A MICROPATTERNED CULTURE WITH HUMAN HEPATOCYTES AND KUPFFER MACROPHAGES FOR STUDYING INFLAMMATION-DRUG INTERACTIONS

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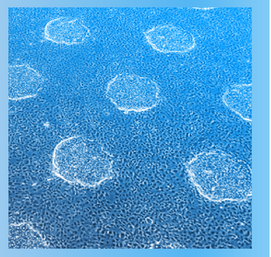

ABSTRACT

The appearance or relief of inflammation through drug therapy (i.e. therapeutic proteins) could differentially affect levels of enzymes involved in metabolism of co-administered drugs with potential pharmacological and toxicological consequences. An *in vitro* model that mimics liver inflammation could provide better predictive data in preclinical testing. We have developed a micropatterned co-culture of primary human hepatocytes and embryonic fibroblasts (HepatoPac™) that retains high levels of phenotypic functions such as drug metabolism enzymes for 4 weeks *in vitro*. Here, we augment the HepatoPac platform with primary Kupffer macrophages in order to mimic one component of inflammation. Kupffer cells were added to human HepatoPac at multiple ratios (to mimic both the normal and inflamed state of the liver) after stabilization to generate a tri- culture with human hepatocytes and embryonic fibroblasts (HepatoPac-Kupffer co-culture). Stimulation of this model with lipopolysaccharide (LPS) caused secretion of IL-6 and TNFα (not shown) for 4 days in culture at levels similar to those in LPS-stimulation of cultures of Kupffer cells alone indicating the presence of functional Kupffer cells. Assessment of hepatocyte metabolic competence in the presence or absence of Kupffer cells showed comparable production of albumin and urea and Cyp3A4 activity suggesting the presence of functional hepatocytes in co-cultures. We then showed cytokine-mediated suppression of Cyp3A4 activity and gene expression in HepatoPac/Kupffer co-cultures towards first steps in modeling inflammation-drug interactions. We are now characterizing release of other relevant cytokines from this model, impact on other major CYP450 isoenzymes in hepatocytes, and assessment of inflammation-drug interactions. In the future, this *in vitro* model could be useful for mechanistic study of aspects of xenobiotic-inflammation interactions and serve as a unique preclinical testing tool for biotech and pharmaceutical companies engaged in biologics drug discovery.

INTRODUCTION

Species-specific variations in organ functions between animals and humans can be significant, especially in liver-specific metabolic pathways (i.e. CYP450).This, along with other factors, may severely limit the utility of animal models for predicting human-specific responses. Isolated primary human hepatocytes in adherent culture are widely considered to be the most suitable for *in vitro* testing [1].They are relatively simple to use and maintain an intact cellular architecture with complete, undisrupted enzymes and cofactors. Conventional culture models utilized for drug screening expose hepatocytes to tumor-derived Matrigel™ and/or collagen-I gels (sandwich cultures). When utilized with near confluent monolayers, these models allow better retention of hepatocyte cyto-architecture and activities of specific CYP450s for 3-5 days over hepatocytes on rigid collagen substratum. However, sandwich cultures are inherently unstable in their phenotypic functions and their short-term functionality does not allow for clinically-relevant chronic drug dosing regimens to be thoroughly tested. Accordingly, there is a need for better *in vitro* models of primary human liver tissue that are more predictive of clinical outcomes and can be used with existing industrial automation for high-throughput screening in industry-standard multi-well formats. We have utilized microtechnology tools to both optimize and miniaturize, in a multi-well format (up to 96-well), *in vitro* models of human and animal (i.e. rat, dog, monkey) livers called HepatoPac [2]. Specifically, primary hepatocytes are organized into colonies of prescribed, empirically-optimized dimensions and subsequently surrounded by supportive stromal cells. Hepatocytes in HepatoPac retain their *in vivo*-like morphology, express liver genes, metabolize compounds using active Phase I/II drug metabolism enzymes [3], secrete diverse liver-specific products, and display functional bile canaliculi for 4-6 weeks *in vitro*. Furthermore, HepatoPac outperforms conventional culture models with respect to magnitude and longevity of liver-specific functions. **In the current study, we augmented the HepatoPac model with primary human Kupffer cells (Figure 1 below) for utility in evaluating inflammation-drug interactions [4]. We found that Kupffer cells did not compromise hepatic functionality in our model and remained functional (as assessed via phagocytosis and cytokine release) for up to 10 days post addition to HepatoPac. Finally, we evaluated the utility of our HepatoPac-Kupffer co-culture model for cytokine-mediated CYP450 inhibition studies.**

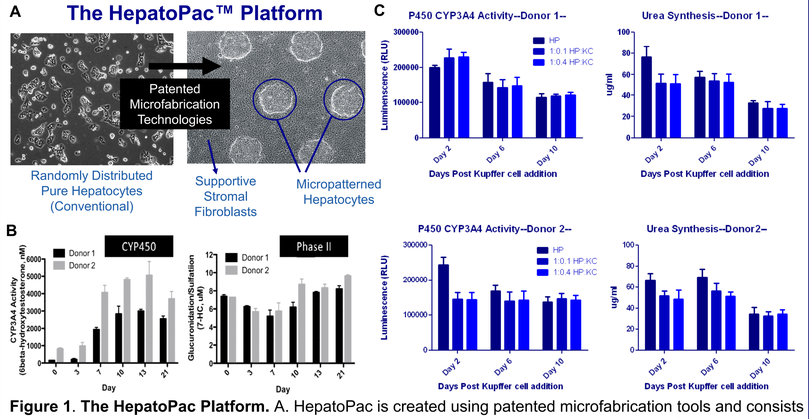

Figure 1. The HepatoPac Platform. A. HepatoPac is created using patented microfabrication tools and consists of primary hepatocytes arranged in optimized domains and surrounded by stromal fibroblasts. B. Human HepatoPac cultures created using multiple cryopreserved human hepatocyte donors retain long- term functionality for several weeks *in vitro* [1]. Cyp3A4 activity (via testosterone 6β-hydroxylation) is shown but other major CYP450 enzymes show similar stability. Phase II metabolism was assessed via glucuronidation/sulfation of 7- hydroxycoumarin. C. Addition of primary human Kupffer cells to HepatoPac does not significantly affect functionality of hepatocyte donors as determined by Cyp3A4 activity and urea synthesis. *HP = HepatoPac alone, HP:KC = HepatoPac/Kupffer co-cultures at various ratios.*

MATERIALS & METHODS

- Cryopreserved human hepatocytes and fresh human Kupffer cells were obtained from Celsis In Vitro Technologies. The cytokines used in these studies (IL-2, IL-1β, TNF-α, or IL-6) were obtained from R&D Systems. Cytokine ELISA kits were purchased from BD Biosciences while pHrodo™ S.*aureus* Bioparticles were obtained from Life Technologies. Anti-Human CD68 antibody was purchased from Biolegend.
- Human HepatoPac cultures utilizing cryopreserved human hepatocytes and 3T3-J2 murine embryonic fibroblasts were manufactured following Hepregen's proprietary protocols and quality controls. HepatoPac cultures were stabilized in serum-supplemented medium for 7 days prior to addition of Kupffer cells at hepatocyte:Kupffer cell ratios of 1:0.1 and 1:0.4 to mimic physiological and inflamed liver states, respectively. Lipopolysaccharide (LPS) stimulation of cultures was carried out for 20 hours at 50ng/ml after which supernatants were analyzed for cytokine secretion using OptEIA™ ELISA kits (BD Biosciences)
- HepatoPac/Kupffer cell co-cultures were exposed to different concentrations of IL-2, IL-1β, TNF-α, or IL-6 for up to 4 days in serum-free medium. IL-6 secretion, Cyp3A4 activity (P450-Glo Assay; Promega), and cellular ATP content (Cell-Titer Glo Assay; Promega) were measured 2 and 4 days post addition of cytokines. RNA was extracted (Qiagen RNeasy Kit) and CYP450 mRNA levels were evaluated using Taqman® primer-probes from Life Technologies. Statistical analyses were performed via a two-tailed t-test.

RESULTS

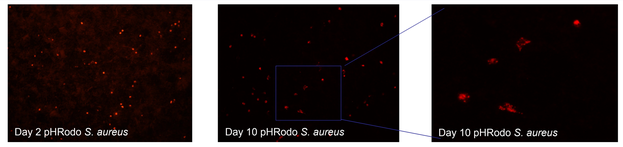

Figure 2. Validation of Kupffer cell functionality in HepatoPac/Kupffer cell co-cultures. As shown above in red, Kupffer cells in co-culture are specifically able to phagocytose pHrodo red labeled *S.aureus* bioparticles. Phagocytosis, which is an important marker for functional Kupffer cells, is detected at 2 days and as late as 10 days post Kupffer cell addition to HepatoPac. Immuno-staining with anti-CD68 [5] also confirmed the presence of Kupffer cells in co-cultures (data not shown).

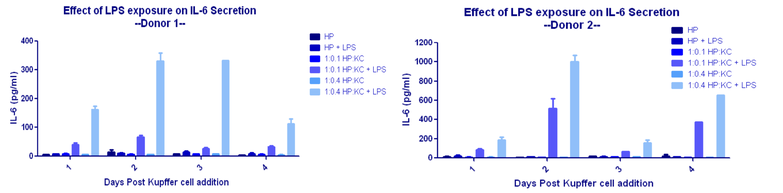

Figure 3. LPS stimulation causes release of IL-6 in HepatoPac/Kupffer cell co-cultures. Co-cultures from multiple donors were stimulated for 20 hours with 50 ng/mL of LPS on Day 1, 2, 3 and 4 post addition of Kupffer cells. LPS-mediated stimulation of IL-6 secretion was found to be dependent on number of Kupffer cells in co-cultures indicating the presence of functional Kupffer cells in the system. *HP = HepatoPac alone, HP:KC = HepatoPac/Kupffer co-cultures at various ratios.*

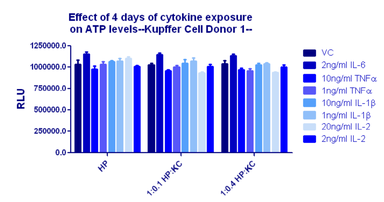

Figure 4. Effect of cytokine exposure on viability in HepatoPac/Kupffer co-cultures. ATP levels were determined after exposure to cytokines for 4 days. Exposure to cytokines over the concentration ranges used here did not affect the viability of the co-cultures. Similar trends were seen in a separate donor as well (data not shown). *HP = HepatoPac alone, HP:KC = HepatoPac/Kupffer co-cultures at various ratios.*

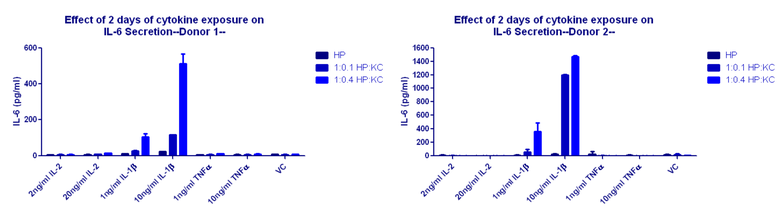

Figure 5. IL-1β stimulation causes release of IL-6 in HepatoPac/Kupffer co-cultures. Co-cultures from multiple donors were exposed to IL-2, IL-1β and TNF-α for 2 days at multiple doses. Secretion of IL-6 into the culture supernatant was then measured. Exposure to IL-1β caused IL-6 secretion in a dose- and Kupffer cell number-dependent manner. IL-2 and TNF-α had no effect on IL-6 secretion at the doses tested. *HP = HepatoPac alone, HP:KC = HepatoPac/Kupffer co-cultures at various ratios.*

RESULTS CONTINUED

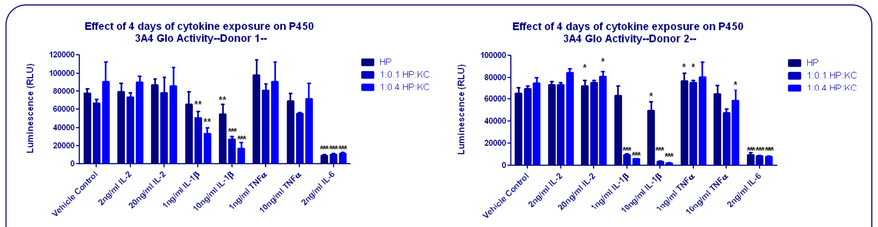

Figure 6.Cytokine-mediated Cyp3A4 activity inhibition in HepatoPac/Kupffer cell co-cultures. Treatment of multi-donor co-cultures with IL-1β caused a dose-dependent and Kupffer cell-enhanced suppression of Cyp3A4 activity potentially due to Kupffer-mediated secretion of IL-6 and other cytokines. Donor-dependent differences were observed in magnitude of such suppression. Treatment of co-cultures with IL-6 caused suppression of Cyp3A4 activity in the presence and absence of Kupffer cells. *HP = HepatoPac alone, HP:KC = HepatoPac/Kupffer co-cultures at various ratios. Endpoints were compared to their vehicle control using a two-tailed t-Test; *p<0.05, **p<0.01, ***p<0.001.*

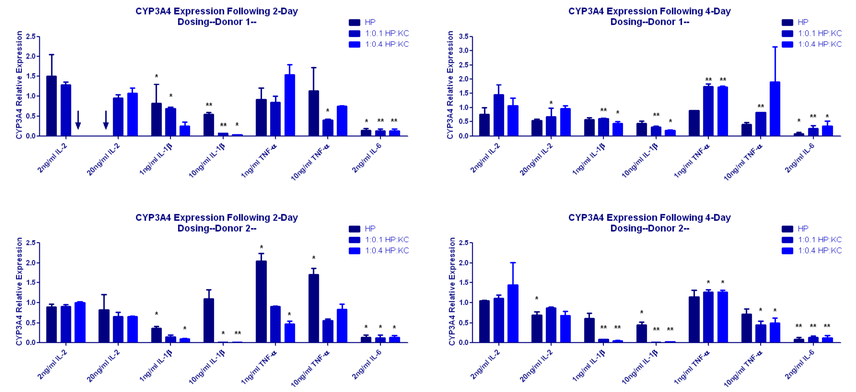

Figure 7.Cytokine-mediated CYP3A4 mRNA repression in HepatoPac/Kupffer cell co-cultures. Trends parallel those seen at the level of enzymatic activity as seen in **figure 6**. Relative expression of genes were normalized to their respective vehicle controls using the ΔΔCt method followed by a two-tailed t-test. Arrows indicate that no data was available.

CONCLUSIONS & FUTURE DIRECTIONS

- Human HepatoPac is a multi-well (i.e. 96-well plates) platform that consists of primary hepatocytes organized in empirically optimized clusters and subsequently surrounded by 3T3-J2 murine embryonic fibroblasts. Here, HepatoPac was augmented with primary human Kupffer cells for utility in assessment of inflammation-drug interactions**.**
- Kupffer cells seeded at physiologically-relevant or inflamed ratios in HepatoPac (1:0.1 or 1:0.4 hepatocyte:Kupffer, respectively) did *not* affect hepatocyte functionality as assessed via Cyp3A4 activity and urea synthesis.
- Kupffer cells in HepatoPac/Kupffer co-cultures remained viable and functional for up to 10 days post addition to the wells as assessed via CD68 immunostaining and positive phagocytosis of pH-sensitive S.*aureus* bioparticles. IL-6 secretion remained consistent for 5 days following stimulation with endotoxin (LPS) and pro-inflammatory cytokine IL-1β.
- Suppression of both Cyp3A4 activity and CYP3A4 expression were observed over four days in response to IL-1β in a dose-dependent and Kupffer-cell enhanced manner. IL-6 exerted suppressive effects on Cyp3A4 activity and gene expression independent of Kupffer cells.
- Future studies on the HepatoPac/Kupffer co-culture platform will seek to characterize the release of other relevant cytokines and their influence on the expression and activity of additional CYP450 isoenzymes in hepatocytes.
- The HepatoPac/Kupffer co-culture platform could find utility in assessment of clinically-relevant interactions between therapeutic biologics sand small molecule drugs.

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