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Review

Liver fibrosis *in vitro*: Cell culture models and precision-cut liver slices

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Abstract

Chronic liver injury of various etiologies can cause liver fibrosis, which is characterized by the progressive accumulation of connective tissue in the liver. As no effective treatment for liver fibrosis is available yet, extensive research is ongoing to further study the mechanisms underlying the development of disease- or toxicity-induced liver fibrosis and to identify potential pro- or anti-fibrotic properties of compounds. This review gives an overview of the *in vitro* methods that are currently available for this purpose. The first focus is on cell culture models, since the majority of *in vitro* research uses these systems. Both primary cells and cell lines as well as the use of different culture matrices and co-culture models are discussed. Second, the use of precision-cut liver slices, which recently came into attention as *in vitro* model for the study of fibrosis, is discussed. The overview clearly shows that continuous optimization and adaptation have extended the potential of *in vitro* models for liver fibrosis during the past years. By combining the use of the different cell and tissue culture models, the mechanisms underlying multicellular fibrosis development can be studied *in vitro* and potential pro- or anti-fibrotic properties of compounds can be identified both on single liver cell types and in human liver tissue.

Keywords: Fibrosis; Cell lines; Precision-cut liver slices; Hepatic stellate cells; Extracellular matrix

Contents

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1. Introduction

Chronic liver injury of various etiologies can cause liver fibrosis, which is characterized by the progressive accumulation of connective (scar) tissue in the liver. The most common causes of liver fibrosis are infection with the hepatitis B or C virus, autoimmune diseases, and metabolic disorders, but also alcohol abuse, and drug toxicity can cause liver fibrosis (Friedman, 2003). Liver fibrosis will eventually lead to cirrhosis and liver failure and is one of the top ten causes of death in the Western world. The development of fibrosis in the liver is the result of a multicellular process, which implicates that different cell types are involved. The involvement of many of the cell types present in the liver in development of fibrosis has been extensively reviewed previously (Bataller and Brenner, 2005; Friedman, 2003; Hui and Friedman, 2003). Briefly, injured liver cells, mainly damaged hepatocytes, produce several mediators, such as reactive oxygen species and fibrogenic cytokines that initiate the activation and proliferation of hepatic stellate cells (HSC) and/or other fibrogenic cells (Guyot et al., 2006) and the production of excess extracellular matrix (ECM). In addition, liver injury leads to the recruitment of immune cells into the liver and to the activation of local Kupffer cells, the macrophages of the liver, which can further promote the fibrotic process via secretion of inflammatory and fibrogenic cytokines (Fig. 1).

The increased deposition of ECM is mainly caused by the increased production of matrix proteins by activated HSC. In addition, matrix breakdown is decreased due to alterations in the expression levels of metalloproteinases and their inhibitors (Arthur, 2000). These processes result in accumulation of ECM and in profound changes in its protein composition, which in turn can further promote the activation and proliferation of HSC and thus contribute to the progression of fibrosis (Bedossa and Paradis, 2003). The extent of fibrosis and inflammation depends on the type and duration of the liver injury. When injury is acute, the fibrotic response is taken over by regeneration, with replacement of apoptotic or necrotic cells, removal of scar tissue, and resolution of inflammation. However, when injury is sustained, the regeneration process is insufficient and liver tissue is gradually replaced by scar tissue. As this process progresses, morbidity and mortality due to disease-related complications will increase unless the causal factor is removed.

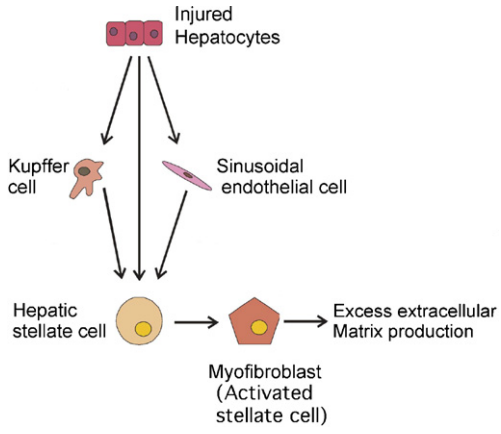

Fig. 1. Schematic representation of the interplay between the four major liver cell types, hepatocytes, Kupffer cells, stellate cells, and endothelial cells, in the development of liver fibrosis in response to chronic liver injury. Liver injury mainly affects hepatocytes, as these cells represent approximately 80% of the liver cells. Injured hepatocytes produce several mediators, such as reactive oxygen species and inflammatory and pro-fibrogenic cytokines, which activate hepatic stellate cells, both directly and indirectly via Kupffer cells and sinusoidal endothelial cells. Activated stellate cells transform into myofibroblast-like cells, which are the main producers of the excess extracellular matrix that is characteristic for liver fibrosis.

As no effective treatment for liver fibrosis is available yet, extensive research is ongoing to further study the mechanisms underlying the development of disease- or toxicity-induced liver fibrosis and to identify potential pro- or anti-fibrotic properties of compounds (Bataller and Brenner, 2005; Lotersztajn et al., 2005).

To date, studies on the development of anti-fibrotic drugs rely mostly on *in vivo* animal experiments, whereas *in vitro* models have contributed to the understanding of the mechanisms involved in the development of liver fibrosis. Several *in vivo* animal models are used, which are designed to mimic different causes of the development of liver fibrosis in humans as closely as possible, e.g., by chronic administration of toxic compounds such as carbon tetrachloride, thioacetamide, dimethylnitrosamine, and galactosamine (French, 2001; George et al., 2001; Jenkins et al., 1985; Jonker et al., 1994; Muller et al., 1988; Pierce et al., 1987), infection with Schistosoma (Dunn et al., 1977), or ligation of the bile-duct (Hinz et al., 1997; Kountouras et al., 1984). These *in vivo* models allow to take into account the possible effects of the immune system, the central nervous system and other organs which are suggested to be important in the development of liver fibrosis. Importantly, the etiology of the disease and the effects of anti-fibrotic compounds can be studied for different types of fibrosis, which closely mimic the causes of fibrosis development in patients, and take the pharmacokinetic profile and tissue exposure of potential drugs into account. An important disadvantage of experimental animal models, however, is that these models are largely restricted to rodents and may be of limited predictive value for human disease and drug toxicity leading to fibrosis in man due to interspecies differences. In addition, *in vivo* fibrosis models give high discomfort to the experimental animals and should therefore be avoided when possible.

The main advantage of *in vitro* models, apart from reduction of animal use due to the more efficient use of experimental animals, is the possibility to use of human cells or tissue to address species differences, and the results are of higher relevance to toxicity and disease in man.

This review gives an overview of the *in vitro* methods that are currently available for this purpose. The first focus is on cell culture models, since in the majority of *in vitro*

research on HSC activation and fibrogenesis these systems are used. Second, the use of precision-cut liver slices, which recently came into attention as *in vitro* model for the study of HSC activation and fibrosis, will be discussed.

2. Cell culture models

As discussed above, on a cellular level HSC are considered to be the key players in the development of liver fibrosis. The study of fibrosis *in vitro* is therefore mainly focused on HSC. In normal liver, HSC encompass ≈5–8% of the total cell population. Their main functions are the uptake, storage, and release of retinoids among which vitamin A, the regulation of sinusoidal blood flow, and the synthesis and degradation of ECM proteins (Geerts, 2001). During liver fibrosis HSC get activated, leading to genotypic and phenotypic alterations, such as the loss of Vitamin A storage, increased expression of α smooth muscle actin (αSMA), and their transformation into myofibroblast-like cells. In addition, the number of HSC drastically increases due to proliferation of the activated cells. Activated HSC are mainly responsible for the increased deposition of scar tissue in response to fibrogenic stimuli, although also other (myo)fibroblast populations in the liver may play a role in this process (Guyot et al., 2006).

2.1. Primary HSC

The first approach used to study HSC activation *in vitro* was the use of primary isolated cells. In the first attempts, some forty years ago, to isolate cells from a whole liver mainly mechanical force was used to separate the cells, which resulted in extensive cellular damage (Berry and Simpson, 1962). Therefore, efforts were made to digest the liver into single cells by using enzymatic methods (Howard et al., 1967). Berry and Friend (1969) succeeded in developing a high yield method to isolate liver cells from rat liver using perfusion with collagenase and hyaluronidase. To date, this principal is still widely applied to isolate hepatocytes and has been extended for the isolation of pure fractions of the different non-parenchymal cells (Tokairin et al., 2002; Valatas et al., 2003; Weiskirchen and Gressner, 2005). These methods use density gradient centrifugation to separate the different liver cell types, often preceded by pronase treatment to selectively kill hepatocytes. However, the thus obtained HSC fractions are still contaminated by other non-parenchymal cells and cell debris (De Bleser et al., 1997; Geerts et al., 1998), and culturing for 1–3 days with subsequent removal of contaminants is required for further purification. The purity of primary HSC cultures can be further increased by using side-scattering cell sorting, (Geerts et al., 1998) or centrifugal elutriation (Weiskirchen and Gressner, 2005) to separate the cells, or by selective killing of Kupffer cells *in vivo* (Yata et al., 1999) resulting in cell purities of >95%. However, none of these methods can completely prevent contamination of the cell fractions by other liver cell types.

Primary isolated HSC closely resemble their *in vivo* state and offer an important tool to study HSC activation *in vitro*. However, isolation procedures for primary HSC are time-consuming, yields are relatively low, and considerable variation between the different batches of isolated primary HSC exists. In addition, the possibility to subculture primary HSC is limited and human liver tissue suitable for the isolation of HSC is scarce.

2.2. HSC cell lines

As a result of these abovementioned limitations, also cell lines have been developed for the research of HSC biology.

Currently available HSC cell lines, and their characteristics are listed in Table 1. The first method used to generate these cell lines was selection of spontaneous immortalized primary cells in culture. Using this method, several cell lines were developed from primary HSC or myofibroblasts isolated from normal or pathological human or rodent liver tissue. A second method used to develop cell lines is genetic manipulation of HSC in culture. In general these cell lines are derived from primary HSC isolated from normal human or rodent liver tissue, with the exception of TWNT-1, which was derived from a cell line. Genetically induced immortalization can be achieved by transfection with the large T antigen of simian virus 40 or polyoma virus (Kim et al., 1998; Kitamura et al., 1997; Weill et al., 1997; Xu et al., 2005). In addition, stabilization of the telomere length by transfection with human telomerase reverse transcriptase can extend the life span of primary HSC or cell lines (Schnabl et al., 2002; Watanabe et al., 2003).

All HSC cell lines have an activated phenotype with a fibroblast-like appearance, but many also possess retinoid related parameters and/or inducible vitamin A storage, which are characteristics of the fat-storing phenotype of HSC. The fat-storing phenotype of HSC *in vivo* is considered as a quiescent phenotype, however, in the case of cell lines this is less well established. It was suggested that the fat-storing phenotype of these cell lines is either an alternative state of HSC activation (Mermelstein et al., 2001) or uncoupled to the activation state of the cells (Vogel et al., 2000). Therefore, when using HSC cell lines it is important to accurately establish their cellular and molecular features. Several cell lines have been characterized for their applicability for the study of retinoid metabolism (Borojevic et al., 1990; Matsuura et al., 1999; Sauvant et al., 2002; Vogel et al., 2000), but only LX cells have been extensively characterized with respect to HSC activation and fibrogenesis related parameters (Xu et al., 2005). The absence of thorough characterization of a cell line concerning its cellular and molecular features makes interpretation of experimental results difficult. It must be taken into consideration that the process of immortalization and culturing of cell lines most likely affects multiple cellular and molecular functions. The use of cell lines for the study of HSC activation and fibrogenesis can therefore, at best, give an indication of the responses of HSC in man.

Table 1
Characteristics of the cell lines used for the study of hepatic stellate cells and fibrosis

Cell line	Species	Source	Method	Retinoid metabolism	References
GRX	Mouse	MFB from fibrotic lesions induced by schistosomiasis	Spontaneous immortalization	Inducible	Borojevic et al. (1990), Guma et al. (2001), Margis and Borojevic (1989), Margis et al. (1992), Pinheiro-Margis et al. (1992)
A640-IS	Mouse	Primary HSC from normal liver	Transformation with temperature-sensitive SV40 large T antigen	n.r.	Kitamura et al. (1997)
CFSC	Rat	Primary HSC from CCl_4-treated cirrhotic liver	Spontaneous immortalization	n.r.	Greenwel et al. (1993, 1991)
NFSC	Rat	Primary HSC from normal liver	Spontaneous immortalization	n.r.	Greenwel et al. (1991)
HSC-T6	Rat	Primary HSC from normal liver	Transformation with SV40 large T antigen	Inducible	Kim et al. (1998), Vogel et al. (2000)
PAV-1	Rat	Primary HSC from normal liver	Spontaneous immortalization	n.r.	Sauvant et al. (2002)
MG-2	Rat	Primary HSC from normal liver	Spontaneous immortalization	n.r.	Lee et al. (2003)
LI90	Human	Hepatic mesenchymal tumor	Spontaneous immortalization	Inducible	Matsuura et al. (1999), Murakami et al. (1995)
GREF-X	Human	Primary MFB from normal liver	Transformation with polyoma virus large T antigen	n.r.	Weill et al. (1997)
hTERT-HSC	Human	Primary HSC from normal liver	Retroviral transfer of human telomerase reverse transcriptase	n.r.	Schnabl et al. (2002)
TWNT-1	Human	LI90-cells	Retroviral transfer of human telomerase reverse transcriptase	n.r.	Watanabe et al. (2003)
LX1	Human	Primary HSC from normal liver	Transformation with SV40 large T antigen	Inducible	Xu et al. (2005)
LX2	Human	Primary HSC from normal liver	Low serum selection pressure of LX-1 cells	Inducible	Xu et al. (2005)

HSC – hepatic stellate cells; MFB – myofibroblasts; CCl_4 – carbon tetrachloride; SV40 – Simian virus 40; n.r. – not reported.

2.3. Culture matrices

It is well known that when culturing freshly isolated HSC on uncoated plastic, these cells spontaneously get activated. In addition, as discussed above, HSC cell lines in general have an activated rather than a quiescent phenotype. Although, further stimulation of these cells can still be achieved, this spontaneous activation makes it difficult to analyze HSC activation induced by drugs or toxic compounds. Therefore, to prevent the activation of HSC during culture, several culture matrices have been developed.

Morphologically, quiescent and activated HSC can be easily distinguished. Quiescent HSC have cytoplasmic lipid droplets and long cytoplasmic processes whereas activated HSC lack both the droplets and the processes, have a fibroblast-like appearance, and express αSMA (Geerts, 2001; Sato et al., 2003). Depending on the culture matrix, both primary HSC and HSC cell lines in culture show different phenotypes. When cultured on uncoated polystyrene or on collagen type I, III, IV or V, laminin or fibronectin-coated culture dishes, HSC have a flattened fibroblast-like shape without cellular processes, indicating an activated phenotype. This is largely explained by the induction of stellate cell activation by scar-tissue components such as collagen I and III in contrast to the presence of basement membrane constituents which may maintain the stellate cells in a more quiescent state When cultured on Matrigel, a commercially available gel containing basement membrane components that closely resembles the extracellular matrix of HSC *in vivo*, HSC are round-shaped with no cellular processes, low proliferation rate, and a similar appearance to freshly isolated, quiescent HSC (Friedman et al., 1989, 1985; Kojima et al., 1998; Sato et al., 1998), however, it should be noted that this phenotype is not similar to the *in vivo* phenotype. Importantly, culturing activated rat or human primary HSC on, or in this gel deactivates these cells, as shown by a reduction of αSMA and collagen expression and low proliferation rates (Gaca et al., 2003; Sohara et al., 2002). HSC cultured on collagen gels have long cytoplasmic processes, and lipid droplets, a distinct phenotype from the flattened fibroblast-like phenotype seen when culturing HSC on plastic or collagen-coated culture dishes (Kojima et al., 1998; Miura et al., 1997; Sato et al., 1998; Senoo et al., 1996). These cells produce less collagen and are less proliferative than HSC cultured on uncoated plastic (Senoo et al., 1996) and it was suggested that HSC cultured on, or in collagen gels adapt their quiescent, normal *in vivo* phenotype.

2.4. Co-cultures

Cell cultures of a single liver cell type lack a physiological cellular context that can incorporate the cell–cell interactions that are involved in multicellular toxicity and fibrosis development in the liver. This limitation can be addressed by using co-cultures of different liver cell types. Several methods have been developed to obtain co-cultures consisting of two or more liver cell types (Bhatia et al., 1999). The conventional method of making co-cultures is to isolate different liver cell types and plate them together in monolayer culture. By varying the seeding-densities of the cell types, the degree of cellular interactions can be modulated. In addition, by plating one cell type on culture inserts or coverslips, direct cell–cell interactions in the co-culture can be prevented in order to study soluble factors influencing cell behavior. Using these methods, co-culturing of primary HSC or cell lines with Kupffer cells, endothelial cells, or hepatocytes is possible. In addition, in several studies this method was used to co-culture all these liver cell types, however, the exact cellular composition of these cell mixtures during incubation was not described (Karam and Ghanayem, 1997; Mitaka et al., 1999; Shimaoka et al., 1987; Villafuerte et al., 1994). Another approach used to obtain a co-culture containing all liver cell types, was to culture hepatocytes in a collagen gel, and plate a mixture of non-parenchymal cells on top of this gel, thus obtaining a 3D co-culture but loosing direct cellular interactions of the non-parenchymal cells with hepatocytes (Bader et al., 1996). In addition co-culture models are described that use a confluent layer of pre-cultured non-parenchymal cells, plating hepatocytes on top of this layer to achieve a co-culture of the four liver cell types (Naughton et al., 1995; Ries et al., 2000).

2.5. Cell culture models as in vitro model for liver fibrosis

As quiescent and activated HSC can be easily distinguished by their phenotype and by different expression levels of cellular markers and genes associated with fibrogenesis (Geerts, 2001), cell cultures of primary HSC or HSC cell lines provide a simple tool to study HSC biology and to determine potential promoting or inhibiting effects of drugs or toxic compounds on HSC activation and fibrogenesis. Although primary HSC in culture and HSC cell lines in general have an activated phenotype, further stimulation of these cells can still be achieved, e.g., by incubation with growth factors or cytokines (Di Sario et al., 2002; Isbrucker and Peterson, 1998; Kim et al., 1998; Sugimoto et al., 2005; Xu et al., 2005), or by incubation with fibrogenic toxic compounds (Fontana et al., 1997; Svegliati-Baroni et al., 1999). In addition, inhibitory effects of compounds on HSC activation, proliferation, and fibrogenesis can be easily determined. To measure such effects on HSC in culture mainly proliferation rate or expression levels of αSMA are used. In addition, gene and protein expression of factors associated with fibrosis such as certain cytokines and their receptors, extracellular matrix proteins, and matrix metalloproteinases and their inhibitors are indicative for HSC activation and fibrogenesis.

The applications of cell cultures of one single liver cell type are somewhat limited because indirect effects via other liver cell types cannot be taken into account. Because *in vivo* the activation of HSC and the development of liver fibrosis is the result of a complex interplay of all different liver cell types (Friedman, 2003; Hui and Friedman, 2003), this implicates that multicellular toxicity and fibrosis development cannot be adequately mimicked in these cell cultures. Similarly, when inhibitory or stimulatory effects of compounds on HSC are detected in these models, this does not necessarily mean that these compounds will have pro- or anti-fibrotic effects *in vivo*, as counteracting effects on other liver cell types are not measured and metabolism of the compounds, which is mainly mediated by hepatocytes, is not taken into account. By testing compounds also in cell cultures of other non-parenchymal cells and hepatocytes the responses of all different liver cell types can be studied separately, however, cell–cell and cell–ECM interactions are not incorporated and the combined response is difficult to predict. The use of co-culture models of different liver cell types can at least partly overcome this limitation and a number of studies are described investigating the effects of intercellular interactions on HSC activation and fibrogenesis using co-cultures of different liver cell types (Arnaud et al., 2003; Ikeda et al., 1999; Loreal et al., 1993). In addition, ethanol-induced collagen synthesis, which requires metabolic conversion of ethanol into acetaldehyde by hepatocytes, was successfully studied in a co-culture model of hepatocytes and HSC (Fontana et al., 1997; Gutierrez-Ruiz et al., 2001). Although current co-culture models cannot accurately mimic the *in vivo* cellular composition of the liver and its acinar structure, these models are promising for the development of an *in vitro* model that more closely mimics the *in vivo* situation, with respect to intracellular interactions.

A final aspect that must be taken into account when using cell culture models is the activated phenotype of primary HSC in culture and of HSC cell lines, which makes it difficult to study HSC activation in a regulated manner. In this respect, the use of matrices for the culturing of HSC is promising for the development of an *in vitro* model in which activation of HSC can be more closely regulated. In addition, the possibility to induce deactivation of HSC (Gaca et al., 2003; Sohara et al., 2002) might be helpful in elucidating mechanisms underlying the regression of fibrosis. During the development of liver fibrosis, the composition of the ECM alters (Friedman, 2003; Hui and Friedman, 2003) and the use of this system to study the effects of different matrix components on HSC might be valuable to give further insight in the process of HSC activation and fibrogenesis. However, the culture matrix does not merely determine the phenotypic appearance of HSC, but also several other aspects of the behavior of (activated) HSC, like proliferation and the production of

extracellular matrix (degrading) proteins (Gaca et al., 2003; Li et al., 1999; Senoo et al., 1996; Wang et al., 2004). Since these processes are closely related to the activation state of HSC, interpretation of responses of cells cultured on matrix to pro-, or anti-fibrogenic compounds might be difficult.

3. Precision-cut liver slices

Recently, precision-cut liver slices came into the attention as a potential model for the study of HSC activation and liver fibrosis because, unlike current *in vitro* models, they enable to study these processes *in vitro* in a multicellular system in which cell–cell and cell–ECM interactions are maintained. Precision-cut liver slices are extensively used for the study of drug metabolism and toxicity (Lerche-Langrand and Toutain, 2000; Olinga and Groothuis, 2001) and have even made it into commercial services, indicating their usefulness for general toxicity testing. However, studies describing liver slices as a tool to study HSC activation and fibrogenesis are still limited.

3.1. Preparation and culturing of liver slices

To obtain precision-cut slices, tissue cylinders with a diameter of 5–8 mm are made that are subsequently placed in a tissue slicer to be cut into slices with a reproducible thickness. Two tissue slicers are developed for this purpose, the Krumdieck tissue slicer and the Brendel–Vitron tissue slicer, which in general perform equally well (Lerche-Langrand and Toutain, 2000). Ideally, liver slices must have a thickness of less than 250 μm to allow oxygen and nutrients to diffuse to the inner cell layers, but more than 175 μm to keep the ratio of damaged cells at the outer layers to the living cell mass as favorable as possible (Olinga and Groothuis, 2001). However, recent research showed that for studies on hepatic metabolism even slices with a thickness of 100 μm can be used successfully (De Graaf et al., 2006), indicating that probably damaged cells do not influence the functioning of the remaining cells.

Various systems for the culturing of precision-cut liver slices are in use, which can be divided in continuously submerged culture systems and dynamic culture systems (Olinga and Groothuis, 2001). In continuously submerged culture systems the slices are floating within the culture medium in 6-, 12-, or 24-wells-plates or in flasks while the system is gently shaken, or the slices are placed on a stainless-steel grid while the culture medium is magnetically stirred. In the dynamic organ culture system slices are alternately exposed to the gas phase and the culture medium by placing the slices on inserts in a glass vial or 6-wells culture plate, which is rolled or rocked, respectively, during incubation. Irrespective of the culture system, the liver slices are incubated at 37 °C in a humidified incubator in the presence of oxygen concentrations varying between 95% oxygen/5% CO_2 and 20% oxygen (air)/5% CO_2. It was suggested that for short-term studies, incubation in 20% O_2/5% CO_2 is sufficient to retain slice viability (Lerche-Langrand and Toutain, 2000), whereas oxygen concentrations of at least 40% are essential for prolonged incubation of liver slices (Drobner et al., 2000; Olinga and Groothuis, 2001). In addition, for long-term incubation, nutrient-rich culture medium is required. There is no clear consensus regarding the maximum period that liver slices can be cultured while maintaining slice viability, mainly due to differences in incubation methods and viability parameters used. For incubations up to 24 h, provided that oxygen concentrations are high enough, slice viability is retained equally well in the incubation systems described above, with the exception of the stirred 24-well system in which the liver slices showed a decreased viability (Olinga et al., 1997). Several studies are described using longer incubation times than 24 h (Toutain et al., 1998; Verrill et al., 2002; Vickers et al., 2004; Yoshida et al., 2004), but no thorough comparison has been made on the influence of culture conditions on slice viability after longer incubation intervals. Although slices are easily maintained in culture for up to 48 h, for prolonged incubation of the slices careful monitoring of the culture conditions is necessary. It was suggested that for prolonged incubation a dynamic culture system as well as enriched culture medium are needed to retain slice viability (Behrsing et al., 2003; Lerche-Langrand and Toutain, 2000; Vickers et al., 2004). In addition, liver slice viability may be improved by inserting flow conditions in the culture system. With optimal culture conditions, currently liver slices remain viable in culture for 3–4 days.

3.2. Cell functionality and intercellular interactions in liver slices

The presence and functionality of all liver cell types provides the possibility to study liver function *in vitro* in a multicellular context. Several studies on the viability and functionality of the different cell types present in liver slices have shown intact cellular functions of hepatocytes (Lerche-Langrand and Toutain, 2000; Olinga et al., 2001), Kupffer cells (Elferink et al., 2004; Neyrinck et al., 2004; Olinga et al., 2001), endothelial cells (Oudar et al., 1998), and HSC (Beljaars et al., 2001; Van de Bovenkamp et al., 2005, 2006b) during incubation. Importantly, unlike co-culture models of isolated cells, the cells are maintained in their original extracellular matrix, with the same relative cell number and orientation towards other cells. In addition, the acinar structure of the liver is maintained, although it remains uncertain how long zonal differences between cells are retained because blood flow is lost. When used for prolonged incubation it must be noted that during culturing of liver slices after 48 h of incubation some degree of cell proliferation may occur (Vickers et al., 2004). Different regenerative capacities and proliferation rates of several liver cell types may result in an altered cellular composition of the liver slices. Besides, the demonstration of intact cellular functions does not imply that all cellular functions are

preserved during culture of the liver slices. For instance the drug metabolism activity of hepatocytes in liver slices shows a time-dependent decrease during incubation, in particular for cytochrome P450 mediated processes (Hashemi et al., 2000; Wright and Paine, 1992), as is also observed in hepatocyte cell cultures (Wright and Paine, 1992). In addition, although the different cell types remain functionally active in the liver slices, their activities may change during incubation, which is for example reflected by the increased expression of fibrogenic markers during incubation of rat (Van de Bovenkamp et al., 2006a; Vickers et al., 2004) and human (Van de Bovenkamp, 2006) liver slices. Nevertheless, the presence and functionality of all liver cell types in their original, three-dimensional multicellular milieu is one of the main advantages of the use of liver slices over the use of cell culture models. This feature of liver slices allows the testing of multicellular processes and interactions in the liver in a milieu closely resembling the *in vivo* situation. Indeed, a number of studies have demonstrated intact interactions between Kupffer cells and hepatocytes in the liver slice (Elferink et al., 2004; Neyrinck et al., 2004; Olinga et al., 2001). In addition, the carbon tetrachloride-induced early activation of HSC in liver slices (Van de Bovenkamp et al., 2005, 2006b) is also most likely caused by multicellular toxicity, which besides HSC involves both hepatocytes and Kupffer cells. Liver slices thus provide an *in vitro* system that closely resembles the *in vivo* situation in the liver.

3.3. The extracellular matrix in liver slices

The extracellular matrix in the liver consists of several types of collagens, glycoproteins, and proteoglycans, which surrounds the different liver cell types and thereby both assures the coherence of the liver and regulates cellular functions. The composition of the extracellular matrix can influence the differentiation, proliferation and activation state of liver cells (Berthiaume et al., 1996; Imai et al., 2000; Shakado et al., 1995). This effect of extracellular matrix on cell function is mainly mediated via integrin receptors, which are located on the cell membrane and bind to extracellular matrix molecules (Giancotti and Ruoslahti, 1999). In addition, the extracellular matrix can bind and release several growth factors, cytokines, and hormones, which in turn influence cellular behavior (Hui and Friedman, 2003; Mohammed and Khokha, 2005).

Given the important role of the extracellular matrix in regulating cellular function, the presence of the physiological extracellular matrix in liver slices provides a major advantage compared to cell culture models in mimicking the *in vivo* situation of the liver. However, during incubation of both human and rat liver slices pro-collagen 1a1 mRNA expression and collagen protein content increased (Van de Bovenkamp et al., 2006a; Vickers et al., 2004). An increased collagen production in liver slices during incubation and increased expression of genes associated with extracellular matrix deposition was reported previously (Vickers et al., 2004). Although the expression of matrix components other than collagens was not analyzed, it cannot be excluded that the extracellular matrix in the liver slice may be subject to alteration. Because the protein composition of the extracellular matrix can influence cell function, this should be taken into account in liver slice studies. In addition, proteolysis of the extracellular matrix in liver slices might result in the release of cytokines and growth factors such as TGFβ which may induce a fibrogenic response in the liver slices.

3.4. Studying fibrosis in liver slices

As mentioned previously, activation of HSC is generally considered to be the key event in the development of liver fibrosis. Therefore, studies on the applicability of liver slices for the study of fibrosis were primarily focused on HSC. During liver fibrosis, HSC become activated and transform into myofibroblasts-like cells, which are the main producers of the excess extracellular matrix leading to liver dysfunction (Friedman, 2003). Thus, increased HSC activation in liver slices can be considered an indication for the early manifestation of fibrosis. To detect HSC activation in the liver slices, mRNA and protein expression levels of HSC specific markers can be studied. In addition, immunohistochemical methods can provide information about the localization of the cells expressing the marker proteins in the liver slice, which is often the only way to differentiate activated HSC from other (myo)fibroblasts in the liver. In this respect, the use of laser capture microscopy may be beneficial to specifically isolate HSC or other liver cell types from the liver slice and study gene and protein expression in the specific cell types.

Several cellular markers can discern HSC from hepatocytes, Kupffer cells, and endothelial cells in normal liver. Among these markers are the cytoskeletal proteins desmin and glial fibrillary acidic protein (GFAP) and vinculin, heat shock protein 47 (HSP47), the retinol-handling protein retinaldehyde dehydrogenase 1, and the neuronal protein synaptophysin (Geerts, 2001; Kawai et al., 2003). The phenotypic and genotypic alterations that take place when HSC become activated result in a different expression pattern of cellular markers (Geerts, 2001). Most of the markers that are present in quiescent HSC are also expressed in activated cells. Some of these markers, such as desmin in rat liver (Ballardini et al., 1988), αSMA and αB-crystallin in human liver (Cassiman et al., 2001; Geerts, 2001), and HSP47 in both rat and human liver (Brown et al., 2005; Kawada et al., 1996; Masuda et al., 1994), show increased expression in activated HSC as compared to quiescent cells. In rat liver some cellular markers are expressed in activated HSC that are not, or rarely present in quiescent HSC, like αB-crystallin (Cassiman et al., 2001; Lang et al., 2000) and αSMA (Ballardini et al., 1988; Geerts, 2001). Table 2 gives an overview of cellular markers that were used to study HSC activation in liver slices, and indicates the relative

Table 2
Relative expression of the cellular markers in quiescent HSC (qHSC), activated HSC (aHSC), and (myo)fibroblast populations other than activated HSC (MFB) in rat and human liver

		Rat liver			Human liver			References
		qHSC	aHSC	MFB	qHSC	aHSC	MFB	
HSP47	Heat shock protein	+	++	++	+	++	++	Brown et al. (2005), Masuda et al. (1994)
αB-crystallin	Heat shock protein	–	++	++	±	++	++	Cassiman et al. (2002, 2001), Lang et al. (2000)
Desmin	Cytoskeletal protein	+	++	++	–	–	–	Cassiman et al. (2002), Geerts (2001)
αSMA	Cytoskeletal protein	–	++	++	±	++	++	Geerts (2001)
Vinculin	Cytoskeletal protein	+	+	n.r.	+	+	n.r.	Kawai et al. (2003)
Synaptophysin	Membrane protein	+	+	–	+	+	–	Cassiman et al. (2002, 1999)
Fibulin-2	ECM protein	–	–	++	n.r.	n.r.	n.r.	Knittel et al. (1999a,b)

n.r. – not reported and HSP47 – heat shock protein 47.

expression levels in quiescent and activated HSC in human and rat liver.

In theory, increased expression of HSP47, αB-crystallin, desmin, and αSMA in liver slices can be used as a marker for HSC activation in the slices. However, the markers that are available to detect activated HSC are general (myo)fibroblast markers and consequently are also present in other types of (myo)fibroblasts that are present in the liver. Although some markers, such as synaptophysin (Cassiman et al., 1999) and fibulin-2 (Knittel et al., 1999a,b) can be used to discern HSC from (myo)fibroblasts, to our knowledge, there is no marker that can specifically indicate the presence of activated HSC without measuring either other (myo)fibroblasts or quiescent HSC (Table 2). Thus, when studying HSC activation in liver slices, it must be taken into account that increased expression of HSC activation markers in the liver slice may also reflect the presence of an increased number of (myo)fibroblasts or HSC. On the other hand, although the activated HSC play the major role in the development of liver fibrosis, the population of fibrogenic cells involved in this process is likely more heterogeneous and can also include activated portal fibroblasts and possibly second-layer cells located around centrolobular veins and smooth muscle cells (Guyot et al., 2006). When liver slices are used to study the development of fibrosis and to test the effects of potential pro- or anti-fibrotic compounds, they provide the advantage that the response of all fibrogenic cells in the liver can be studied together in a milieu that closely resembles the *in vivo* situation.

Finally, to study the involvement of the other liver cell types in processes leading to HSC activation and fibrogenesis in liver slices, specific inhibitors of cellular functions can be used. In addition, expression of genes and proteins associated with fibrosis, such as certain cytokines and their receptors, collagens, and matrix metalloproteinases and their inhibitors, are indicative of fibrogenesis and may provide information on the involvement of the different liver cell types in fibrotic processes in the liver slices.

3.5. Liver slices as in vitro model for fibrosis

Although precision-cut liver slices may provide a promising tool for the study of human liver fibrosis *in vitro*, only a limited number of studies describe the use of liver slices for the study of fibrosis and to test effects of pro- and anti-fibrotic compounds. First, in two studies the development of a technique to induce early HSC activation in rat (Van de Bovenkamp et al., 2005) and human (Van de Bovenkamp et al., 2006b) liver slices via a multicellular mechanism was described. This was achieved by incubation of the liver slices with the pro-fibrotic compound carbon tetrachloride, which *in vivo* induces fibrosis via its conversion into free radicals by hepatocytes and the subsequent formation of lipid peroxides and other mediators that activate hepatic stellate cells, both directly and indirectly via Kupffer cells (Basu, 2003; Weber et al., 2003). Incubation of rat and human liver slices with carbon tetrachloride resulted in early HSC activation, as was determined by the expression levels of HSP47 and αB-crystallin. Given the mechanism of fibrosis development due to carbon tetrachloride *in vivo*, this HSC activation can likely only occur because all liver cell types are present in the liver slice and cell–cell interactions are maintained. This *in vitro* model thus provides a system to study multicellular mechanisms underlying toxicity-induced early HSC activation in rat as well as human liver tissue.

Second, studies evaluating the mRNA expression of αSMA and pro-collagen 1a1 as markers for HSC activation and fibrogenesis during incubation of rat and human liver slices showed a yet unexplained decreasing expression level of these markers during the first hours of incubation followed by increasing gene expression of the markers. The latter suggests that a fibrotic process is initiated in the liver slices after prolonged incubation (Van de Bovenkamp et al., 2006a; Vickers et al., 2004). Whether this process reflects a normal wound healing response or the initiation of (pathological) fibrosis, and whether there is a fundamental difference between these processes, remains to be established. Similar to fibrogenesis *in vivo*, which is likely mediated by different populations of (myo)fibroblasts (Guyot et al., 2006), both activated HSC and other (myo)fibroblast populations appeared to be involved in this process, as was concluded from the expression pattern of synaptophysin and fibulin-2 (Van de Bovenkamp, 2006). HSC activation and fibrogenesis occurring in liver slices generated from normal liver tissue, suggests that the induc-

tion is intrinsically related to the preparation or culturing method used. Common factors in the methods 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 indicated that oxidative stress or 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. The latter may be explained by the removal of dead cells and soluble factors that can potentially activated the stellate cells during the period of pre-incubation, which is generally applied prior to incubation of the liver slices. In addition, several short-term studies suggest that during incubation up to 7 h accumulation of bile salts, which is potentially fibrogenic, does not occur (Barth et al., 2006; Thompson et al., 1993). Rather, the degree of stellate cell activation occurring in the liver slices appeared to be correlated with the thickness of the liver slices, which suggests that some degree of cell damage occurs in the center of the liver slices resulting in subsequent stellate cell activation.

Incubation of rat and human liver slices in the presence of the anti-fibrotic compound pentoxifylline resulted in significant inhibition of the spontaneous fibrotic process (Van de Bovenkamp, 2006). Taken together, this model provides a simple system to study fibrogenesis in the liver and to test anti-fibrotic drugs in a multicellular environment, closely resembling the *in vivo* situation. The latter is especially of importance when compounds are tested that exert their effects on stellate cell activation and fibrogenesis in an indirect manner, e.g., via effects on Kupffer cells. Further elucidation of the processes underlying both the initial decreased expression of HSC activation and fibrogenesis markers and their subsequent increased expression after prolonged incubation is necessary to fully characterize the model.

Third, liver slices generated from fibrotic rat liver tissue were evaluated as a tool to study fibrosis and test anti-fibrotic compounds (Van de Bovenkamp et al., 2006a). Fibrotic rat liver slices were generated from rat liver three weeks after bile-duct ligation (BDL). These livers showed clear signs of fibrosis compared to control rat liver, with prominent collagen protein content and proliferated HSC and bile duct epithelial cells, which are characteristic for BDL-induced fibrosis (Hinz et al., 1997). These features were retained after the slicing procedure, indicating that the liver slices still were fibrotic. During incubation up to 48 h, the viability of the fibrotic liver slices and the different liver cell types therein was maintained, with retained cell–cell interactions (Van de Bovenkamp et al., 2006a). Incubation of fibrotic rat liver slices resulted in similar changes in the expression of markers for HSC activation and fibrogenesis as described above for normal rat liver slices, with a more pronounced increase of these markers after prolonged incubation, which was likely mediated by both activated HSC and other (myo)fibroblast populations. This activation and/or proliferation of fibrogenic cells was accompanied by an increased production of collagen protein and could be inhibited by pentoxifylline, gleevec, and dexamethasone, drugs that were previously shown to possess anti-fibrotic activity *in vitro* (Desmouliere et al., 1999; Preaux et al., 1997; Windmeier and Gressner, 1996; Yoshiji et al., 2005) and/or *in vivo* (Desmouliere et al., 1999; Dos Santos Pyrrho et al., 2002; Ensminger et al., 2004; Neef et al., 2006; Peterson and Neumeister, 1996; Raetsch et al., 2002; Yoshiji et al., 2005). Fibrotic liver slices thus provide the unique opportunity to study progressed stages of fibrosis and to test the effects of anti-fibrotic compounds in a multicellular and, importantly, fibrotic environment, which is not possible using other *in vitro* models or normal rat liver slices. This approach can be used for different types of disease- or toxicity-induced fibrosis, not only for liver but likely also for other organs. In addition, it may also be applicable for diseased human liver tissue.

Finally, one study showed that liver slices can be used to study the mechanisms involved in the development of portal fibrosis due to bile acid cytotoxicity, as was concluded from the bile-acid induced proliferation of biliary epithelial cell and portal fibroblast in liver slices (Clouzeau-Girard et al., 2006).

4. Conclusions

In vitro models for the study of HSC activation and fibrogenesis are valuable tools in addition to *in vivo* experiments. Although *in vitro* models can never completely mimic the *in vivo* situation, continuous optimization and adaptation have extended their potential during the past years. Cell cultures of single liver cell types provide an easy system to study effects of pro- and anti-fibrotic compounds *in vitro*, and can be used to study responses of the different liver cell types separately. This provides the opportunity to unravel multicellular processes leading to liver toxicity and fibrogenesis with respect to the potential role that different liver cell types play in these processes. By using co-cultures of different liver cell types also cell–cell interactions can be incorporated to some degree, which allows studying indirect effects of potential pro- or anti-fibrotic compounds on HSC activation. In addition, the use of culture matrices can be used to study the effects of ECM proteins on HSC activation and fibrogenesis. However, it must be taken into account that co-culture models and culture matrices cannot accurately mimic the *in vivo* situation with respect to the cellular and ECM composition. Precision-cut liver slices provide a multicellular *in vitro* model for the liver in which *in vivo* cell–cell and cell–ECM interactions are maintained. The first studies exploring the use of precision-cut liver slices as *in vitro* model for liver fibrosis clearly indicate that liver slices provide a promising model to study liver fibrosis, filling the gap between *in vivo* and cell culture models. In addition, recently the engineering and culturing of sinusoid-like structures *in vitro* was described, which may also

represent a promising development for the study of liver fibrosis *in vitro* (Nahmias et al., 2006).

Taken together, by combining the use of these different cell and tissue culture models, the mechanisms underlying multicellular fibrosis development can be studied and potential pro- or anti-fibrotic properties of compounds can be identified both on single liver cell types and in human liver tissue. The development of these *in vitro* cell and tissue culture models can contribute substantially to the reduction, refinement, and possibly to the replacement of animal experiments. In addition, the use of human liver cells or (fibrotic) human liver tissue in these *in vitro* models may improve the predictive value for man.

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