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Precision-Cut Liver Slices as a New Model to Study Toxicity-Induced Hepatic Stellate Cell Activation in a Physiologic Milieu

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Hepatic stellate cell (HSC) activation is a key event in the natural process of wound healing as well as in fibrosis development in liver. Current *in vitro* models for HSC activation contribute significantly to the understanding of HSC biology and fibrogenesis but still fall far short of recapitulating *in vivo* intercellular functional and anatomic relationships. In addition, when cultured on uncoated plastic, HSC spontaneously activate, which makes HSC activation difficult to regulate or analyze. We have examined whether the use of precision-cut liver slices might overcome these limitations. Liver slices (8 mm diameter, 250 μm thickness) were generated from normal rat liver and incubated for 3 or 16 h with increasing doses of carbon tetrachloride (CCl_4). Rat liver slices remained viable during incubation, as shown by minimal enzyme leakage. Expression of markers for HSC activation and the onset of fibrogenesis in the liver slices was studied using real-time PCR and Western blotting. In unstimulated liver slices, mRNA and protein levels of desmin, heat shock protein 47, and αB-crystallin remained constant, indicating quiescence of HSC, whereas Krüppel-like factor 6 expression was increased. In contrast, incubation with CCl_4 led to a time- and dose-dependent increase in mRNA expression of all markers and an increased αB-crystallin protein expression. In conclusion, we have developed a technique to induce activation of quiescent HSC in rat liver slices. This model permits the study of toxicity-induced HSC activation within a physiological milieu, not only in animal but ultimately also in human tissue, and could contribute to the reduction of animal experiments.

Key Words: **hepatic stellate cells; carbon tetrachloride; precision-cut liver slices.**

Hepatic stellate cell (HSC) activation is a key event in the natural process of scar tissue formation as well as in fibrosis development in the liver (Friedman, 2003; Hui and Friedman, 2003). Current *in vitro* models for HSC activation have contributed significantly to the understanding of HSC biology and fibrogenesis but still fall far short of recapitulating *in vivo* intercellular functional and anatomic relationships. In addition, when cultured on uncoated plastic, HSC spontaneously activate (Friedman *et al.*, 1992; Sato *et al.*, 2003), which makes HSC activation difficult to regulate or analyze. Based on these limitations, there persists a need for an *in vitro* system in which HSC remain in their *in vivo* quiescent state within a more native milieu and can be activated upon introduction of an injurious stimulus. Precision-cut liver slices could provide such a system, since in the liver slice all liver cell-types are present in their natural environment (Olinga and Groothuis, 2001), thereby preserving the cell–cell and cell–extracellular matrix interactions that are vital for recapitulating HSC activation and fibrogenesis in a more relevant manner. The liver slice system described here has already been used successfully in studies of metabolism and (multicellular) toxicity (De Kanter *et al.*, 2002; Melgert *et al.*, 2000; Olinga *et al.*, 2001b), but has not previously been applied to the study of HSC activation and the onset of fibrogenesis.

Cellular and molecular features of quiescent and activated HSC have been well characterized (Friedman, 2003; Geerts, 2001; Hui and Friedman, 2003) and provide useful markers to study HSC. Two accessible features include expression of heat shock protein 47 (HSP47) and desmin. HSP47 is a collagen-specific chaperone (Nagata, 1998) that is expressed in the liver exclusively in HSC, myofibroblasts, and vascular cells (Kawada *et al.*, 1996). Expression of HSP47 in HSC is increased during spontaneous HSC activation in culture as well as during CCl_4-induced fibrosis *in vivo* (Ikejima *et al.*, 2001; Kawada *et al.*, 1996; Masuda *et al.*, 1994), as is expression of the cytoskeletal protein desmin (Geerts, 2001; Niki *et al.*, 1999). In addition to these HSC-specific markers, αB-crystallin and Krüppel-like transcription factor 6 (KLF6) can also be used as markers for HSC activation and the onset of fibrogenesis. αB-Crystallin is a small heat shock protein that is expressed in activated HSC and in a minority of hepatocytes in response to cellular stress (Cassiman *et al.*, 2001). Expression of αB-crystallin and of the ubiquitously expressed transcription factor KLF6 is increased early in the process of HSC activation and fibrogenesis both

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during spontaneous activation of HSC on uncoated plastic and during *in vivo* HSC activation induced by galactosamine and CCl_4, respectively (Cassiman *et al.*, 2001; Lang *et al.*, 2000; Ratziu *et al.*, 1998). Since increased expression of HSP47, αB-crystallin, and KLF6 is observed within the first 24 h of spontaneous HSC activation *in vitro* (Kawada *et al.*, 1996; Lang *et al.*, 2000; Ratziu *et al.*, 1998), in the present study these markers were preferred to more frequently used markers to determine HSC activation (e.g., pro-collagens and alpha smooth muscle actin) that are expressed later during the activation process (Mathurin *et al.*, 2002; Uchio *et al.*, 2002).

The aim of this study was to investigate the utility of liver slices in studying toxicity-induced HSC activation as an onset to fibrogenesis. Since spontaneous activation of HSC is a limitation of many *in vitro* models for the study of HSC, we first investigated whether HSC remain quiescent in unperturbed culture. Next, we evaluated whether HSC activation is induced following incubation with carbon tetrachloride (CCl_4), a compound whose toxicity and fibrogenic activity requires conversion into a free radical by hepatocytes. Its fibrogenic activity, therefore, has been linked to oxidative damage to hepatocytes, yielding lipid peroxides and other mediators that activate HSC (Basu, 2003; Ikejima *et al.*, 2001).

The development of this *in vitro* model enables the study of HSC activation within a physiological milieu and, importantly, provides the opportunity to study this process not only in animal, but ultimately also in human liver tissue.

MATERIALS AND METHODS

Liver slice preparation. Rat livers were isolated from adult nonfasted male Wistar rats (Harlan PBC, Zeist, the Netherlands) and stored at 4°C in University of Wisconsin organ preservation solution (UW, DuPont Critical Care, Waukegan, IL). To avoid interexperimental variations due to the circadian rhythm of the rats, isolation of the livers was always performed between 9 and 11 A.M. Precision-cut liver slices (diameter 8 mm, thickness 250 μm) were prepared in ice-cold Krebs-Henseleit buffer saturated with carbogen (95% O_2 / 5% CO_2) and containing 25 mM glucose (Merck, Darmstadt, Germany), 25 mM $NaHCO_3$ (Merck), and 10 mM HEPES (ICN Biomedicals, Inc. Aurora, OH) using the Krumdieck tissue slicer (Krumdieck *et al.*, 1980; Olinga *et al.*, 2001a). Slices were prepared from the whole liver and randomly selected for incubation and sampling. Slices were stored at 4°C in UW until the start of the experiments.

Incubations. Slices were preincubated for 1 h in Williams Medium E with glutamax-I (Gibco, Paisly, Scotland) supplemented with 25 mM D-glucose and 50 μg/ml gentamycin (Gibco) (WEGG) under carbogen atmosphere at 37°C in six-well culture plates, while gently shaken (90 times/min). Preincubation enables the slices to restore their ATP-levels (Olinga and Groothuis, 2001). After preincubation, slices were transferred to 25-ml Erlenmeyer flasks containing 5 ml WEGG saturated with carbogen and incubated at 37°C for 3 or 16 h, while gently shaken (90 times/min). At the start of the incubation period 0–10 μl carbon tetrachloride (CCl_4, Fluka Chemie, Steinheim, Switzerland) was added in the 20-ml headspace of the flask to a paper attached to the stopper. During incubation CCl_4 evaporates, and equilibrium is reached between the gas phase and the medium (Azri *et al.*, 1990a,b).

Viability. Lactate dehydrogenase, aspartate transaminase, and alanine transaminase leakage into the culture medium was determined by routine clinical chemistry.

Real-time PCR. RNA (3 μg total), isolated from three snap-frozen slices using TRIzol reagent (Invitrogen, Carlsbad, CA), was used to synthesize cDNA using the Promega Reverse Transcription System (Promega, Madison, WI). In real-time PCR reactions 1.25 μl cDNA was used, with SYBRgreen reaction mixture (Applied Biosystems, Warrington, UK) and the appropriate primers (αB-crystallin: 5′-CTGACCTCTTCTCTACAGCCACT and 5′-CGTGCACCTCAATCACGTCTCC; heat shock protein 47: 5′-ACAAGAGTCCACTCCGTTAGACA and 5′-TTAAAGGGAATCAGAGGTGAACA; Krüppel-like factor 6: 5′-AATCTTCTCAACTATGGGGCTGT and 5′-GAGGTAACTGGGTAGGAAGGCAAT; desmin: 5′-AGGAACAGCAGGTCCAGGTA and 5′-AGAGCATCAATCTCGCAGGT; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-CCATCACCATCTTCCAGGAG and 5′-CCTGCTTCACCACCTTCTTG). Agarose gel electrophoresis and dissociation curves confirmed homogeneity of the PCR products. The comparative threshold cycle (C_T) method was used for relative quantification. C_T is inversely related to the abundance of mRNA transcripts in the initial sample. Mean C_T of duplicate measurements was used to calculate the difference in C_T for target and reference GAPDH gene (ΔC_T), which was compared to the corresponding ΔC_T of the control experiment ($\Delta\Delta C_T$). Data are expressed as fold induction or repression of the gene of interest according to the formula $2^{-(\Delta\Delta CT)}$.

Western blot. Liver slice homogenates (40 or 70 μg protein) were electrophoresed in a 10% acrylamide gel and transferred electrophoretically to a polyvinylidene difluoride membrane (Roche). After blocking, the membrane was successively incubated with the primary antibody, washed, and incubated with peroxidase-conjugated secondary antibody. After washing, Luminol (Sigma-Aldrich) and enhancer (4-iodophenol, Acros Organics, Fair Lawn, NJ) were added, and signals were visualized on a Kodak Biomax Light film. Peak density of the bands was measured using Quantity One (Bio-Rad Laboratories). Coomassie blue staining confirmed equal protein loading. For the primary antibodies against αB-crystallin, HSP47 (mouse-monoclonals, both from StressGen Biotechnology, Victoria, BC, Canada), desmin (mouse polyclonal, Affinity BioReagents, Golden, CO), and the horseradish-peroxidase labeled secondary antibodies (Dakocytomation, Denmark), a 1:1000 dilution was used. The primary antibody against Krüppel-like factor 6 (rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:250.

Statistics. Experiments were performed with 3–4 livers using slices in triplicate from each liver. Data were compared using a multiple comparisons ANOVA with post hoc least square difference correction. Real-time PCR results were compared using mean $\Delta\Delta C_T$ values and are presented as mean fold induction $2^{(-\Delta\Delta CT)}$. A p-value<0.05 was considered significant. Data are presented as mean ± standard error of the mean (SEM).

RESULTS

Viability of Liver Slices during Culture

Rat liver slices remained viable in the incubation system used for at least 16 h, as shown by minimal liver-enzyme leakage (Fig. 1). Addition of 1 or 5 μl CCl_4 during incubation of rat liver slices did not result in significant changes in lactate dehydrogenase, aspartate transaminase, or alanine transaminase leakage compared to slices incubated without CCl_4, indicating that these concentrations were subtoxic. Incubation with 10 μl CCl_4 led to a significant increase in enzyme leakage only after 16 h of incubation, but not after 3 h (Fig.1).

Effect of Control Incubation on Liver Slices

mRNA and protein expression of HSP47, desmin, αB-crystallin, and KLF6 in rat liver slices after 3 and 16 h of

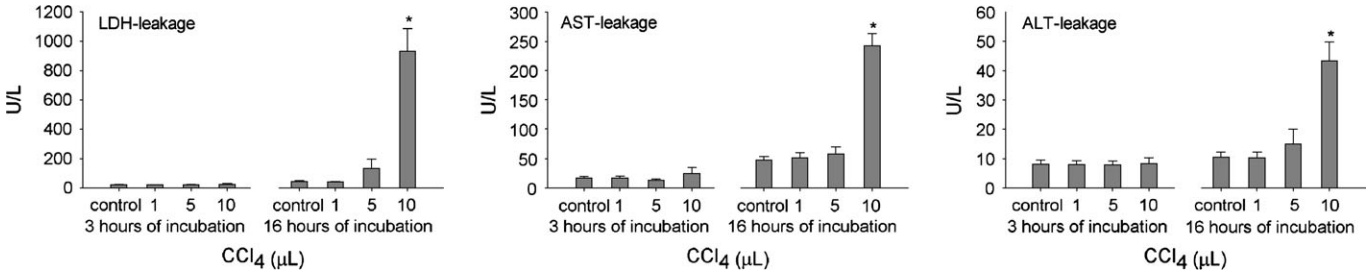

FIG. 1. Lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) leakage from rat liver slices incubated with increasing doses of CCl_4 for 3 or 16 h. The means of 4 independent experiments ± SEM are shown. # − $P < 0.005$ compared to leakage from control slices incubated without CCl_4.

incubation intervals were compared to expression levels directly after slicing (Fig. 2). Expression of desmin and αB-crystallin mRNAs remained constant during incubation up to 16 h in unstimulated culture. Expression of HSP47 mRNA was slightly decreased in slices incubated for 3 h and normalized after 16 h of incubation (Fig. 2A). Expression of KLF6 mRNA increased significantly after preincubation (52.9 ± 9.2 fold, $p < 0.005$), and subsequently decreased approximately 65% after 3 and 16 h of incubation to fold inductions of 16.4 ± 5.7 and 19.5 ± 9.6 respectively (Fig. 2B).

Western blot analysis of unstimulated rat liver slices showed no changes in protein expression of HSP47 and desmin after incubation up to 16 h (Fig. 3). Protein expression of αB-crystallin was increased modestly after 16 h of incubation (2.35 ± 0.55 fold). KLF6 protein expression in unstimulated rat liver slices was only detectable when 70 μg of total protein from the liver slices was analyzed. A minor increase of KLF6 protein expression was observed after 16 h of incubation.

Effect of Incubation with CCl_4 on Liver Slices

Marker expression in rat liver slices following 3 or 16 h of incubation with CCl_4 was determined and compared to expression levels in slices incubated without CCl_4.

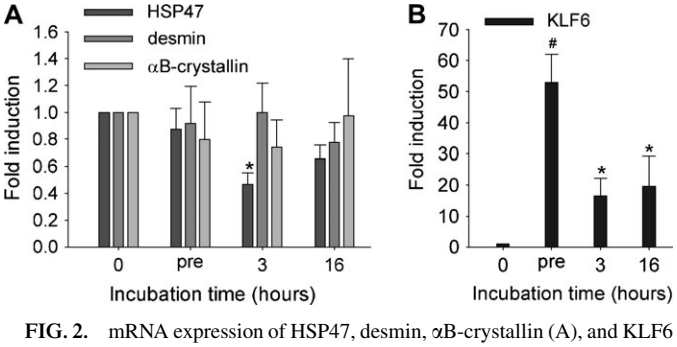

FIG. 2. mRNA expression of HSP47, desmin, αB-crystallin (A), and KLF6 (B) in rat liver slices after preincubation (pre) and after 3 or 16 h of incubation as determined by real-time PCR. Data are expressed relative to mRNA expression in rat liver slices directly after the slicing procedure (0). The means of three independent experiments ± SEM are shown. *$p < 0.05$ compared to the control (0). #$p < 0.005$ compared to the control (0).

After 3 h of incubation with 10 μl CCl_4, HSP47 mRNA was significantly increased (2.2 ± 0.2 fold, $p < 0.005$) (Fig. 4A). In addition, αB-crystallin and KLF6 mRNAs were dose-dependently increased to fold inductions of 3.9 ± 0.7 ($p < 0.005$) and 30.0 ± 24.5 ($p < 0.05$), respectively. Differences in desmin mRNA expression after 3 h of incubation with CCl_4 were not significant.

A further induction of the mRNA expression of all markers was observed after 16 h of incubation with CCl_4 compared to expression levels in slices incubated for 3 h with CCl_4 (Fig. 4B). Desmin, αB-crystallin, and KLF6 mRNA expression were dose-dependently induced up to 1.4 ± 0.1 fold ($p < 0.005$), 164.1 ± 113.3 fold ($p < 0.005$), and 22.5 ± 16.1 fold ($p < 0.05$), respectively, after 16 h of incubation with CCl_4. HSP47 mRNA expression increased significantly in liver slices incubated for 16 h with 5 μl CCl_4 (2.1 ± 0.1 fold; $p < 0.05$).

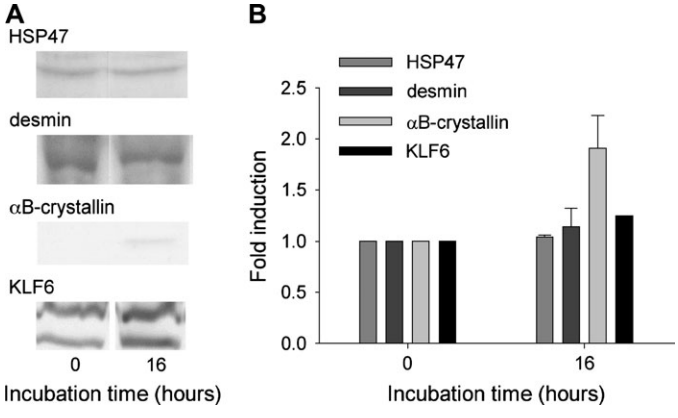

FIG. 3. (A) Protein expression levels of HSP47, desmin, αB-crystallin, and KLF6 in rat liver slices after 0 or 16 h of incubation as determined by Western blot analysis. Results of representative experiments are shown for HSP47, desmin, and αB-crystallin. For KLF6, a pool of protein samples from four independent experiments was analyzed. (B) HSP47, desmin, αB-crystallin, and KLF6 protein expression in rat liver slices after 16 h of incubation relative to expression in rat liver slices directly after the slicing procedure as determined by peak density of the corresponding bands obtained with Western blot analysis. The average of at least three independent experiments ± SEM is shown, except for KLF6: results represent expression in pooled protein samples from four independent experiments. *$p < 0.05$ compared to slices incubated for 16 h without CCl_4.

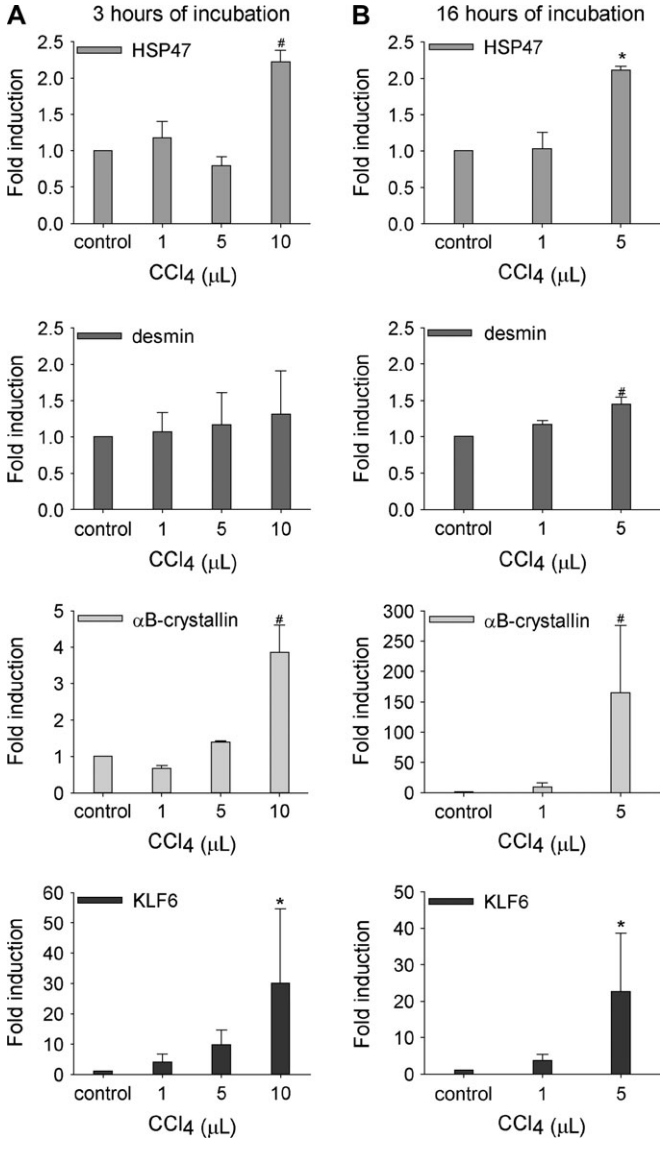

FIG. 4. mRNA expression of αB-crystallin, HSP47, desmin, and KLF6 in rat liver slices after 3 h (A) and 16 h (B) of incubation with increasing doses of CCl_4 as determined by real-time PCR. Data are expressed relative to mRNA expression in rat liver slices incubated without CCl_4. The means of three independent experiments ± SEM are shown. $*p < 0.05$ compared to the control; $\#p < 0.005$ compared to the control.

Western blot analysis showed no significant differences in HSP47 and desmin protein expression (Fig. 5). Protein expression of αB-crystallin was dose-dependently increased in liver slices following 16 h of incubation with increasing doses of CCl_4 up to 3.41 ± 0.51 fold ($p < 0.05$) compared to slices incubated for 16 h without CCl_4. KLF6 protein expression was very low and only detectable when 70 μg of total protein from the liver slices was used for Western blotting. A small increase of KLF6 protein expression was observed in liver slices incubated with CCl_4 compared to slices incubated without CCl_4.

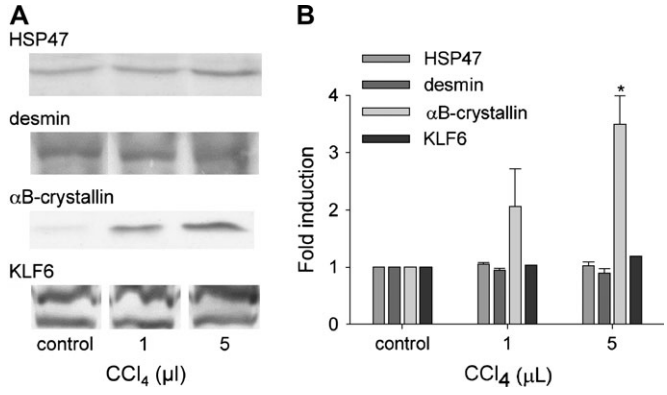

FIG. 5. (A) Protein expression levels of HSP47, desmin, αB-crystallin, and KLF6 in rat liver slices after 16 h of incubation in the presence of 0, 1, or 5 μl CCl_4 as determined by Western blot analysis. Results of representative experiments are shown for HSP47, desmin, and αB-crystallin. For KLF6, a pool of protein samples from four independent experiments was analyzed. (B) HSP47, desmin, αB-crystallin, and KLF6 protein expression in rat liver slices after 16 h of incubation in the presence of 1 or 5μl CCl_4 relative to expression in rat liver slices incubated without CCl_4 as determined by peak density of the corresponding bands obtained with Western blot analysis. The average of at least three independent experiments ± SEM is shown, except for KLF6: results represent expression in pooled protein samples from four independent experiments. $*p < 0.05$ compared to slices incubated for 16 h without CCl_4.

DISCUSSION

We have previously shown that precision-cut liver slices can be used to study drug metabolism and (multicellular) toxicity (De Kanter *et al.*, 2002; Melgert *et al.*, 2000; Olinga *et al.*, 2001b). Here we have successfully adapted this approach to investigate toxicity-induced HSC activation in rat liver slices.

Viability of Liver Slices during Culture

Liver slices remained viable for at least 16 h as determined by enzyme leakage, a parameter generally used as indicator of viability (Olinga and Groothuis, 2001). Additional experiments showed that rat liver slices remain viable in culture for at least 96 h (Vickers *et al.*, 2004), which enables extension of the period in which we can follow the responses to toxic and fibrogenic stimuli. The constant mRNA expression of the HSC-specific markers desmin and HSP47 during unstimulated incubation, and their increased expression in response to incubation with CCl_4 (Fig. 4) indicate that HSC in the liver slices remain viable during incubation. Previously, we have shown that hepatocytes, Kupffer cells, and endothelial cells are similarly viable in rat liver slices during incubation up to 24 h (De Kanter *et al.*, 2002; Olinga *et al.*, 2001a,b). Since HSC activation *in vivo* is a multicellular process, the presence and functionality of all liver cell types in slices offers a major advantage over current *in vitro* models for the study of HSC. However, we realize that liver slices do not encompass the immune system or the neuronal system, which may also play an important role in HSC activation and fibrogenesis and may

influence cell functionality (Akiyoshi and Terada, 1998; Friedman, 2003; Oben *et al.*, 2004).

Effect of Control Incubation on Liver Slices

The effect of incubation of rat liver slices on HSC activation was studied by comparing marker expression in unstimulated liver slices after different incubation intervals with expression levels directly after slicing. The early increase of KLF6 mRNA expression in unstimulated liver slices likely reflects cellular stress during preparation, consistent with KLF6's role as an immediate-early gene induced in response to stress or injury (Bieker, 2001; Ratziu *et al.*, 1998). Moreover, KLF6 levels diminished thereafter, indicating substantial recovery. Given the predominance of hepatocytes within the liver slice, these cells are likely the major source of the ubiquitous expressed early stress gene KLF6. Unlike KLF6, expression of the markers specific for HSC activation remained constant in the liver slices during incubation. In contrast, in monolayer culture spontaneous activation of HSC is associated with increased αB-crystallin within 6 h after isolation and maximum HSP47 protein expression after 1 day of culture (Kawada *et al.*, 1996; Lang *et al.*, 2000). Taken together, we conclude that in rat liver slices HSC remain quiescent up to 16 h of culture. However, we cannot exclude the possibility that the loss of vascular tone in the vessels and the presence of culture medium rather than blood in the extracellular matrix may influence cell behavior. In addition, preparation of the liver slices results in cellular damage at the cutting edges, which may lead to a tissue-repair response. Although this does not result in activation of HSC within the time frame of our experiment, after prolonged incubation complete quiescence of HSC in the liver slices may not be achieved. Additional experiments showed HSC activation in liver slices after 48 h of incubation (Vickers *et al.*, 2004). In this study, however, the culture medium contained growth factors and hormones that may induce this activation. In the present system the liver slices are incubated in culture medium without addition of these compounds, which may prevent spontaneous activation of HSC.

Effect of Incubation with CCl_4 on Liver Slices

To evaluate the utility of liver slices in studying toxicity-induced HSC activation, the expression levels of HSP47, desmin, αB-crystallin, and KLF6 in liver slices incubated with CCl_4 was compared to those in unstimulated slices. Increased expression of HSP47, αB-crystallin, and KLF6 is observed within the first 24 h of spontaneous HSC activation *in vitro* (Kawada *et al.*, 1996; Lang *et al.*, 2000; Ratziu *et al.*, 1998), and these markers were therefore preferred to more frequently used markers like pro-collagens and alpha smooth muscle actin that are expressed later during activation of HSC (Mathurin *et al.*, 2002; Uchio *et al.*, 2002).

CCl_4 was added in the headspace of the culture flasks to a paper attached to the stopper. During incubation, CCl_4 evaporates and dissolves into the medium until equilibrium is reached between the gas phase and the medium. This way of administration yields relative constant concentrations of CCl_4 in the culture medium, and the liver slice and is more reproducible than administering CCl_4 directly into the medium (Azri *et al.*, 1990a,b). The concentrations of CCl_4 used were based on a study by Azri *et al.*, in which similar doses of CCl_4 induced decreased potassium retention in liver slices after 3 to 9 h of incubation but did not increase liver enzyme leakage (Azri *et al.*, 1990a). Thus, CCl_4 did induce cellular damage in the slices, likely by conversion of CCl_4 in hepatocytes into free radicals, but did not cause overt toxicity. Therefore, we hypothesized that these concentrations of CCl_4 could activate HSC within the liver slices via lipid peroxides and other mediators yielded by CCl_4-derived free radicals, as has been described after CCl_4 treatment *in vivo* (Basu, 2003; Ikejima *et al.*, 2001). Liver slices treated for 16 h with 10 µl of CCl_4 were excluded from further analysis because of too severe toxicity.

In rat liver slices, mRNA expression of the collagen-specific chaperone protein HSP47 was significantly induced in response to incubation with CCl_4 (Fig. 4). This is in accordance with *in vivo* CCl_4 models (Ikejima *et al.*, 2001; Masuda *et al.*, 1994) and is an indication of HSC activation. HSP47 protein expression was increased in mouse liver after 3 weeks of CCl_4 treatment (Kawada *et al.*, 1996). In the present study HSP47 protein was slightly, although nonsignificantly increased in liver slices incubated for 16 h with CCl_4. Similarly, protein expression of desmin in liver slices incubated with CCl_4 was unchanged, while desmin mRNA expression was significantly increased. This could be ascribed to differences in sensitivity between real-time PCR and Western blot analysis or the relatively short incubation times used. The dose-dependent increase of desmin mRNA expression in the liver slices in response to CCl_4 could indicate that HSC in the liver slices became activated and is in agreement with the increased desmin expression observed in rat liver after treatment with CCl_4 *in vivo* (Niki *et al.*, 1999).

Expression of αB-crystallin and KLF6 mRNA in the rat liver slices was dose-dependently increased after addition of CCl_4. This increase was accompanied by a significant increase in αB-crystallin protein expression and a slightly increased expression of KLF6 protein. Since KLF6 and αB-crystallin expression in the liver is not restricted to HSC (Cassiman *et al.*, 2001; Ratziu *et al.*, 1998), we cannot exclude the possibility that this increased expression also reflects responses of other liver cell types to CCl_4. Currently we are optimizing laser microdissection microscopy techniques to specifically capture HSC from within liver slices to more accurately determine the mRNA expression of these cells. Our present results are in accordance with *in vivo* and *in vitro* studies showing induction of αB-crystallin and KLF6 during spontaneous activation of HSC cultured on uncoated plastic, as well as during *in vivo* HSC activation induced by galactosamine and

CCl_4, respectively (Cassiman *et al.*, 2001; Lang *et al.*, 2000; Ratziu *et al.*, 1998).

Onset of Fibrogenesis

Overall, our data indicate that, while quiescent during control incubation, HSC in rat liver slices are rapidly activated in response to addition of CCl_4 during the incubation period. Since activation of HSC is the key event in the natural process of wound healing and scar tissue formation, as well as in the development of liver fibrosis, we argue that the HSC activation in liver slices incubated with CCl_4 also reflects the onset of fibrogenesis. This is further supported by a study showing that in the bladder HSP47 expression is induced by fibrosis-inducing stimuli but not by nonfibrogenic stressors (Shackley *et al.*, 2002). Importantly, in liver slices incubated with increasing doses of the nonfibrogenic hepatotoxin acetaminophen, no increased expression of HSP47 was observed after different incubation intervals (manuscript in preparation). In addition, a time-dependent link between KLF6 expression, TGFβ, and fibrogenesis has been suggested in a study on steatohepatitis (Starkel *et al.*, 2003), and KLF6 expression was shown to precede the expression of several genes known to be involved in the development of liver fibrosis (Botella *et al.*, 2002; Kim *et al.*, 1998).

In Conclusion

We have developed a technique to induce activation of quiescent HSC in a physiological milieu using rat liver slices. Importantly, in contrast to many *in vitro* models for the study of HSC, preparation and incubation does not lead to spontaneous HSC activation in the rat liver slices. In addition, all major liver cell types are both present and functional in the liver slices, which is an advantage over current *in vitro* models for HSC activation, since it enables studying this process in its multicellular context. The changes observed in marker expression in rat liver slices in response to CCl_4 are consistent with those reported during HSC activation and fibrogenesis *in vivo*. Since our results indicate that precision-cut liver slices can be used to study toxicity-induced HSC activation, we will now start experiments with increased incubation intervals to investigate more progressed stages of fibrogenesis in liver slices. The development of this model could contribute substantially to the reduction, refinement, and potential replacement of animal experiments. It provides the unique opportunity to (1) track activation of quiescent HSC while maintained within a physiological milieu, (2) analyze pathways of cell–cell signaling during early fibrogenesis, and (3) ultimately study these processes in human tissue.

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