevertheless, exposure of PCLS to a mixture of PDGF and TGF-β resulted in a significant induction of both HSP47 and PCOL1A1 gene expression as compared to control by 1.7-fold and 2.4-fold, respectively (Fig. 2D). These results suggest that both pathways might contribute to the development of fibrosis in PCLS. This notion is corroborated by the observed reduction in HSP47 and PCOL1A1 gene expression following treatment with the MAPK inhibitor SB203580 (Fig. 2E). In contrast, NSC23766 solely decreased HSP47 expression 1.3-fold, while Sis3 had no effect.

3.3. Impact of antifibrotic drugs on the gene expression of fibrosis markers

Since both the PDGF and TGF-β pathway appear to induce fibrogenesis in human PCLS, we investigated the capacity of multiple potential antifibrotic compounds, associated with either pathway, to mitigate the observed increase in expression of the fibrosis markers. The inhibitors mainly targeting the PDGF pathway were not toxic for PCLS at the tested concentrations, except for the highest concentration of imatinib (10 μM; Fig. 3A). Quantitative PCR revealed that sunitinib, in a concentration range of 0.5 μM to 5 μM, reduced the gene expression of HSP47 and PCOL1A1 with more than 27%, and up to 65%, compared to the control slices incubated for 48 h without inhibitor, whereas, imatinib and sorafenib did not influence the gene expression of both markers (Fig. 3B).

Regarding the TGF-β inhibitors, toxicity was only observed for pirfenidone at the highest concentrations tested (2.5 mM) illustrated by a reduction in ATP levels of more than 30% (Fig. 4A). As shown in Fig. 4B, 0.5 mM valproic acid decreased the expression level of both HSP47 and PCOL1A1 with 64% and 43%, respectively, while rosmarinic acid, tetrandrine and pirfenidone reduced the gene expression of PCOL1A1 but not that of HSP47. In contrast, perindopril did not affect gene expression levels of any of the fibrosis markers. Thus, both classes of antifibrotic compounds can influence fibrogenesis in PCLS, yet the TGF-β inhibitors seem to be more effective than the PDGF-inhibitors.

4. Discussion

Here, we describe the onset and molecular mechanism of fibrosis in healthy human PCLS. In addition, we evaluated the antifibrotic efficacy of a broad range of different compounds using this unique model. The expected therapeutic targets of the tested compounds and the obtained results are summarized in Fig. 5.

In this study, we report the early onset of fibrosis in healthy human PCLS during culture, as illustrated by a clear increase in the gene expression of both HSP47 and PCOL1A1. However, the fibrotic process in human PCLS was not associated with an increased expression of the well-known fibrosis and myofibroblast marker αSMA (Hinz et al., 2012). The observed gene expression profile is in agreement with a previous study from our group using human PCLS (van de Bovenkamp et al., 2008). As suggested previously, the decrease of αSMA expression might indicate a loss of myofibroblasts in human PCLS, since fibulin-2 expression also decreased while gene levels of the hepatic stellate cell marker, synaptophysin, remained stable during culture (van de Bovenkamp et al., 2008). Although PCLS represent a good and
multicellular model for fibrotic disease, the specific role of myofibroblasts in this model requires further study.

As mentioned before, there are clear species differences in the fibrotic process as well as the effectiveness of antifibrotic compounds. For instance, using rat PCLS, we previously demonstrated that the early onset of fibrogenesis in this rodent model was accompanied by an increased gene expression of HSP47, PCOL1A1 and αSMA (Westra et al., 2014a). Moreover, contrary to the current study, expression of these markers could be augmented in rat PCLS by treatment with either PDGF or TGF-β (Westra et al., 2014a). With regard to antifibrotic compounds, we now report that TGF-β pathway inhibitors are more effective in dampening fibrosis in human PCLS as compared to PDGF-inhibitors. This is in disparity to rat PCLS, where the early onset of fibrosis could be more effectively countered with PDGF pathway inhibitors (Westra et al., 2014a). These results might explain why several drugs, with proven antifibrotic effects in animal studies, lack efficacy in man. To illustrate, imatinib, a well-known PDGF receptor tyrosine kinase inhibitor (Buchdunger et al., 2000), was demonstrated to be a potent antifibrotic

Fig. 2. Pathways involved in fibrogenesis in human PCLS. Human PCLS were cultured for 48 h in the presence or absence of PDGF, TGF-β or several inhibitors. Afterwards, gene expression was studied using qPCR. GAPDH was used as reference gene and relative expression levels were calculated using the 2^(-ΔΔCT) method. (A) Gene expression of TGF-β (black bars) or PDGF (white bars) in human PCLS during culturing (n = 5). Impact of (B) PDGF (n = 4), (C) TGF-β (n = 4) or (D) PDGF + TGF-β (n = 5) on the gene expression of HSP47 (black bars), αSMA (white bars) and PCOL1A1 (red bars) in PCLS. (E) Impact of several inhibitors on the gene expression of HSP47 (black bars) and PCOL1A1 (red bars) in PCLS (n = 4). Results are presented as mean ± SEM of minimally three experiments performed in triplicate. * indicates p < 0.05, # indicates p = 0.059. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
compound in a broad array of rodent fibrosis models affecting several organs such as the lung, kidney and liver (Westra et al., 2014a, 2014b; Daniels et al., 2004; Wang et al., 2005; Yoshiji et al., 2005). Moreover, imatinib has been shown to induce apoptosis of hepatic stellate cells in vitro (Kuo et al., 2012). Yet, in the current study, imatinib was ineffective as antifibrotic compound in human PCLS. This finding is in agreement with a randomized double-blind clinical trial showing that the compound was ineffectual as antifibrotic drug in humans, since the compound did not affect survival or lung function in patients with idiopathic pulmonary fibrosis (Daniels et al., 2010). In addition, it is well known that imatinib can induce hepatotoxicity (Mindikoglu et al., 2007; Lyseng-Williamson and Jarvis, 2001), similar to our observations in human PCLS. Taken together, it is clear that there are species-specific effects of potential antifibrotic compounds, further illuminating the pressing need for reliable and reproducible human in vitro fibrosis models for, amongst others, drug development.

Our results further demonstrate that pirfenidone, valproic acid and rosmarinic acid, amongst others, had a clear impact on the gene expression of fibrosis markers in human PCLS. These results are in line with our previous observations in rodent PCLS (Westra et al., 2014a; Iswandana et al., 2016). Pirfenidone is a well-established antifibrotic compound shown to reduce TGF-β expression in a wide variety of animal models of fibrosis (Schaefer et al., 2011). Also, using human LX-2 cells, it was shown that pirfenidone diminished αSMA and COL1 gene expression (Zhao et al., 2009). Furthermore, Armendariz-Borunda et al. (2006) reported that twelve months of pirfenidone treatment caused a reduction in both the gene- and protein expression of COL1A, TGF-β and TIMP-1 in liver biopsies from patients with advanced liver fibrosis (Armendariz-Borunda et al., 2006). Moreover, treatment with pirfenidone was also demonstrated to be beneficial in patients with diabetic nephropathy and idiopathic pulmonary fibrosis (Sharma et al., 2011; King et al., 2014). Yet, in our hands, toxicity was observed with the highest tested concentration of pirfenidone, although this did not result in a reduction of HSP47 gene expression. Therefore, the true antifibrotic potential of pirfenidone requires further scrutiny; nevertheless, the compound shows great potential as a therapeutic agent against fibrotic diseases in humans, partially supported by our results in human PCLS.

Valproic acid markedly decreased gene expression of fibrosis markers in human PCLS, although this effect might be due to hepatotoxicity, since a non-significant decrease of ATP content was observed at concentrations ≥0.5 mM. Nonetheless, at the lowest concentration we also observed a clear reduction in PCOL1A1 gene levels. This suggests that valproic acid might be useful as antifibrotic drug. This notion is supported by the study of Watanabe et al. (2011) showing that valproic acid diminished TGF-β-induced collagen Iα1 gene and protein expression, as well as the number of αSMA fibers in LI90 cells (Watanabe et al., 2011). These results warrant further investigation into the use of HDAC inhibitors for the treatment of (liver) fibrosis.

Rosmarinic acid is a caffeic acid ester present in a myriad of plants (Petersen et al., 2009). Several studies report antifibrotic effects of this compound (Li et al., 2010; Zhang et al., 2011; Domitrovic et al., 2013). Yet, to the best of our knowledge, our study is the first to report the antifibrotic properties of rosmarinic acid using a human fibrosis model. Previously, we demonstrated that rosmarinic acid decreased the expression of fibrosis markers in fibrotic rat PCLS as well as healthy murine PCLS (Westra et al., 2014a, 2014b; Iswandana et al., 2016). Therefore we postulate that antifibrotic effects of rosmarinic acid in human liver fibrosis may be expected.

Next to testing the efficacy of antifibrotics in human PCLS we also strove to unravel the molecular pathways underlying fibrogenesis in the model. Our results indicated that the MAPK pathway plays a vital role during the onset of the fibrotic process in human PCLS, in agreement with our observations in rat PCLS (unpublished data). This notion is further supported by the study from Varela-Rey et al. (2002) demonstrating that the specific p38 MAPK inhibitor SB203580 abated the induction of COL1A1 gene expression by TGF-β in hepatic stellate cells (Varela-Rey et al., 2002). In addition, our findings suggested that induction of fibrosis in human PCLS by TGF-β goes via the MAPK pathway and is independent of Smad3. The Smad-independent activity of both pathways in regulating collagen gene expression has been reported before by Tsukada et al. (2005). The trigger for MAPK activation in human PCLS remains unclear. It is known that this pathway can be induced by reactive oxygen species (ROS) (Adachi et al., 2005), and even though...
PCLS are cultured in an atmosphere with high oxygen levels prior literature demonstrated that there is no evidence for the presence of ROS in the model (Schaffert et al., 2010; Klassen et al., 2008; Vickers et al., 2004). Moreover, previous work from our group revealed that accumulation of waste products or bile salts is not involved in the observed fibrotic response in PCLS (van de Bovenkamp et al., 2008). Thus, the causative agent(s) for fibrogenesis by culture activation in human PCLS remain to be elucidated. Of note, culture conditions have a profound impact on PCLS and should be tailored to fit the study type (e.g. drug metabolism or toxicity) (Starokozhko et al., 2015).

The current study only delineated the pathways underlying the early onset of fibrosis in human PCLS as well as the efficacy of antifibrotic compounds during this stage of the disease process. To gain more insight in the complete pathophysiological mechanism of liver fibrosis studies are currently underway using PCLS prepared from human cirrhotic livers. These studies will hopefully grant us a better understanding of fibrotic disease and provide new leads for therapeutic targets.

In conclusion, in this study we demonstrate that TGF-β pathway inhibitors effectively hamper incipient fibrosis in human PCLS. Moreover, it became apparent that the MAPK signaling cascade plays a vital role in the fibrotic process. Our results provide additional insight into the mechanism of liver fibrosis and establish human PCLS as a good model for this disease.

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Transparency document

The Transparency document associated with this article can be found, in online version.
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