Liver fibrosis in vitro
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Chapter 4

Effect of pentoxifylline on stellate cell activation in human liver slices

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Abstract

Introduction: Precision-cut liver slices are a promising alternative to cell culture models for the study of hepatic stellate cell (HSC) activation and fibrogenesis since HSC are maintained in their original extracellular matrix and multicellular milieu. In the present study we evaluated human liver slices as a tool to study fibrogenesis and to test anti-fibrotic drugs.

Methods: Firstly we determined whether spontaneous fibrogenesis occurred in human liver slices (250 µm thick, diameter 8 mm) during 1-48 hours of incubation and whether the anti-fibrotic compound pentoxifylline could prevent this. Secondly, the effect of pentoxifylline on carbon tetrachloride (CCl₄)-induced early HSC activation was determined. To assess HSC activation and fibrogenesis mRNA expression of several markers was measured.

Results: After prolonged incubation of human liver slices (> 24 hours), αSMA and procollagen 1α1 mRNA expression started to increase, which was significantly inhibited by pentoxifylline. Analysis of synaptophysin and fibulin-2 mRNA expression during incubation with or without pentoxifylline indicated that likely both HSC and other (myo)fibroblasts are involved in this fibrotic process. CCl₄-induced early HSC activation, which was reflected by increased HSP47 and αB-crystallin mRNA expression, was not inhibited by pentoxifylline.

Conclusions: 1) Preparation and/or culturing of human liver slices induced fibrogenesis after prolonged incubation, which may be mediated by both activated HSC and resident liver (myo)fibroblasts. 2) Our results suggest that pentoxifylline can only inhibit relatively late processes of HSC activation. 3) Human liver slices are a promising in vitro system to study both early HSC-activation and fibrogenesis, and to test anti-fibrotic drugs in a multicellular environment, closely resembling the in vivo situation in man.
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Introduction

Liver fibrosis due to viral, metabolic, or alcohol-induced liver injury is one of the leading causes of death worldwide [1]. To date no curative treatment for liver fibrosis is available and patients are dependent on liver transplantations. In order to develop effective anti-fibrotic pharmaco-therapies, it is of importance to elucidate the mechanisms underlying fibrosis development in human liver. In addition, there is a great need for representative experimental models to test the effects of potential anti-fibrotic compounds. In vivo animal models for liver fibrosis are time-consuming and implicate high discomfort for the experimental animals. Therefore, the first stage in the testing of a potential anti-fibrotic drug is to study its efficacy in in vitro models. As hepatic stellate cell (HSC) activation is a key event in fibrosis development [1, 2], current in vitro models for fibrosis mainly encompass cultures of primary HSC or HSC cell lines, occasionally co-cultured with other liver cell types. These models have contributed significantly to the understanding of HSC biology, but cannot accurately incorporate in vivo cell-cell and cell-extracellular matrix interactions, which play an important role in the multicellular fibrosis process. In addition, although activated HSC are thought to play the major role in fibrosis development, the (myo)fibroblast population involved in fibrosis development in vivo, is likely more heterogeneous, and may also involve resident myofibroblasts, portal fibroblasts, second-layer cells, and bone marrow derived myofibroblasts [3, 4]. Current cell culture models do not account for this heterogeneous population of fibrogenic cells. Thus, there persists a need for an in vitro model that can mimic the in vivo situation more closely. Recently, precision-cut liver slices came into attention as a promising alternative to cell culture models for the study of fibrosis [5-9]. The main advantage of liver slices is that all liver cell types are present and the cells are maintained in their original extracellular matrix and multicellular milieu.

The applicability of liver slices for the study of the processes underlying HSC activation and fibrogenesis was suggested in previous studies, which showed that during incubation of rat liver slices spontaneous HSC activation and fibrogenesis occurs [6, 8]. In addition, we showed that the known fibrogenic compound carbon tetrachloride (CCl₄) induced early HSC activation in liver slices [5, 9]. The aim of the present study was to evaluate human liver slices as a tool to study fibrogenesis and to test the effects of anti-fibrotic drugs. For this purpose, first we studied whether spontaneous fibrogenesis occurs during the incubation of human liver slices and evaluated the involvement of HSC and (myo)fibroblasts in this process. Secondly, we determined the effects of pentoxifylline on mRNA expression of several markers of fibrogenesis and stellate cell activation during incubation of human liver slices. Finally, we studied whether the previously described CCl₄-induced early HSC activation in human liver slices [9], which provides a model to study the mechanisms underlying toxicity-induced HSC activation, can be inhibited by pentoxifylline.

As anti-fibrotic model compound pentoxifylline was chosen. Pentoxifylline is a phosphodiesterase inhibitor, which inhibits proliferation and collagen synthesis in cultured HSC and myofibroblasts [10-14], and reduces HSC activation and proliferation in experimental animal models for fibrosis [15, 16]. In addition, pentoxifylline has anti-
cytokine effects, mainly as inhibitor of interleukin 1 and tumor necrosis factor α expression and activity [17, 18]. To assess the effects of control incubation and incubation in the presence of CCl₄ and/or pentoxifylline on HSC activation and fibrogenesis in human liver slices, heat shock protein 47 (HSP47) and αB-crystallin mRNA expression was used as an early marker for HSC activation, and expression of α smooth muscle actin (αSMA) and pro-collagen 1 α1 mRNA as a late marker for HSC activation and fibrogenesis. Besides, to discern HSC related phenomena from those in (myo)fibroblasts, synaptophysin and fibulin-2 mRNA expression was studied. Synaptophysin is reported to be exclusively expressed in HSC, both quiescent and activated [19]. Recently, in rat liver, fibulin-2 was reported to be almost exclusively present in (myo)fibroblasts and rarely in activated HSC [20]. Therefore, in the present study we also measured fibulin-2 mRNA expression in human liver slices, assuming that this marker mainly reflects (myo)fibroblasts rather than activated HSC.

Methods

Human liver tissue

Human liver tissue was obtained from multi-organ donors (Tx-livers) or from patients after partial hepatectomy because of metastasis of colorectal carcinoma (PH-livers). Consent from the legal authorities and from the families concerned was obtained for the use of Tx-livers for transplantation-related research. The Tx-liver was perfused with cold University of Wisconsin organ storage solution (UW, DuPont Critical Care, Waukegan, IL, USA) in situ before explantation, and stored in cold UW until the liver was reduced in order to perform reduced-size or split liver transplantation. During reduction the liver was immersed in UW cooled with ice slush. The liver tissue remaining after bipartition was stored in cold UW solution until the start of the slicing procedure. In case of PH-livers, consent to use liver tissue for research purposes was obtained from the patients concerned. The technique of partial hepatectomy was performed as described earlier [21] after which a wedge from the resected liver lobe was cut at distance from the metastases. Immediately after excision the biopsy wedge was perfused with cold UW, and transported to the laboratory were the slicing procedure was started within 30 min. The medical ethical committee of the University of Groningen approved the research protocols.

Preparation of liver slices

Precision-cut liver slices (8 mm diameter, 250 µm thickness) were prepared in ice-cold Krebs-Henseleit buffer saturated with carbogen (95% O₂/ 5% CO₂) and containing 25 mM glucose (Merck, Darmstadt, Germany), 25 mM NaHCO₃ (Merck) and 10 mM HEPES (ICN Biomedicals, Inc. Aurora, OH, USA) using the Krumdieck tissue slicer [22, 23]. Slices were stored at 4°C in UW until the start of the experiments.

Incubation of liver slices

Slices were pre-incubated for 1 hr in 3.2 ml Williams Medium E with glutamax-I (Gibco, Paisly, Scotland) supplemented with 25 mM D-glucose and 50 µg/ml gentamycin (Gibco) (WEGG) under carbogen (95% O₂/5% CO₂) atmosphere at 37°C in 6-well culture plates (Greiner bio-one, Frickenhausen, Germany) while gently shaken. Pre-incubation enables the slices to restore their ATP-levels [24]. After pre-incubation slices were transferred to 25 ml Erlenmeyer flasks containing 5 ml carbogen-saturated WEGG, and incubated at 37°C for 16 hours, while gently shaken. At the start of the incubation period 0, 5, or 10 µl carbon tetrachloride (CCl₄, Fluka Chemie, Steinheim, Switzerland) was added in the 20 ml headspace of the flask to a paper attached to the stopper. Simultaneously 0 or 1mM pentoxifylline was added to the culture medium. During incubation CCl₄ evaporates, and equilibrium is reached between the gas phase and the medium. Alternatively, to study marker expression profile in the liver slices during control incubation, after preincubation slices were transferred to 6-wells culture plates containing fresh carbogen-saturated WEGG and incubated for 3-48 hours. In case of 48 hours of incubation, after 24 hours of incubation, slices were again transferred to 6-well culture plates containing fresh WEGG and incubated for another 24 hours.
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Viability
Each slice was transferred to a sonication solution containing 70% ethanol and 2 mM EDTA, snap frozen in liquid nitrogen, and stored at -80°C until ATP determination. ATP was determined in the supernatant that was obtained after sonication the samples 15 s. and centrifuging the homogenate 2‘ at 13000 rpm using the ATP Bioluminescence assay kit CLSII (Roche diagnostics, Mannheim, Germany).

Real-time PCR
RNA was isolated from three snap-frozen slices using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 2 µg total RNA, using the Promega Reverse Transcription System (Promega, Madison, WI, USA). 0.63 µl cDNA was used in real-time PCR reactions using Taqman reaction mixture (Applied Biosystems, Warrington, UK), and the appropriate primers and probes (Assays-On-Demand, Applied Biosystems, Foster City, CA, USA). The comparative threshold cycle (Ct) method was used for relative quantification. Ct is inversely related to the abundance of mRNA transcripts in the initial sample. Mean Ct of duplicate measurements was used to calculate the difference in Ct for target and reference GAPDH gene (∆Ct), which was compared to the corresponding ∆Ct of the control (∆∆Ct). Data are expressed as fold-induction or repression of the gene of interest according to the formula $2^{-\Delta\Delta C_t}$.

Collagen protein determination
5 µm cryostat sections were stained with saturated picric acid containing 0.1% Sirius-Red for collagen protein staining (Chroma, Stuttgart, Germany) and 0.1% Fast-Green for non-collagen protein staining (Sigma-Aldrich). To quantify the collagen content the color was eluted from the sections with 0.1 N NaOH diluted 1:1 with methanol and measured at 540 nm (Sirius-Red) and 605 nm (Fast-Green). Calculations were performed according to Lopez-De Leon [25]. Firstly, the absorbance of Sirius-Red was corrected for the contribution of Fast Green (29.1%). Secondly, the absorbances were divided by their color equivalences (2.08 and 38.4) to obtain values representing the amount of collagen and non-collagenous protein that were present in the sections. Finally, the percentage of collagen per total protein was calculated.

Statistics
Experiments were performed with at least 3 livers using slices in triplicate from each liver. Data were compared using an unpaired two-tailed Student’s-t-test. A P-value < 0.05 was considered significant. Data are presented as mean ± standard error of the mean (SEM).

Results
Control incubation
Human liver slices remained viable during incubation, as indicated by essentially constant ATP levels during the first 24 hours of incubation and a small, but significant decrease after 48 hours of incubation (ATP retention 73 ± 10%) (Figure 1).

Figure 1. ATP content of human liver slices directly after the slicing procedure (0) after pre-incubation (pre) and after 3-48 hours of incubation. ATP content is expressed relative to the ATP content in liver slices at the start of the incubation period. The average of at least 5 independent experiments ± SEM is shown. *: P< 0.05 as compared to slices after pre-incubation.
To study the fibrotic process in human liver slices during incubation, the mRNA expression level of several markers was measured. Expression of HSP47 and αB-crystallin mRNA, two early markers for HSC activation, was significantly increased at the start of the incubation period and tended to normalize after prolonged incubation. In contrast, mRNA levels of the late markers for HSC activation and fibrogenesis, αSMA and pro-collagen 1α1, decreased during the first hours of incubation, whereas after prolonged incubation expression started to increase as compared to the expression levels after 16 and 6 hours of incubation, respectively. Fibulin-2 mRNA expression, which was used as a marker for resident (myo)fibroblasts, also decreased during the first hours of incubation, however, expression did not increase after prolonged incubation up to 48 hours. Synaptophysin mRNA expression, which is a marker for HSC, remained relatively constant during incubation, but significantly decreased after 48 hours of incubation (Figure 2). Collagen protein content of the liver slices was determined in two independent experiments and increased on average 1.5-fold during incubation (Figure 3).

Figure 2. mRNA expression of HSP47, αB-crystallin, αSMA, pro-collagen 1α1, synaptophysin, and fibulin-2 in human liver slices directly after the slicing procedure (0), after pre-incubation (pre), and after 3-48 hours of incubation. Expression levels are indicated relative to expression levels in liver slices directly after the slicing procedure. The average of at least 3 independent experiments ± SEM is shown. *: P< 0.05 compared to liver slices directly after slicing.
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The effect of pentoxifylline on the spontaneous fibrotic process in the liver slices was determined by measuring marker expression after 16 and 24 hours of incubation with or without pentoxifylline (Figure 4). mRNA expression of HSP47, αB-crystallin, and synaptophysin was not inhibited by pentoxifylline at these time-points. In contrast, mRNA expression of pro-collagen 1a1 and αSMA was significantly inhibited by pentoxifylline both after 16 and 24 hours of incubation. Fibulin-2 mRNA expression was inhibited after 24 hours of incubation with pentoxifylline but not after 16 hours of incubation (Figure 4). ATP content of the liver slices was not influenced by pentoxifylline (not shown).

Incubation in the presence of CCl₄
Incubation of human liver slices in the presence of CCl₄ resulted in a dose-dependent decrease of the ATP content of the liver slices (Figure 5A). In slices incubated in the presence of 10 µl CCl₄, addition of pentoxifylline during the incubation period seemed to increase the ATP content slightly, whereas ATP content of slices incubated with 0 or 5 µl CCl₄ was unaffected by pentoxifylline (Figure 5B).

Previously we showed that CCl₄ induces early HSC activation in human liver slices [5]. Similarly, in the present study CCl₄ induced an increase in mRNA expression of HSP47 and αB-Crystallin, two early markers for HSC activation. mRNA expression of the late markers for HSC activation and fibrogenesis αSMA and pro-collagen 1a1, was not induced by CCl₄. Synaptophysin mRNA expression, a marker for HSC, was not changed after incubation with CCl₄, whereas mRNA expression of fibulin-2, which was used as a marker for (myo)fibroblasts, was significantly decreased after 16 hours of incubation with CCl₄ (Figure 6).

To determine whether CCl₄-induced early HSC activation in human liver slices can be inhibited, the liver slices were incubated simultaneously with CCl₄ and the anti-fibrotic compound pentoxifylline, and effects on mRNA expression of the above-mentioned markers was measured. Pentoxifylline did not inhibit the CCl₄-induced increased mRNA expression of αB-crystallin or HSP47 in human liver slices, whereas expression of αSMA and pro-collagen 1a1 mRNA was significantly reduced. mRNA expression of synaptophysin and fibulin-2 in liver slices incubated with CCl₄ was not affected by pentoxifylline (Figure 6). The effects of pentoxifylline on liver slices incubated without CCl₄ are indicated in figure 4.
Figure 4. mRNA expression of HSP47, αB-crystallin, αSMA, pro-collagen 1a1, synaptophysin, and fibulin-2 in human liver slices incubated for 16 or 24 hours in the presence or absence of 1 mM pentoxifylline (PTX). Expression levels are indicated relative to expression levels in liver slices incubated without pentoxifylline. The average of at least 5 independent experiments ± SEM is shown. *: P< 0.05 compared to slices incubated without pentoxifylline.
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Figure 5. A) ATP content of human liver slices after 16 hours of incubation in the presence of pentoxifylline (PTX, 1 mM) or CCl₄ relative to the ATP content in liver slices after 16 hours of control incubation B) ATP content of human liver slices after 16 hours of incubation with 5 or 10 µl of CCl₄ in the presence of 1 mM pentoxifylline (PTX) relative to ATP levels in liver slices incubated with CCl₄ alone. The average of at least 5 independent experiments ± SEM is shown. *: P< 0.05 compared to control slices.

Figure 6. mRNA expression of HSP47, αB-crystallin, αSMA, pro-collagen 1α1, synaptophysin, and fibulin-2 in human liver slices incubated for 16 hours in the absence (control) or presence (CCl₄) of 5 µl CCl₄, and in liver slices incubated simultaneously with 5µl CCl₄ and 1 mM pentoxifylline (CCl₄+PTX). Expression levels are expressed relative to expression levels in liver slices incubated with CCl₄. The average of 6 independent experiments ± SEM is shown. *: P< 0.05.
**PDGF receptor mRNA expression in human liver slices**

Because the anti-fibrotic effects of pentoxifylline are generally attributed to its effects on platelet derived growth factor (PDGF) signaling, mRNA expression levels of the receptor for PDGF in human liver slices was determined as an indication of the responsiveness to PDGF. During control incubation of human liver slices PDGF receptor mRNA levels initially decreased and started to increase after prolonged incubation (Figure 7A). Incubation in the presence of CCl₄ for 16 hours did not influence PDGF receptor mRNA expression in human liver slices (Figure 7B).

![Graph A](image1)

**Figure 7.** A) mRNA expression of the PDGF receptor in human liver slices directly after the slicing procedure (0), after preincubation (pre), and after 3-48 hours of incubation. Expression levels are indicated relative to expression levels in liver slices directly after the slicing procedure (0). B) mRNA expression of the PDGF receptor in human liver slices incubated for 16 hours in the presence of 5 µl CCl₄ relative to expression levels in slices incubated without CCl₄ (control). The average of at least 3 independent experiments ± SEM is shown. *: p<0.05 compared to liver slices directly after slicing #: P<0.05 compared to liver slices after 3 hours of incubation.

**Discussion**

Recently the applicability of liver slices for the study of the processes underlying HSC activation and fibrogenesis was suggested in several studies [5-9]. The main advantage of liver slices over current available *in vitro* models for fibrosis is that all liver cell types are present in a physiologic extracellular matrix. In the present study we aimed to evaluate human liver slices as a tool to study fibrogenesis and as a test system for anti-fibrotic drugs. For this purpose we first studied spontaneous fibrogenesis in the liver slices and the involvement of HSC and/or resident (myo)fibroblasts, and determined the effects of pentoxifylline on this process. Secondly, we studied whether CCl₄-induced early HSC activation in human liver slices could be inhibited by pentoxifylline.

**HSC activation and fibrogenesis in human liver slices during control incubation**

During incubation of human liver slices an initial decrease in αSMA and pro-collagen Iα1 mRNA expression was observed in the liver slices, which was also described previously in rat liver slices [6, 8], but is yet unexplained. In the present study this
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decreased expression was also observed for fibulin-2 mRNA expression. In rat liver this marker is mainly present in (myo)fibroblasts [20], and we assumed that also in human liver this marker mainly reflects (myo)fibroblasts rather than activated HSC. In contrast to fibulin-2 mRNA expression, synaptophysin mRNA expression, which is specific for HSC [19], did not show an initial decrease during incubation. These results suggest that (myo)fibroblasts are at least partly responsible for the decrease of αSMA and pro-collagen 1α1 mRNA expression during incubation and that the decreasing expression of αSMA is not due to loss of HSC, which are known to express αSMA in normal human liver [26]. However, a specific effect on αSMA expression in HSC cannot be excluded. In contrast to the late markers for HSC activation and fibrogenesis, mRNA expression of the early markers HSP47 and αB-crystallin was increased in human liver slices during the first hours of incubation. During the incubation period the mRNA expression of αB-crystallin remained approximately 2-fold increased, although non significantly, whereas HSP47 mRNA expression normalized after prolonged incubation. The latter may be related to the observation that in progressed fibrosis in patients HSP47 protein, rather than mRNA expression is increased in HSC [27]. After prolonged incubation of human liver slices also the mRNA expression of αSMA and pro-collagen 1α1 as well as the collagen protein content in human liver slices started to increase. Similar changes in expression of fibrogenic markers in liver slices were also described previously in rat liver slices [6, 8] and suggest that spontaneous activation of HSC and/or fibroblasts and fibrogenesis occurs in the liver slices. Alternatively, it may reflect an increased number of (myo)fibroblasts or (activated) stellate cells in the liver slices. Because fibulin-2 and synaptophysin mRNA expression did not increase during incubation this seems less probable, however, the inhibitory effect of pentoxifylline on fibulin-2 expression after 24 hours of incubation could indicate some involvement of (myo)fibroblasts in the spontaneous fibrotic process in human liver slices. Pentoxifylline did not inhibit the early HSC activation reflected by the initial increased expression of αB-crystallin and HSP47 mRNA in human liver slices, but significantly inhibited the increase in mRNA expression of αSMA and pro-collagen 1α1 in the liver slices after prolonged incubation. These results suggest that pentoxifylline can only inhibit relatively late stages of HSC activation, which may be related to the mechanism of action of the compound. The anti-fibrotic effects of pentoxifylline are generally attributed to inhibition of platelet derived growth factor (PDGF) signaling via the elevation of intracellular cyclic adenosine mono-phosphate levels due to inhibition of phosphodiesterases [17]. PDGF is the most potent inducer of HSC and (myo)fibroblast proliferation in culture and plays a role in the perpetuation of HSC activation [2]. Since only activated HSC express the PDGF receptor, responsiveness of HSC to PDGF may require that the cells are fully activated [28]. If the inhibitory effect of pentoxifylline is solely mediated via its effect on PDGF signaling, this could explain why it is unable to inhibit early stages of HSC activation. Indeed, PDGF receptor mRNA expression in human liver slices paralleled the mRNA expression of the late markers for fibrosis αSMA and pro-collagen 1α1, rather than that of the early markers HSP47 and αB-crystallin. However, it was also reported that pentoxifylline inhibits HSC activation
independent of its phosphodiesterases inhibitory activity [15], thus its effect may not exclusively be mediated via interfering with PDGF signaling. The inhibitory effect of pentoxifylline on αSMA and pro-collagen 1α1 mRNA expression in human liver slices was observed at a time-point at which mRNA expression was not yet increased compared to expression levels in slices prior to incubation. This suggests that the fibrogenic process in human liver slices occurs already early during incubation, but is masked by the simultaneously occurring process that leads to decreased expression of αSMA and pro-collagen 1α1 mRNA. This hypothesis is supported by the early increase of HSP47 and αβ-crystallin mRNA and the slightly increased collagen protein content of the liver slices after 6 hours of incubation. The underlying mechanism of these processes remains to be elucidated but is likely intrinsic to the preparation and culturing of the slices. Factors in the method used that might trigger the fibrotic process are ischemia-reperfusion injury, the presence of high oxygen tension during incubation, cell death during incubation, or accumulation of (waste) products in the slices. Pilot experiments suggested that the damage to the cells on the surface caused by the cutting of the slices does not play a major role in triggering the spontaneous fibrotic process in liver slices and that accumulation of bile salts, which is potentially fibrogenic, did not occur during incubation of the liver slices. Additional evaluation of the liver slices is necessary to further elucidate this matter.

**CCL4-induced early HSC activation in human liver slices**

Previously, we showed that early HSC activation could be induced in human liver slices via a multicellular mechanism by incubation with the fibrogenic compound CCL4 [9]. To evaluate this model as a system to test the effects of anti-fibrotic compounds, in the present study the liver slices were incubated simultaneously with CCL4 and the anti-fibrotic compound pentoxifylline. Firstly, the effect of pentoxifylline on the CCL4-induced decrease of ATP content was determined, showing a slightly, though not significantly, improved ATP content when liver slices were incubated with 10 µl CCL4 in the presence of pentoxifylline. In vivo liver toxicity of CCL4 is caused by cytochrome P450 mediated conversion of CCL4 into free radicals in the hepatocytes [29]. Pentoxifylline has anti-oxidant properties [30], which could explain this small protective effect of pentoxifylline on CCL4-induced toxicity. To avoid that the possible effect on CCL4-toxicity interferes with the measurement of potential inhibitory effects of pentoxifylline on CCL4-induced early HSC activation, we choose to study the latter in liver slices incubated with 5 µl CCL4. At this concentration no beneficial effect of pentoxifylline on CCL4-toxicity in human liver slices was observed.

As reported previously [9], incubation of human liver slices with the fibrogenic compound CCL4 resulted in an increase in mRNA expression of HSP47 and αβ-crystallin, two early markers for HSC activation [27, 31]. The mRNA expression of αSMA and pro-collagen 1α1, which is induced later in the process of HSC activation [32, 33], was not changed after 16 hours of incubation with CCL4 as compared to control incubation. Synaptophysin mRNA expression, which is a marker for quiescent as well as activated HSC, was also not affected by CCL4. These results indicate that the increased expression of the early HSC activation markers is not caused by an increased number of
HSC or (myo)fibroblasts present in the liver slices and that early HSC activation was induced. The reduced expression of fibulin-2 after incubation with CCl₄ may be explained by cell death. In rat liver, fibulin-2 is expressed in (myo)fibroblasts in the portal area as well as in cells surrounding the central vein [20], the latter is the primary localization of CCl₄ toxicity [29]. Pentoxifylline did not inhibit the CCl₄-induced increased expression of the early markers for HSC activation HSP47 and αB-crystallin, nor did it inhibit synaptophysin or fibulin-2 mRNA expression after 16 hours of incubation. In contrast, mRNA expression of αSMA and pro-collagen 1α1 was significantly inhibited by pentoxifylline, despite the absence of CCl₄-induced increased mRNA expression of these markers. These results show that pentoxifylline is unable to inhibit early HSC activation in human liver slices, and suggest that pentoxifylline does have inhibitory effects on fully activated HSC and/or other (myo)fibroblast populations in human liver slices. This corresponds with the effects of pentoxifylline on the spontaneously occurring fibrotic process in human liver slices. As discussed above, the inhibitory effects of pentoxifylline on HSC may require that the cells express the PDGF receptor. In human liver slices PDGF receptor mRNA expression was not increased after 16 hours of incubation with CCl₄, which could indicate that the PDGF receptor is not yet expressed. If the inhibitory effect of pentoxifylline is solely mediated via its effect on PDGF signaling, this could explain why pentoxifylline is unable to inhibit the CCl₄-induced early stages of HSC activation.

In conclusion

Our results show that preparation and/or culturing of human liver slices induced fibrogenesis after prolonged incubation, which may be mediated by both activated HSC and resident liver (myo)fibroblasts. Pentoxifylline inhibited this spontaneous fibrogenic process in human liver slices but did not inhibit CCl₄-induced early activation of HSC. These results suggest that pentoxifylline can only inhibit relatively late processes of HSC activation. Therefore, to confirm that CCl₄-induced early HSC activation can also be used as a model to study effects of anti-fibrotic drugs, other types of inhibitors, which act on different pathways involved in HSC activation, should be tested. Taken together, human liver slices provide a promising in vitro system to study both early HSC activation and fibrogenesis and to test anti-fibrotic drugs in a multicellular environment, closely resembling the in vivo situation in man.

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