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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.

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

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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 1A1 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 (Elferink 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 $NaHCO_3$ (Merck), 10 mM HEPES (MP Biomedicals, Aurora, OH, USA) and saturated with carbogen (95% O_2, 5% CO_2) 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% O_2, 5% CO_2 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 (Bioconnect, Huissen, The Netherlands), the Smad 3 inhibitor Sis3 (Bioconnect) 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, Bioconnect) and TGF-β1 (1 – 5 ng/ml; hTGF-β1, Roche Applied Science, Mannheim, Germany). Nonspecific 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 Shandon 2LE Processor. Afterwards, slices were vertically embedded in paraffin, sectioned (4 μm) and stained with hematoxylin and eosin. On microscopical examination, viability was determined by estimating the percentage of viable cells in the cross-section, taking nuclear shape/staining and cytoplasmatic staining into account.

Table 1
Characteristics of liver donors.

Number	27
PH/TX	10/17
Female/Male	20/7
Age (years)	51 (10–82)

Values are shown as mean (range).
PH: tissue obtained from partial hepatectomy.
TX: tissue obtained from transplantation liver.

Table 2
Experimental treatment.

Inhibitor	Compound	Concentration (μM)	Cmax (μM), reference
PDGF	Imatinib	1 –10	5, (Leveque and Maloisel, 2005)
	Sorafenib	0.5 – 2	9, (Strumberg et al., 2007)
	Sunitinib	0.5 – 5	14, (Minkin et al., 2008)
TGF-β	Perindopril	10 – 100	0.04[a], (Devissaguet et al., 1990)
	Valproic acid	100 –1000	1000, (Chavez-Blanco et al., 2005)
	Rosmarinic acid	120 – 270	–
	Tetrandrine	1 – 10	–
	Pirfenidone	500 – 2500	85, (Rubino et al., 2009)
Smad 3	Sis3	0.3 – 3	–
MAPK	SB203580	5 – 10	–
Rac1	NSC23766	5 – 50	–

[a] After oral administration of 8 mg perindopril. Needs to be metabolized *in vivo* into the active form perindoprilat (Devissaguet et al., 1990).