Co-culture of Hepatocytes and Kupffer Cells as a Model for Liver Inflammation.

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ABSTRACT

Introduction: Kupffer cells are activated liver resident macrophages that play an important role in liver immunity and in the modulation of xenobiotic metabolism during liver inflammation. These cells suppress the expression of multiple cytochrome P450 enzymes through the exchange of soluble factors, following activation with either lipopolysaccharide (LPS) or other pro-inflammatory cytokines. Since those interactions lead to severe pharmacological and toxicological consequences, there is an evident need to develop a commercially available model mimicking the liver inflammatory state that could be used for in vitro drug metabolism and discovery ADME/Tox applications. We use a liver co-culture system consisting of plated primary hepatocytes and Kupffer cells in a physiologic 2:1 ratio, which is relevant to inflammation, and compare these to monocultures of Kupffer cells or hepatocytes. **Material and Methods**: Primary rat Kupffer cells and hepatocytes were isolated using a modified two step collagenase perfusion. Purity of isolated Kupffer cells was determined by culturing cells for 2 days and analyzing them by immunostaining for ED1 and ED2 expression. To create inflammatory co-cultures, Kupffer cells were seeded at a density approximating half that of hepatocytes and cultured for 48hr prior to all experiments. Kupffer cell activation was induced by treating hepatocyte cultures, Kupffer cell cultures, or co-cultures with LPS (1µg/mL) or IL2 (200ng/mL) for a durations of 24, 48 and 72hrs. Following treatment, all cultures were analyzed for morphology, cytokine production by ELISA, and P450 mRNA expression (CYP3A and CYP1A2). **Results**: Isolated Kupffer cells expressed ED1 at 88.7% (±1.05) and ED2 at 75.5% (±5.4) after 2 days in culture. Following 6 days in culture, expression of ED1 remained at 85.6% (±1.9) and ED2 expression significantly increased to 93.5% (±0.4) suggesting Kupffer cell activation. As expected, following plating and activation, primary Kupffer cells significantly increased production of pro-inflammatory cytokines IL-6 and TNFa in co-cultures and Kupffer cell monocultures. However, at 72hr after treatment with either LPS or IL2, only co-cultures showed upregulated expression of IL6 by nearly 2-fold compared with monocultures of Kupffer cells. This suggests auto-assembly of cellular interactions between both types of cells. After 72hr treatment, LPS and corresponding IL-6 up-regulation correlated with approximately 20% down-regulation of CYP3A and CYP1A2 expression. IL2 treatment correlated with approximately 80% down-regulation of both enzymes. **Conclusion:** Our data suggests that Kupffer cell and hepatocyte co-cultures can self-assemble within 72hr of treatment with pro-inflammatory cytokines or LPS, and can function by effectively modulating P450 expression in co-cultured hepatocytes. As such, co-culture of primary hepatocytes and Kupffer cells mimicking liver inflammatory state offer a powerful in vitro ADME/Tox tool to assess the effects of xenobiotics on drug metabolizing enzymes and more closely model more complex hepatic cell biology in vitro.

INTRODUCTION

The largest solid organ in the human body, the liver is responsible for diversity of function with most important being metabolism, detoxification and protein synthesis. Most of those functions take place within hepatocytes which represent a major cell type of the hepatic parenchyma; however, liver also contains a large proportion of non-parenchymal cells (NPCs) which include liver sinusoidal endothelial cells, hepatic stellate (ito) cells, cholangiocytes and Kupffer cells. In general, NPCs provide physical and biochemical structure to the liver. Out of all NPCs, Kupffer cells, which are the liver resident macrophages, play an important role in liver physiology and homeostasis by participating in the acute and chronic responses of the liver to toxic compounds during liver inflammation.

In the liver, Kupffer cells are located on the sinusoidal side of hepatic parenchyma and use their stellate like cytoplasmic extensions for direct cell-to-cell contact with hepatocytes. This contact is essential for proper modulation and the development of a fulminant hepatic inflammatory response. In addition to cell contact, activation of Kupffer cells results in the release of a variety of inflammatory cytokines and growth control mediators that suppress the expression of multiple cytochrome P450 enzymes. Two of those factors, Interleukin 6 (IL6) and Tumor Necrosis Factor-a (TNFa) are known to induce the synthesis of acute phase proteins and suppress CYP1A2, CYP2C19 and CYP3A activities. Those three P450 enzymes represent the main group of metabolic enzymes involved in detoxification of xenobiotic substances.

MATERIALS AND METHODS

Rat Kupffer cell isolation: Primary rat Kupffer cells were isolated using proprietary enzymatic digestion method developed at Life Technologies. Using this technique, Kupffer cells can be isolated at viabilities higher than 98% and purities of approximately 90% or higher as determined by immune stain for ED1 (CD68) and ED2 (CD163) markers. Contaminating cells represent small populations of endothelial cells ~3%, fibroblasts ~2%, hepatocytes 1%, and Kupffer cells that did not stain sufficiently to be counted). Isolated Kupffer cells attach within 15-30min and display macrophage morphological characteristics within 24hr. They become activated and ready for experiments within 48hr after plating. Kupffer cells are cultured in supplemented WEM.

Rat Hepatocyte isolation: Primary rat hepatocytes are isolated using two step collagenase perfusion. Cells with viability of at least 85%, as determined by trypan blue exclusion, are used in these studies. Supplemented WEM was used during culture and changed every 24 h.

Co-Cultures: Hepatocytes and Kupffer cells were co-cultured in 24-well dishes at ratios of 1:2 of Kupffer cells/hepatocytes to approximate physiological inflammatory liver state. Additional 1:4, 1:8 and 1:16 ratios were used to determine linearity of inflammatory response. Supplemented WEM was used in co-culture and changed every 24 h.

Data Analysis: To activate inflammatory response in Kupffer cells, co-cultures were treated with either LPS (1ug/ml) or IL2 (200ng/ml). Response was analyzed at 24, 48 or 72hr for IL6 and TNFa production using ELISA assays. For those assays, media was collected from each well at predetermined time points and stored at -80ºC until analysis.
To perform qRT-PCR, at terminal time point, cells were washed with HBSS and total RNA was collected. Following cDNA synthesis, TaqMan analysis was performed using probes for rat CYP1A2 and CYP3A23

RESULTS

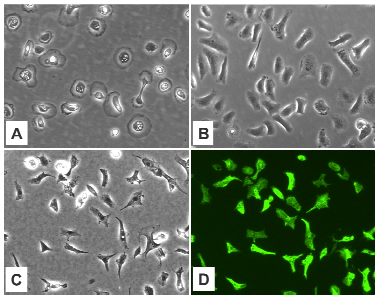

Figure 1. Morphological characterization of primary rat Kupffer cells. **A**. At 2hr after plating, rat Kupffer cells display fried egg like morphology of immature macrophages; **B**. Within 24hr after plating, rat Kupffer cells expand and activate to their mature macrophage-like phenotype; **C-D**. After 48hr to 96hr after plating and thereafter, Kupffer cells become fully activated to their stellate-like phenotype that is characteristic of liver resident macrophages and further validated by ED2 (CD163) immune stain.

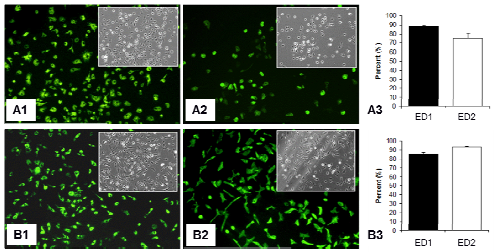

Figure 2. Activation of primary rat Kupffer cells in culture. **A1-A3**. Immune characterization of Kupffer cells at 48hr after plating for presence of ED1 (A1) and ED2 (A2) macrophage markers reveals their expression at 88.7% and 75.5%, respectively (A3). **B1-B3**. Following activation at 6 days after plating, Kupffer cells express ED1 (B1) at 85.6% and ED2 (B2) at 93.5% (B3).

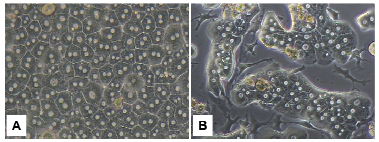

Figure 3. Primary rat Kupffer cell co-cultures with hepatocytes. **A**. Representative image of primary rat monoculture of hepatocytes plated for 48hr displaying characteristics of epithelial monolayer; **B**. Representative image of inflammatory co-culture representing the 1:2 ratio of Kupffer cells to hepatocytes. Note stellate like morphology of Kupffer cells and their proximity to hepatocytes.

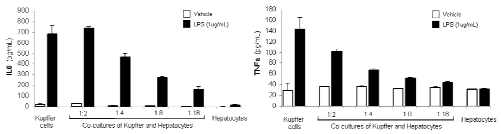

Figure 4. Progressive media accumulation of IL6 and TNFa in co-cultures of Kupffer cells and hepatocytes. Cells were plated at different ratios corresponding to those of normal (1:16) and inflamed (1:8 – 1:2) liver at 24hr after treatment with 1ug/ml of LPS. .

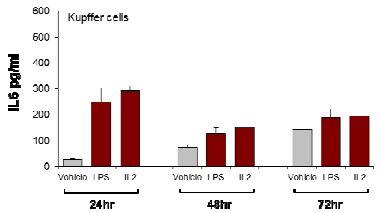

Figure 5. IL6 production in Kupffer cells after LPS and IL2 stimulation for 24, 48 and 72hr. As expected, following activation with LPS treatment, IL6 production is significantly up-regulated at 24hr and then it progressively decreases suggesting desensitization of Kupffer cells to chronic stimulation with LPS.

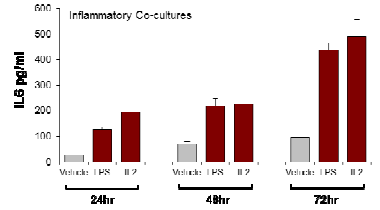

Figure 6. IL6 production in Kupffer cells and hepatocyte co-cultures after LPS and IL2 stimulation for 24, 48 and 72hr. Note that IL6 is significantly up-regulated in co-cultures at all time points of 24, 48 and 72hr. This suggests cellular self assembly between Kupffer cells and hepatocytes that synergistically allows those cells to function together during resolution of inflammation.

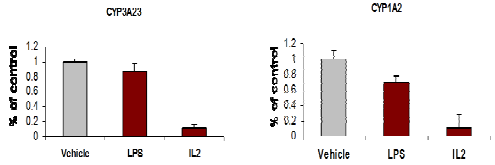

Figure 7. Modulation of CYP3A2 and CYP1A2 enzyme activities in co-cultures after LPS and IL2 stimulation for 72hr. Note near 80% decrease of CYP3A23 and CYP1A2 after IL2 treatment.

CONCLUSIONS

-Kupffer cells secrete potent mediators of the inflammatory response that control liver inflammation and hepatocyte metabolic rates through direct interactions with phase I and phase II enzymes.
-Kupffer cells can be used to create co-culture liver inflammatory system when plated together with hepatocytes
-Isolated primary rat Kupffer cells are >90% pure as determined by immune stain for ED1 and ED2.
-Plated Kupffer cells culture activate as determined by increase expression of resident macrophage marker ED2.
-Kupffer cells and hepatocytes self-assembly in culture
-IL2 or LPS activated Kupffer cells modulate CYP3A and CYP1A2 activities

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