Precision-cut tissue slices: a novel ex vivo model for fibrosis research

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Chapter 7

General discussion and perspectives

Fibrosis is a condition, which is characterized by the excessive accumulation of extracellular matrix (ECM). It is well recognized that fibrosis is the result of chronic and recurrent injury, for instance due to inflammation. Fibrosis can occur in multiple organs, including, liver, kidney, intestine and lung (Wynn, 2008). However, the pathophysiological mechanisms leading to fibrosis are still poorly understood and the pathways leading to fibrosis may be different in each organ (Dugina et al., 1998). One of the main hurdles to unravel the mechanism of fibrosis, and finding potential antifibrotic compounds, is the lack of suitable human *in vitro* models for disease development and progression. Animal models for some types of fibrosis, have been established, yet, these models do not, or only partly, mimic human fibrosis. Therefore, human *in vitro* models that can unravel the pathophysiology of fibrosis are needed, which will greatly aid in finding effective pharmacological therapies.

This thesis consists of two sections: the first section delineates the development of a novel *ex vivo* model for the early onset of intestinal fibrosis (IF) by using precision-cut intestinal slices (PCIS). The second section focuses on the use of precision-cut tissue slices (PCTS) to evaluate the efficacy of various compounds for the treatment of fibrosis, including IF. Furthermore, the organ-specific toxicity and species-specific antifibrotic effect of rosmarinic acid (RA) was assessed in both IF and liver fibrosis. Lastly, precision-cut kidney slices (PCKS) were used to study, amongst others, the efficacy of a novel an IFNγ conjugate which is targeted to the platelet-derived growth factor (PDGF) receptor with antifibrotic properties.

IBD and intestinal fibrosis

Inflammatory bowel disease (IBD) affects more than 3.5 millions people worldwide (Kaplan, 2015). Ulcerative colitis (UC) and Crohn's disease (CD) are the most common types of IBD. Furthermore, 60 % of all CD patients will finally progress to IF, which is characterized by the remodeling of intestinal tissue and the formation of strictures (Verstockt and Cleynen, 2016). Although the pathophysiological mechanism underlying IF is not completely clear, it is becoming apparent that profibrotic cytokines, especially transforming growth factor β1 (TGFβ1) and PDGF-BB, play an important role in IF pathogenesis. These cytokines are released upon injury and when persistent, this will ultimately lead to development of fibrosis.

Identify markers for intestinal fibrosis

In order to develop a good (*ex vivo*) model of IF relevant biomarkers of disease development and progression are a must. Chapter 1 provides a timely overview of the available markers for IF. Unfortunately, only a limited number of relevant and specific disease markers are available. Therefore, we build upon our prior knowledge gained from studies with PCTS prepared from other organs, *e.g.* liver, to identify potential markers of fibrosis in PCIS. The fibrogenesis markers used in this thesis are, in general, directly related to ECM formation. Furthermore, we included some markers of profibrotic pathway activation and downstream signaling to capture the gamut of fibrosis development. Of note, the majority of the markers are directly regulated by TGFβ1.

Activation of TGFβ1 signaling will induce, amongst others, plasminogen activator inhibitor 1 (PAI-1) and connective tissue growth factor (CTGF) (Kutz, Hordines, McKeown-Longo, & Higgins, 2001) via the phosphorylation of Smad proteins (Ghosh and Vaughan, 2012). This will subsequently result in the induction of pro-collagen1a1 (COL1A1), the precursor of collagen 1 which is an important constituent of fibrotic lesions. Furthermore, TGFβ1 stimulates the expression of Serpinh1, also known as heat shock protein 47 (HSP47), a chaperone protein that plays an essential role in collagen maturation, biosynthesis and secretion (Taguchi, Nazneen, Al-Shihri, Turkistani, & Razzaque, 2011; Taguchi & Razzaque, 2007). All in all, activation of TGFβ signaling will results in excessive ECM deposition. The fibrotic lesions in IF mainly contain collagen 1, collagen 3 and fibronectin (Dendooven et al., 2011; Latella et al., 2013; Speca, 2012). Interestingly, the type of collagens in the lesions as well as the ratio of various collagens depends on the region of the intestine that is affected and the state of disease (Graham et al., 1988; Rieder et al., 2014).

The main cell type that produces ECM in IF are activated myofibroblasts (Burke et al., 2007), which express α-smooth muscle actin (α-SMA) (Brenner et al., 2012). Whereas in liver fibrosis, hepatic stellate cells are the main ECM-producing cells (Cassiman et al., 1999; van de Bovenkamp et al., 2006). Interestingly, Fiocchi et al. have indicated that stellate cells are also present in the intestine (Fiocchi and Lund, 2011). Therefore, we utilized synaptophysin in our studies as a marker for intestinal stellate cells.

Profibrotic stimuli in intestinal fibrosis

In chapter 3, 4 and 5 we successfully used the above-mentioned markers to study fibrogenesis in rodent PCIS. In chapter 3, fibrosis was induced by either culture activation or TGFβ1 in both rat and murine PCIS, and we could clearly demonstrate that the slices acquired a fibrotic phenotype during the experiments. Conversely, in human PCIS, only *HSP47* was increased during culture, while no regulation of *COL1A1* was found, even in the presence of TGFβ1. We postulated that the TGFβ signaling pathway is already activated during or before procurement of the human intestine or that the TGFβ pathway cannot be activated. Interestingly, gene expression of *PAI-1*, a marker of TGFβ pathway activation, was elevated during culture. Yet, expression levels of this marker were not influenced by TGFβ1. These results indicate that the TGFβ pathway is activated during culture, but further stimulation is not feasible. Thus, the trigger for TGFβ1 signaling in human PCIS remains unknown. In future studies SMAD phosphorylation will also be studied to obtain more information regarding downstream signal propagation (Rieder et al., 2016).

A major difference between human and rodent PCIS is the presence of the intestinal muscle layer during culture. During preparation of human PCIS the mucosa and muscle layer of the intestine are separated before the slices are prepared. This is in contrast to animal PCIS described in chapter 3, 4 and 5. Thus, the observed differences in the fibrotic response in human and rodent PCIS suggests that the surrounding muscle layer may play a role in fibrogenesis (Rieder and Fiocchi, 2008). However, this hypothesis requires further scrutiny and studies in this direction are currently being performed in our lab.

Also in murine PCIS differences can arise, mainly due to strain-specific responses. In this thesis, murine PCIS were mainly prepared from C57BL/6J and our results demonstrated that the TGFβ signaling pathway could be activated, which resulted in fibrogenesis (chapter 3, 4 and 5) and this process could be inhibited by TGFβ inhibitors (chapter 4 and 5). These results indicate that PCIS from C57BL/6J mice can be used to study fibrosis. However, in PCIS prepared from BALB/c mice, TGFβ1 does not stimulate fibrogenesis (Pham et al., 2013a). Therefore, murine models for IF should be developed in C57BL/6J, and these strain and species differences should be taken into account when setting up new fibrosis models.

In chapter 4, we showed that several inhibitors, could mitigate the fibrotic response induced by TGFβ1. In contrast, chapter 5 delineates that RA did not impact TGFβ1 induced gene transcription in human PCIS, while it does mitigate liver fibrosis (Li et al., 2010; Westra et al., 2014b). These results illustrate that putative antifibrotic compounds can elicit organ-specific effects which needs to be taken into consideration during the search for novel therapeutic agents.

Various studies have implicated that PDGF works in concert with TGFβ1 in the development of organ fibrosis. As described in chapter 4, incubation of murine PCIS with PDGF BB (up to 50 ng/ml) did not induce fibrogenesis. Noteworthy, the PDGF receptor was present during culture, thus modulation of PDGF signaling should be feasible. Yet, the PDGF-related antifibrotic compounds studied in chapter 4, only showed minor antifibrotic efficacy in murine PCIS. Of all the tested compounds only sunitinib was able to decrease expression of several fibrosis markers. Interestingly, sunitinib also reduced *Pai-1* and *Ctgf* expression, suggesting that sunitinib mitigated TGFβ signaling. Thus, further research, focusing on the downstream signaling proteins of PDGF pathway, *e.g.* pAKT or pErk, is needed to confirm whether the PDGF signaling pathway is present in rodent and human PCIS (Bonner, 2004; Donovan et al., 2013).

Species differences in ex vivo model for intestinal fibrosis

As described in chapter 3, one of the striking differences between slices prepared from different species pertains to the viability of PCIS during culture. In rat PCIS, the ATP content after 24 h of culture was 50% less than compared to freshly prepared PCIS, in contrast to murine and human PCIS, where the viability could be sustained till 72 h. It is striking that rat PCIS can only be used up to 24 h. Dr. M. Oltean (Gothenburg University, Sweden), an expert on intestinal preservation, suggested that rats have less tight junctions between the enterocytes when compared to enterocytes in the human villus (personal communication). This could make the rat villus more vulnerable to the culture conditions. Moreover, as postulated in chapter 3, in rat PCIS xanthine oxidase (XO) will steeply increase during ischemia. Upon re-oxygenation, XO will lead to the production of reactive oxygen species (ROS), and the presence of ROS in combination with a relatively low amount of tight junctions can be the cause for the fast decline in viability of rat PCIS during culture compared

to the viability of human and mouse PCIS (Bianciardi et al., 2004).

Another aspect of our studies clearly influenced by species differences is the efficacy of putative antifibrotic compounds. As reported in chapter 5, RA reduced the gene expression of fibronectin in murine PCIS, while no effect was observed in rat and human PCIS. This finding is in line with other studies reporting species-specific effects of antifibrotic compounds. Previous work from our group revealed that imatinib showed profound antifibrotic effects in rat precision-cut liver slices (PCLS), whereas this effect was completely absent in human PCLS. This corroborated clinical trials in which imatinib also failed to show any antifibrotic effects (Westra et al., 2014b; Westra et al., 2016). Therefore, it is of utmost importance to study antifibrotic compounds in human tissue.

Organ ex vivo models for the early onset of fibrosis

Next to PCIS, it is possible to prepare slices from other organs in order to study fibrosis. In chapter 5 and 6 we successfully used liver and kidney PCTS. PCLS model were already established for fibrosis research (Westra et al., 2014a) and used to test the efficacy of a broad range of potential drug for the treatment of early-stage liver fibrosis as well as cirrhosis. Conversely, the model of PCKS had to be optimized to study fibrogenesis (Chapter 6, Stribos et al., 2016). As illustrated in chapter 6, similar markers of fibrosis can be used to study disease development and progression in PCKS as in PCLS and PCIS. In murine PCKS, TGFβ1 increased both the gene and protein expression of α-SMA and fibronectin. Indicating that PCKS can be used to study TGFβ1 induced fibrogenesis. Interestingly, protein levels of fibronectin could not by induced by TGFβ1 in murine PCIS. This may be due to the fact that in renal fibrosis, fibronectin is the first ECM protein that is deposited (Eddy, 1996; Genovese et al., 2014). These findings suggest that the fibrotic ECM composition might differ slightly between organs.

Antifibrotic drugs

A variety of putative antifibrotic compounds have been tested in this thesis and the most promising drug in our *ex vivo* study of murine IF was pirfenidone. This compound showed promising antifibrotic effects on gene level, warranting further evaluation of this compound in human PCIS. If successful, these studies could be

extended to clinical trials with CD patients. Since pirfenidone is already subscribed to patients who suffer from idiopathic pulmonary fibrosis (Cottin and Maher, 2015), such trials would be relatively easy to perform. This approach could lead to the rapid development of an antifibrotic therapy for IF, which would be extremely beneficial for CD patients at risk for fibrosis.

RA only showed antifibrotic properties in liver slices prepared from rodent and human tissue, but not in PCIS. Thus, even though RA is an interesting candidate for the treatment of liver fibrosis, it does not show any promise to be effective as a therapeutic agent for IF.

PPB-PEG-IFNγ successfully inhibited ECM deposition in murine PCKS, yet it remains to be elucidated whether this construct is effective in IF. Since, the PDGFRβ is expressed in PCIS (chapter 4), one could argue that the construct will also be effective in these slices. Yet, this hypothesis requires further testing. Moreover, to improve translation, it is also worthwhile to develop and test PPB-PEG-IFNγ in human PCTS models.

PERSPECTIVES

The studies described in this thesis are at the basis of the use of precision-cut intestinal and kidney slices in fibrosis research. And, similarly as for liver and lung (Westra et al., 2013), we successfully established both PCIS and PCKS as *ex vivo* models for the early onset of fibrosis. Recently, human PCKS were developed to study the early onset of fibrosis, and it was demonstrated that fibrogenesis could be induced by both culture activation and TGFβ1 (Stribos et al., 2016), similar to murine PCKS (chapter 6). This differs from our studies with human PCIS, in which TGFβ1 did not elicit a fibrotic response. A possible explanation for this discrepancy could be that a second hit is necessary to induce fibrosis in tissue slices. This notion is supported by previous work from our group showing that both PDGF and TGFβ1 were necessary to further induce gene expression of several fibrosis markers in human liver slices (Westra et al., 2016). To further investigate this hypothesis, (gene expression) studies are currently ongoing in which human PCIS are treated with the combination of PDGF and TGFβ1.

In this thesis, the studies pertaining to the efficacy of antifibrotic compounds were solely performed using rodent kidney and intestinal slices. To fully unravel drug efficacy these studies should also be completed in slices prepared from human tissue. Furthermore, our studies mainly focused on the early development of fibrosis. To gain more insight into the pathophysiology of established intestinal and renal fibrosis, it is necessary to develop novel translation models. To this end, preparation and characterization of PCIS and PCKS from animal disease models and from patients are underway.

The PCIS used for the work described in this thesis were all prepared from the jejunum. However, the intestine consists of different regions, i.e. jejunum, ileum and colon, and all these parts have a different function and physiology (Lawrance et al., 2001; Louis et al., 2003; Xavier and Podolsky, 2007). Moreover, CD is heterogeneous in its pathology, which is reflected by the disparate incidence of IF in jejunum, ileum and colon (Cosnes et al., 2002). CD-related IF is mostly presents itself in the ileum and colon (Cosnes et al., 2002; Rieder et al., 2014). Thus, because of the differences in physiology, it is essential to study the effect of antifibrotic compounds in different intestinal regions. Recently, we demonstrated that it is possible to prepare and culture murine PCIS from jejunum, ileum and colon to study the early-onset of

fibrosis. Initial results have shown that there are differences in the effect of TGF-β1 and PDGF on the early onset of fibrosis in different regions of the intestine (Pham et al., 2013b; Pham et al., 2014).

A disadvantage of the PCTS model is the limited time that the slices remain viable and functional in culture. If it were possible to culture the slices for an extended period of time, this would enable us to evaluate the protein expression of fibrosis markers and ECM production, synthesis and breakdown to greater extent, which would be a tremendous asset in the search for effective antifibrotic compounds. Therefore, new culture strategies, such as already implemented for PCLS (Starokozhko et al., 2015), could be used to improve PCIS and PCKS. Moreover, PCTS *ex vivo* model lack the presence of circulating inflammatory cells that contribute to fibrogenesis (Speca, 2012; Wynn, 2008; Wynn and Barron, 2010). To mimic this aspect *ex vivo*, studies are underway to add inflammatory cells *e.g.* T-cells to the culture medium.

Conclusion

PCIS have been extensively used as *ex vivo* model for metabolism and toxicity testing. In this thesis, great progress has been made to optimize and implement the model for the study of fibrosis as well as in the search for effective antifibrotic compounds. An outstanding quality of PCTS pertains to the fact that the model reduces the number of animals needed in fibrosis research and the model can bridge the gap between nonclinical and clinical research.

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