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A long-term three dimensional liver co-culture system for improved prediction of clinically relevant drug-induced hepatotoxicity

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ABSTRACT

Drug-induced liver injury (DILI) is the major cause for liver failure and post-marketing drug withdrawals. Due to species-specific differences in hepatocellular function, animal experiments to assess potential liabilities of drug candidates can predict hepatotoxicity in humans only to a certain extent. In addition to animal experimentation, primary hepatocytes from rat or human are widely used for pre-clinical safety assessment. However, as many toxic responses *in vivo* are mediated by a complex interplay among different cell types and often require chronic drug exposures, the predictive performance of hepatocytes is very limited. Here, we established and characterized human and rat *in vitro* three-dimensional (3D) liver co-culture systems containing primary parenchymal and non-parenchymal hepatic cells. Our data demonstrate that cells cultured on a 3D scaffold have a preserved composition of hepatocytes, stellate, Kupffer and endothelial cells and maintain liver function for up to 3 months, as measured by the production of albumin, fibrinogen, transferrin and urea. Additionally, 3D liver co-cultures maintain cytochrome P450 inducibility, form bile canaliculi-like structures and respond to inflammatory stimuli. Upon incubation with selected hepatotoxicants including drugs which have been shown to induce idiosyncratic toxicity, we demonstrated that this model better detected *in vivo* drug-induced toxicity, including species-specific drug effects, when compared to monolayer hepatocyte cultures.
In conclusion, our results underline the importance of more complex and long lasting *in vitro* cell culture models that contain all liver cell types and allow repeated drug-treatments for detection of *in vivo*-relevant adverse drug effects.

Introduction

Drug-induced liver injury (DILI) is still the leading cause of acute liver failure and post-market drug withdrawals (Kaplowitz, 2005). Studies have shown that different risk factors can contribute to DILI such as genetic susceptibility factors, non-genetic factors including age, sex, diseases and compound factors including daily dose, metabolism characteristics, and drug-drug interactions (Chalasani and Bjornsson, 2010; David and Hamilton, 2010). Preclinical animal studies cannot fully predict drug-toxicity in humans due to species-specific variations between human and animal hepatocellular functions (Pritchard et al., 2003). Human *in vitro* liver models currently used for prediction of drug-induced toxicity include microsomes, cell lines, liver slices and primary hepatocytes (Gebhardt et al., 2003; Guillouzo, 1998; Hewitt et al., 2007; LeCluyse, 2001). Microsomes are used in high-throughput systems to assess drug metabolizing enzymes but lack the cellular machinery required for toxicity testing (Donato et al., 2004). Although hepatoma cell lines such as HepG2 cells can be used for high-throughput screening, they have low levels of CYP activities and lack many key liver-specific functions (Wilkening et al., 2003). Specific hepatoma cell clones such as HepaRG have most of the specific liver functions at levels close to those found in primary human hepatocytes but they do not represent the genetic heterogeneity of human populations (Guguen-Guillouzo and Guillouzo, 2010; Lubberstedt et al., 2011; McGill et al., 2011; Pernelle et al., 2011). Liver slices retain *in vivo* liver architecture but have only short term viability and are not applicable to high-throughput screening (Guillouzo, 1998). Primary hepatocytes growing in monolayer two-dimensional (2D) culture are easy to use but liver specific functions including drug metabolism rapidly decline under standard culture conditions allowing detection of acute drug-induced toxicity only (Guguen-Guillouzo and Guillouzo, 2010; Hewitt et al., 2007; Lecluyse et al., 2012; Sivaraman et al., 2005). Many modifications to standard culture models for primary hepatocytes have been developed to prolong hepatocyte function such as culturing of the cells in collagen type I/IV, fibronectin or other extracellular matrix (ECM)-coated plates (Bissell et al., 1987; Mingoia et al., 2007), or between two layers of collagen type I or matrigel (Dunn et al., 1989; Guguen-Guillouzo and

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Guillouzo, 2010; Hewitt et al., 2007; Lecluyse et al., 2012; Mingoia et al., 2007). However, these modifications, while increasing CYP activities and prolonging the functional lifespan of primary hepatocytes to a certain extent, do not recapitulate all the important functions of the liver, mainly because of the lack of hepatic non-parenchymal cells (NPC; Hasmall et al., 2001; Roberts et al., 2007).

Substantial improvements in hepatocyte *in vitro* models were achieved by the development of more complex human liver systems created by co-culturing of parenchymal cells (PC) with NPC or other cell types. For example, human hepatocytes in a 2D micro-patterned co-culture with mouse 3 T3-J2 fibroblasts (Khetani and Bhatia, 2008) maintained hepatocellular function for several weeks. Yet, the model may not be physiologically relevant for detection of species-specific drug toxicity due to the lack of other liver NPC and the fact that a mouse embryonic fibroblast cell line is used for stabilization of human hepatocyte function (Hasmall et al., 2001; Roberts et al., 2007). In this regard, hepatic stellate cells (HSC) and Kupffer cells play a key role in modulating DILI, including idiosyncratic toxicity and hepatocarcinogenesis, probably due to the release of inflammatory mediators, growth factors and reactive oxygen species after their activation by drugs (Hasmall et al., 2001; Lecluyse et al., 2012; Roberts et al., 2007). More sophisticated models containing hepatocytes and NPC are the 3D liver co-culture bioreactors (Dash et al., 2009; Gerlach et al., 2003; Sivaraman et al., 2005; Zeilinger et al., 2011). These models can be kept in culture for several weeks but due to their complexity may not be suited for drug testing in pharmaceutical industry.

At present only few human co-culture models are available which can be used for drug-safety assessment (Dash et al., 2009; Khetani and Bhatia, 2008; Naughton et al., 1994). There is an urgent need to establish and validate human *in vitro* liver models able to produce clinically-relevant data. We therefore characterized a 3D liver culture model using both human and rat primary cells and evaluated its suitability to assess DILI potential *in vitro*. The model originally described by Naughton and co-workers is based on an industry-standard multiwell format and is therefore amenable to higher-throughput testing (Naughton et al., 1994, 1995). We show that hepatocytes inoculated into a pre-established NPC culture grown on 3D nylon scaffolds can be kept in culture for up to 3 months while maintaining some important hepatic functions and metabolic CYP activities. This allows exposure to compounds over longer time and allows repeated drug-treatments which are not possible using short-term 2D hepatocyte cultures or other currently available 3D models. Thus, we challenged the 3D liver co-culture with known drugs having different hepatotoxic characteristics and assessed its ability to reflect drug-induced species differences in rat and human. We conclude that this model better detects drug-induced acute and chronic liver toxicity observed *in vivo* than monolayer hepatocyte cultures. This underlines the importance of incorporating not only hepatocytes but all liver cell types into such a system and their exposure to compounds over long time to allow *in vitro* assessment of *in vivo*-relevant adverse drug effects.

Materials and Methods

3D liver cell co-cultures. The human and rat 3D liver models were created as a multiwell insert plate system by RegeneMed (San Diego, USA) as follows: All liver NPC, including vascular and bile duct endothelial cells, Kupffer cells and hepatic stellate cells (HSC), were freshly isolated by gradient centrifugation after *in situ* liver perfusion from human or rat livers and expanded in monolayer culture for 3–4 passages (Naughton et al., 1994, 1995). The NPC were then seeded above two interconnected nylon scaffolds (meshes; pore size 140 μm) placed into inserts sitting 1 mm above the bottom of the wells of 24 multi-well plates (Fig. 1A). The nylon scaffolds allow NPC to proliferate in 3D and to express and establish their own ECM components, growth and regulatory factors necessary to sustain long-term survival and function of PC in culture (Naughton et al., 1994, 1995). The bottom of each insert contains a porous membrane (pore size of 12 μm), which allows constant supply of the tissue with medium (Fig. 1A). After one week, when NPC had grown across the majority of the gaps in the screens, the inoculation of human or rat hepatocytes isolated from the same or different donors was performed. Fresh human hepatocytes were isolated always from male donors 30 to 60 years-old (RegeneMed). Rat hepatocytes were isolated from 10 to 14 weeks-old male Wistar rats. Only the hepatocytes which have viability assessed by trypan blue exclusion above 80% were seeded for the creation of 3D liver cultures. The cells were always seeded in proportions similar to the native liver such as 40% NPC and 60% hepatocytes in the 24 well culture inserts following the established protocol from RegeneMed. One week after inoculation of the hepatocytes, a 3D liver tissue, containing NPC and PC, had formed. The cultures were kept in proprietary media (RegeneMed) containing William's E medium (cat #: 35050–079; Life Technologies), serum, 61.5 units penicillin, 61.5 units streptomycin, 50 mg/mL gentamicin, 0.5 mg/mL fungizone and other supplements. The cultures were treated with the drugs at least one week after the inoculation of hepatocytes. Medium was replaced 3 times per week and the cells were kept for more than 3 months in culture.

Hepatocyte isolation and culture. Hepatocytes were isolated from 10 to 14 weeks-old male Wistar rats by a two-step collagenase liver perfusion method as previously described (Roth et al., 2011). After the rats were anaesthetized with sodium pentobarbital (120 mg/kg, i.p.), the liver was first perfused for 5 min with a preperfusing solution consisting of calcium-free, 0.5 mM EGTA-supplemented, 20 mM HEPES-buffered Hank's balanced salt solution (5.36 mM of KCl, 0.44 mM of KH_2PO_4, 137 mM of NaCl, 4.2 mM of $NaHCO_3$, 0.34 mM of Na_2HPO_4, and 5.55 mM of D-glucose). This was followed by a 12 min perfusion with 25 mM $NaHCO_3$-supplemented Hank's solution containing 5 mM $CaCl_2$ and 0.2 U/mL collagenase. Flow rate was maintained at 28 mL/min and all solutions were kept at 37 °C. After in situ perfusion, the liver was excised and mechanically disrupted. The cells were suspended in William's medium E without phenol red and filtered through a set of tissue sieves (30-, 50-, and 80-mesh). Dead cells were removed by a sedimentation step (1 ×*g*, for 15 min at 4 °C) followed by a Percoll centrifugation step (Percoll density: 1.06 g/mL, 50 ×*g*, 10 min) and an additional centrifugation in William's medium E (50 ×*g*, 3 min). Around $(100–300)\times10^6$ cells were obtained from one rat liver. Cell viability was assessed by trypan blue exclusion and ranged between 85% and 95%. Cells were seeded into collagen-coated 24-well Falcon Primaria plates (Fisher Scientific AG, Wohlen, Switzerland), at a density of 3×10^5 cells/well in 0.5 mL of William's medium E supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (0.1 mg/mL), insulin (100 nM), and dexamethasone (100 nM). Different batches of human platetable hepatocytes isolated from several male non-smoking donors 30–50 years-old were obtained from Celsis and seeded on collagen-coated 24-well plates at density of 3×10^5 cells/well in 0.5 ml of William's medium E containing 10% FCS and the same supplements like the medium for the rat hepatocytes. After an attachment period of 3 h, the hepatocyte medium was replaced with serum-free medium and the cells were further kept for a maximum of 3 days at 37 °C in an atmosphere of 5% CO_2/95% air. The media of the hepatocytes was replaced daily. The cells were exposed to drugs in serum-free medium the next day after seeding. We compared the performance of 3D liver cells with the standard hepatocyte monolayer culture, because this is the most common *in vitro* model used in the pharmaceutical industry for drug hepatocyte toxicity screenings, mechanistic studies as well as metabolism experiments (Guillouzo, 1998; Hewitt et al., 2007; Roth et al., 2011; Sivaraman et al., 2005).

Assays of liver-specific function. Culture medium from rat and human 3D liver cells was collected at the indicated time points and stored at −80 °C for albumin, transferrin, fibrinogen and urea measurements.