Novel strategies targeting hepatic stellate cells to reverse liver fibrosis

Shajari, Shiva

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Discussion

Shiva Koets-Shajari, Han Moshage, Klaas Nico Faber
Liver fibrosis is a significant health problem worldwide with an unmet need for effective drug-based treatment. Current therapeutic options are eliminating the disease-causing conditions, and, in advanced cases, liver transplantation. In the past decades, accumulating evidence has identified Hepatic Stellate Cells (HSC) as the major contributors to fibrogenesis in the liver following their transdifferentiation from quiescent into activated myofibroblast-like cells. Although the underlying mechanisms of HSC activation have been studied in detail, no effective drugs are available to halt or reverse hepatic stellate cell activation. Two natural molecules, melatonin and esculetin, have been demonstrated to harbor therapeutic properties in variety of liver diseases, mostly through anti-oxidant effects. (1–8). In this thesis, we established direct anti-fibrotic effects of melatonin and esculetin on HSC activation. Both molecules inhibit 5-lipoxygenase (5-LO) activity, which is an essential enzyme for the production of leukotrienes and lipoxins (9), factors that promote HSC activation. Thus, we propose that melatonin and esculetin may be used for treating liver fibrosis and should be subject of further clinical investigations. One of the hallmarks of HSC activation is their transition to become highly proliferative. In theory, an antiproliferative agent could therefore halt or even reverse liver fibrosis by suppressing HSC proliferation, but should not prevent recovery of functional liver tissue that was lost due to liver disease. We investigated the therapeutic effect of hydroxyurea (HU), an FDA-approved anti-cancer medicine, on liver fibrosis and our data shows that a relatively small dose of HU strongly reduces HSC proliferation and collagen deposition without suppressing hepatocyte proliferation that is essential for liver regeneration. The combination of a safe dose of HU with a 5-LO inhibitor agent, such as melatonin or esculetin, may therefore be a promising drug cocktail that targets various key aspects of HSC activation and leads to regression of liver fibrosis.

In addition, we discovered in the work presented in this thesis that Hormone-sensitive lipase (HSL), an enzyme with retinyl ester hydrolase (REH) activity, is expressed in HSCs and has a role in vitamin A metabolism.

The highlights of our findings are summarized and discussed in more detail below.

In Chapter 2, we discovered that HSL is present in quiescent HSC (qHSC) and partly co-localizes with cytoplasmic lipid droplets that are characteristic for qHSC. Pharmacological super-activation of HSL using isoproterenol accelerated retinyl ester loss during activation, supporting a role for HSL in hydrolyzing retinyl esters in qHSC as a 3rd REH besides adipose triglyceride lipase (ATGL) and patatin-like phospholipase domain-containing protein 3 (PNPLA3). Retinyl content in liver and plasma remained unchanged in ATGL-null mice and HSL-null mice, while retinyl
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Individuals with genetic HSL deficiency are prone to develop hepatic steatosis, systemic insulin resistance and diabetes (15). Xia et al. demonstrated that HSL-null mice (total knock out or adipose tissue-specific knock out) suffer from age-dependent hepatic steatosis. Liver-specific HSL-null mice, on the other hand, do not accumulate triglycerides (TGs) in the liver and do not show any sign of hepatic inflammation or fibrosis. The authors concluded that adipose tissue is a target for therapeutic strategies to prevent and treat hepatic steatosis (16). This may include identification of HSL activators for improving patient’s health based on their HSL expression status (16,17). According to our data, targeting HSL must be evaluated cautiously since HSL has a role in vitamin A homeostasis in HSC. Super-activation of HSL and subsequent release of retinyl may trigger quiescent HSC to undergo activation. Also boosting HSL activity may disturb the retinyl/retinyl esters balance. Therefore, monitoring of serum retinyl levels and/or hepatic vitamin A metabolism is necessary.

HSL is a multifunctional enzyme with a broad range of substrates, including TGs, diacylglycerols (DGs), monoacylglycerols, retinyl esters, cholesterol esters (CEs) and other lipids (18). As previously described, in liver homogenate of HSL-null mice, cholesteryl ester hydrolyzing (CEH) activity is significantly reduced when fed a normal or high-cholesterol diet, while TG and DG hepatic-lipase activities remain intact. Isolated hepatocytes from HSL-null mice contain higher levels of CE, suggesting an important role for HSL as CEH in hepatocytes (19). We found that not only qHSC, but also cholangiocytes and Kupffer cells in the rat liver highly express Lipe, the encoding gene for HSL. Cholangiocytes maintain the cholesterol equilibrium in bile and liver and express proteins involved in cholesterol synthesis (20). Therefore, HSL might be particularly important in controlling cholesterol homeostasis in cholangiocytes. HSL also contributes to hydrolyzing CE in mouse and human macrophages (21,22). In line with this, the primary function of HSL in Kupffer cells, the liver-resident macrophages, may also be as a CEH, although there is no direct evidence for it yet. Altogether, HSL harbors several lipase activities and is present in various hepatic cell types, but its cell type-specific activity will be largely determined by the availability of the specific substrates. Recent single-cell sequencing techniques (23,24) allow us to investigate the cell-type specific function of HSL in more detail. This, together with cell
Chapter 6

Type-specific knock out models will further illuminate the detailed functions of HSL in the liver. Additionally, we observed that the expression of LipE and Pnpla3 drops during HSC activation, while Atgl mRNA levels remain unchanged. This raises the question what is the function of ATGL in fully-activated HSC (aHSC) and, if ATGL hydrolyzes a specific substrate in aHSC, how essential this substrate is for the cells. Further research on the involvement of such metabolic enzymes in aHSC will provide us a better insight in HSC transdifferentiation mechanisms that may lead to new therapeutic strategies.

Melatonin has demonstrated protective effects in various liver disease models induced by chemicals (e.g. benzene), mycotoxins (e.g. ochratoxin A) and drugs, such as adriamycin, cyclosporine and acetaminophen (25–29). Moreover, melatonin ameliorates the harmful effects of radiation (30) and excessive alcohol consumption (7). Various hepatic pathologies are improved by melatonin treatment, including hepatic steatosis (31,32), fibrosis (8), cirrhosis (33) and hepatocellular carcinoma (34). Protective actions of melatonin include prevention of oxidative damage (33), improving mitochondrial function (35), inhibiting hepatic neutrophil infiltration (36), suppressing necrosis (37) and apoptosis (5), and ameliorating liver fibrosis (8). Very recently, melatonin has also been used as a safe and effective drug to alleviate Covid-19-associated symptoms (38–49). Melatonin supplements reduced the levels of pro-inflammatory cytokines in Covid-19 patients (38,39) and ameliorated pulmonary lesions associated with inflammation and oxidative stress (41). Moreover, melatonin decreases the likelihood of a positive Covid-19 laboratory test result (43,50). Despite such an extensive body of evidence on systemic and hepatoprotective effects of melatonin and its strong safety profile, there are no data available on the clinical use of melatonin for treating liver injuries. In Chapter 3, we demonstrated that melatonin directly suppresses HSC activation by modulating the transcription of RAR-related orphan receptor alpha (RORα) leading to downregulation of Alox-5, the encoding gene for 5-LO. Melatonin is available over-the-counter as supplement for regulating the sleep-wake cycle through initiation of sleep (51), which was also used in Covid-19 patients to reduce sleep disturbances and delirium (46). The influence of melatonin on the sleep pattern can be a limiting factor for using it in the treatment of liver fibrosis. However, by identifying the mechanism via which RORα acts we may be able to activate RORα using other ligands that do not affect the sleep-awake cycle. On the other hand, it would be interesting to determine whether people with low endogenous levels of melatonin are more prone to liver fibrosis development, or alternatively, whether people who are using melatonin for a long time show protection against the development of liver fibrosis. Such studies will require large population cohorts, such as Lifelines (52, https://www.lifelines.nl/researcher), and/or in populations with
high use of melatonin, such as in the United Kingdom (53).

RORα is involved in several key physiological processes, such as metabolism and cell proliferation (54,55). In endometrial cancer, stimulation of RORα suppresses proliferation of tumor cells and metastasis by down-regulating beta-catenin (56). RORα is highly expressed in hepatocytes (57). Thus, one can argue that melatonin-induced activation of RORα may decrease cell proliferation of hepatocytes and thereby liver regeneration. However, it has been demonstrated that melatonin actually promotes liver regeneration by reducing hepatocyte apoptosis (7). The selective regulatory function of RORα is not completely understood. According to our data, RORα inhibition also suppresses HSC proliferation. Whether this inhibition is a direct regulatory effect of RORα is an interesting question to explore.

Esculetin is a natural compound present in various herbs and plants, which harbors several pharmacological properties, including anti-inflammatory, anti-diabetic, anti-cancer and anti-fibrotic actions (58,59), in part due to its inhibitory effect on lipoxygenases (60). In Chapter 4, we examined the direct effect of esculetin on human HSC activation in vitro, followed by an in vivo experiment where mice were exposed for 4 weeks to liver fibrosis-inducing CCl₄ and treated with esculetin in the final 2 weeks. Esculetin significantly reversed human HSC proliferation and activation in vitro and suppressed liver fibrosis in the CCl₄-treated mice. In the animal model, esculetin suppressed Collagen1a1 and αSma RNA and protein levels. Moreover, it strongly suppressed expression and serum levels of tissue inhibitor of MMP-1 (TIMP1), which allows collagen degradation by matrix metalloproteinases (MMPs). In addition, esculetin enhanced the hepatic GSH/GSSG ratio in CCl₄-treated mice, indicating that it improved the anti-oxidant status of the liver.

HSC are the major producers of the extracellular matrix (ECM) deposited in liver fibrosis. Turnover of deposited ECM is subsequently regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs). In healthy liver, the production and degradation of ECM are tightly balanced. In injured liver, however, activated HSC overexpress TIMPs that leads to inhibition of MMPs and subsequently impairs ECM degradation (61,62). One way to reverse fibrosis is to increase the degradation of excessive ECM by enhancing MMPs activity, which can be achieved by reducing TIMPs expression (63). We found that esculetin significantly suppressed CCl₄-induced TIMP-1, while MMP9 levels were slightly reduced and MMP2 and MMP13 levels remained unchanged. Thus, the esculetin-induced reduction in collagen deposition in CCl₄-treated mice may not only be result of reduced ECM production, but also of enhanced ECM
Lipoxygenases (LOXs) are lipid peroxidizing enzymes, involved in the production of leukotrienes that initiate and maintain inflammatory responses. Three main LOXs of pharmacological importance are 5-LO, 12-LO and 15-LO (64). Kupffer cells express all these LOXs (65, 66), while hepatocytes have been shown to express epidermal-type lipoxygenase, eLOX3 (encoded by Aloxe3), particularly under enhanced insulin sensitivity condition (67). According to our data, Alox-5 and Alox12/15 are also expressed by rat hepatocytes, however, the protein levels and activities of these enzymes remain to be established. Esculetin is non-competitive inhibitor of 5-LO and 12/15-lipoxygenase (12/15-LO) activity (60). Still, esculetin treatment also significantly suppressed the CCl4-induced Alox12/15 mRNA levels in mouse livers, while it did not affect Alox5 expression. The effect of esculetin on Alox12/15 mRNA levels may be secondary to the suppression of liver injury and fibrosis but may further contribute to improvement of liver health due to esculetin treatment.

Leukotriene B4 (LTB4), a product of the lipoxygenase pathway, is a chemoattractant of neutrophils that promote hepatic inflammation (68). A recent study showed that esculetin reduced LTB4 plasma levels in a rat arthritis model (69). A decrease in LTB4 production might be also a mechanism of esculetin in suppressing inflammation and fibrosis in livers of CCl4-exposed mice. It would therefore be interesting to investigate whether HSC express LTB4 receptors, and if so, whether the migratory capacity of HSC would be impaired by esculetin-mediated reduction of LTB4 production. The leukotriene B4 receptor type 1 (BLT1) and type 2 (BLT2) are expressed by keratinocytes, but not by dermal fibroblasts (70). So far, there are no reports about the expression of these two LTB4 receptors in HSC.

Activated HSC are highly proliferative and suppression thereof is an index for the regression of hepatic fibrosis (71). Inhibition of HSC proliferation can be achieved through targeting two fibrotic factors: platelet-derived growth factors (PDGFs) and transforming growth factor-beta 1 (TGF-β1) (72–74). However, TGF-β1-based therapeutics have not yet been established, mainly because of the complexity of this pathway that makes cell-type targeting of TGF-β1 very challenging (74). Such complications can be associated to PDGF-targeting therapeutic approaches as well, since no medication has been developed yet to inhibit PDGF signaling pathways. Hydroxyurea is an anti-proliferative drug used for the treatment of variety of diseases, including chronic myelogenous leukemia, psoriasis, melanoma, ovarian cancer, polycythemia vera, sickle cell anemia and HIV (75). It is generally well-tolerated with very few side effects. Hydroxyurea prevents proliferation of metastatic cells by suppressing DNA replication through degradation.
inhibition of the enzyme ribonucleotide reductase (RNR) that converts ribonucleotides into deoxyribonucleotides (76). In Chapter 5, we assessed the effect of hydroxyurea on cultured HSC and subjected mice to CCl₄-induced fibrosis, where animals were given a daily dose of hydroxyurea during 4 weeks of CCl₄ treatment. Hydroxyurea significantly and dose-dependently inhibited HSC proliferation in vivo and in vitro. Hydroxyurea did, however, not change the expression of Collagen1a1 and αSma in fully-activated HSC in vitro nor in CCl₄-induced fibrosis in mice. Remarkably, hydroxyurea still attenuated collagen deposition in CCl₄-treated mice, suggesting that the antifibrogenic effect of hydroxyurea is primarily due to its anti-proliferative actions. An effective anti-fibrotic agent should not block liver regeneration, which is essential for the restoration of functional liver tissue following liver injury. We found that, although hydroxyurea strongly suppresses HSC proliferation, it has no significant impact on regenerative hepatocyte proliferation. This cell type-specific effect of hydroxyurea might be due to a difference in uptake and/or metabolism of hydroxyurea between HSC and hepatocyte. Further investigation is required to determine the proper dose of HU and its potential impact on liver parenchymal cells. However, this data suggests the potential efficacy of HU therapy in patients with liver fibrosis.

Given the current view that effective therapies for liver fibrosis may require cocktails of drugs with complementing activities (77,78), the work described in my thesis indicates that the combination of hydroxyurea, with its capacity to suppress HSC proliferation, and melatonin or esculetin, which also suppress HSC activation, could be a promising formula for the treatment of liver fibrosis. All of these compounds, hydroxyurea, melatonin and esculetin, are readily available and low-priced. Although this is beneficiary for patients, especially in low income regions, it is not easy to find sponsors to initiate placebo-controlled double-blinded randomized clinical trials for such compounds. Unfortunately, such inexpensive drugs without patent position do not make strong business models for pharmaceutical companies to pursue such expensive clinical trials. Thus, great opportunities for treating common diseases by natural and affordable compounds are unfortunately lost in this way. Such agents may depend on circumstantial evidence (as from of population cohorts) to get support for their therapeutic action. In an ideal situation, non-profit organizations such as WHO, universities and research centers would invest on such therapeutic potentials based on adequate research results and recommendations. Still, I wonder whether hydroxyurea, melatonin and/or esculetin will ever end up in modern medicine treatments for liver fibrosis.
REFERENCES


52. https://www.lifelines.nl/researcher


