Novel Organotypic 3D Human Liver Tissue Model for Drug Screening and Disease Modeling

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Abstract

Background: In drug development, liver failure is the cause of approximately 30% of post marketing withdrawals of pharmaceuticals. There is a need to develop a human primary cell-based 3D organotypic liver tissue model that exhibits well polarized hepatic morphology, maintains high level expression of major liver associated drug transporters, and expresses metabolizing enzymes with metabolic competence. Models that can be cultured for weeks without phenotypic changes and that allow for repeat exposure and long-term dosing schedules are needed. Such models will recapitulate and predict the important aspects of human response to drugs.

Methods: EpiLiver, a novel human 3D liver tissue model, was produced by seeding adult primary human hepatocytes onto inert cell culture inserts. The tissues were fed with specialized medium to form well differentiated liver tissue with distinct apical and basolateral surfaces. This new model is distinct from the commonly used liver spheroids. Tissues were characterized morphologically by histology. Albumin production was determined using immunohistochemistry (IHC) and ELISA, and tight junction formation (ZO-1) was monitored by IHC. qPCR was used to characterize gene expression in tissues cultured for up to 23 days to monitor changes associated with tissue structure, drug transporters, and Phase I and Phase II drug metabolizing enzymes. Utility of the tissue model for drug toxicity studies was evaluated by dosing the human liver construct with two different concentrations of five model drugs (SN38, Bosentan, Diclofenac, Fialuridine, and Tolcapone) and the extent of liver injury was evaluated. Measurements of liver injury include compromised barrier integrity (TEER), reduction in albumin levels, and increased alanine aminotransferase (ALT) and aspartate aminotransferase (AST) release, which are two biomarkers with clinical relevance in liver function testing. To demonstrate metabolic capabilities of the liver model, tissues were exposed to the drug Midazolam, a substrate for CYP3A4 enzyme for two hours. Culture supernatants from apical and basolateral sides of the tissue were harvested and analyzed by LC/MS for 1-hydroxy midazolam, a metabolite of Midazolam.

Results: Characterization of the EpiLiver tissue model showed hexagonal cellular structure (topical view imaging), 3D columnar hepatocyte tissue formation (histology), albumin production (IHC and ELISA), and tight junction formation. qPCR results demonstrated high level expression of enzymes involved in drug metabolism such as CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP3A7, and CYP4A11. Repeated application of Fialuridine, a drug intended for hepatitis B treatment that was abruptly terminated due to induction of liver failure or causing of severe liver toxicity during human clinical trials, showed compromised barrier, reduced albumin release, and an increase in ALT and AST levels in a time-dependent manner, indicative of drug induced liver injury (DILI) predicting human responses. The positive control SN38, also showed an increase in ALT and AST levels. Following exposure to Midazolam, a significant accumulation of the metabolite 1-hydroxy midazolam was found in the culture supernatant. This novel 3D human liver tissue model creates an opportunity to study liver physiology in an in vitro tissue microenvironment as a stand-alone platform or can be incorporated into organ on a chip device in microphysiological system (MPS) for organ-organ interaction simulations.

Conclusions: The EpiLiver tissue model can: 1) be cultured for a relatively longer time compared to existing practices without changing its functionality, 2) be used for infectious disease modeling, and 3) can play a key role in screening drug candidates during drug development phase or for mechanism of action studies for investigational drugs. Such a model fits well with the FDA modernization Act 2.0 guidelines and will have impact in the development of new approach methodologies (NAMs) intended to identify adverse effects of therapeutic candidates and reduce animal use for experimentation.

Methods

Tissue Preparation: Primary human liver hepatocytes were purchased from commercial vendors. Hepatocytes were thawed, counted, and seeded onto MatTek's PTFE PermaCell inserts (Cat# CCI24-PTFE-0.4, MatTek Corporation, Ashland, MA) coated with human collagen and cultured under submerged condition for three days. On day 3, the inserts were raised to the air-liquid interface and cultured for up to 3 weeks in specially formulated culture medium designed to induce differentiation.

Microscopy, Histology & Immunostaining: Cellular morphology was visualized by brightfield microscopy using an inverted microscope (ECHO Revolve, San Diego, CA) with a 4x objective through the transparent culture membrane (**Figure 1**). Reconstructed liver tissues were fixed in 10% formalin (overnight, room temperature), paraffin embedded, sectioned using a microtome, and stained with hematoxylin and eosin (H&E) according to standard procedures. Immunostaining was performed to visualize albumin production and the tight junction protein, ZO-1 (**Figure 2**).

Test Article Exposure: Reconstructed liver tissues were exposed, for up to 7 days, topically (100 uL) and basolaterally to 2 concentrations of 5 model drugs (SN38, Bosentan, Diclofenac, Fialuridine, and Tolcapone) that are known to have cause hepatoxicity. Outcome measurements for liver toxicity include decreased albumin release (**Figure 3**) and increased ALT (**Figure 4**). Fialuridine, a drug intended for hepatitis B treatment that was abruptly terminated due to induction of liver failure during clinical trial was included to validate the utility of the 3D liver model. SN38, a metabolite of the cancer drug Irinotecan which is known to cause toxicity, was included as a positive control.

ELISA Assays: Albumin and alanine aminotransferase (ALT) release (**Figure 3 & 4**) following drug dosing were characterized by ELISA assay. The albumin ELISA was obtained from ThermoFisher Scientific (Cat. EHALB) and the ALT ELISA was purchased from Abcam (Cat. ab234578/ab263881).

Drug Metabolism Enzyme Expression: Drug metabolism was analyzed by LC-MS method (**Table 1**). qPCR was performed to investigate gene expression levels of Phase I and Phase II drug metabolizing enzymes in the EpiLiver tissue model at different days of the culture period. (**Table 2**).

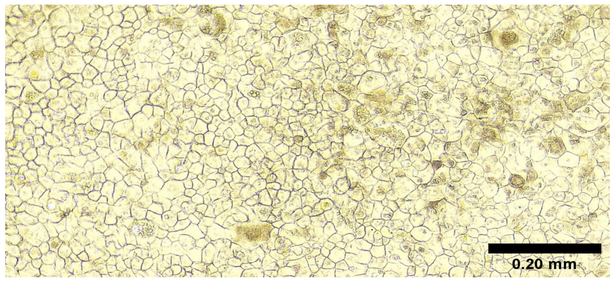

Figure 1: Brightfield microscopy shows that EpiLiver 3D human liver tissue model is formed from hexagonal hepatocytes.

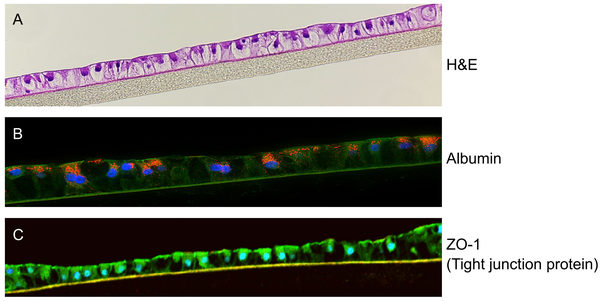

Figure 2: A) H&E-stained histological cross-section of the EpiLiver 3D human liver tissue model showing the hepatocyte polarization and stratification. B) and C) Immunohistochemistry showing albumin production (B; orange) and the tight junction protein, ZO-1 (C; green)

Table 1: Drug metabolism by the EpiLiver model. 1-OH-Midazolam concentration was measured in apical and basolateral supernatants by LC/MS.

Media	Drug	Days of treatment	Calculated concentration (nM)
Apical	Control	12	0.00
	Midazolam	12	148.86
		15	116.81
		22	102.17
Basolateral	Control	12	0.00
	Midazolam	12	257.6
		15	209.3
		22	178.7

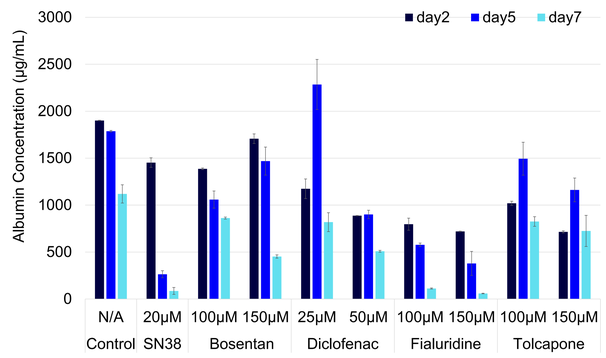

Figure 3: Albumin release by the 3D human liver tissue model following chronic exposure of tissues to different drugs for up to 7 days.

Table 2: qPCR results (RT2 Profiler PCR Array Kit) showing Cq values for Phase I (A) and Phase II (B) drug metabolizing enzymes of hepatocytes in monolayer culture and the human 3D EpiLiver tissue model.

A) Phase I	Monolayer	EpiLiver 3D Tissue Model		
Target	Day 0	Day 9	Day 16	Day 23
CYP1A1	30.31	30.11	29.25	28.98
CYP1A2	25.60	26.30	26.50	26.53
CYP26A1	28.68	36.12	N/A	N/A
CYP2B6	27.47	28.30	28.77	28.63
CYP2C18	28.21	26.29	26.50	26.15
CYP2C19	25.40	22.62	23.02	23.02
CYP2C8	23.66	22.25	23.37	24.62
CYP2C9	24.17	22.93	23.17	23.74
CYP2D6	24.73	24.58	24.19	24.93
CYP2E1	19.48	22.05	23.47	24.19
CYP2S1	37.57	28.78	27.73	27.40
CYP3A4	23.27	27.23	28.17	26.54
CYP3A5	26.02	24.69	24.06	24.29
CYP3A7	24.81	23.06	22.92	22.05
CYP4A11	24.59	22.57	22.56	23.03
CYP4F11	25.78	26.65	26.34	26.47
CYP4F3	25.57	25.57	25.77	26.00
CYP7A1	27.72	27.13	31.10	32.04
CYP7B1	28.11	27.29	27.47	27.10
CYP8B1	24.40	24.22	24.12	24.36
GAPDH	22.09	21.04	21.72	21.34

B) Phase II	Monolayer	EpiLiver 3D Tissue Model		
Target	Day 0	Day 9	Day 16	Day 23
GSTA1	24.13	24.78	27.31	26.17
GSTA3	29.42	30.13	32.33	31.44
GSTA4	29.34	28.50	28.44	28.66
SULT1A1	25.47	27.04	27.04	26.69
SULT1B1	28.59	25.73	26.08	25.52
UGT1A1	25.45	23.78	24.32	24.02
UGT1A4	25.78	28.69	29.19	28.73
UGT1A9	27.29	25.19	25.64	25.04
UGT2A3	27.91	26.18	26.53	26.24
UGT2B10	28.51	24.74	25.91	25.42
UGT2B17	26.71	23.84	24.49	24.24
UGT2B28	24.37	22.27	22.95	22.64
UGT2B4	23.40	23.43	24.08	23.59
UGT2B7	22.28	21.61	22.26	22.00
UGT3A1	29.05	26.52	27.08	26.88
GAPDH	22.48	21.61	22.18	21.73

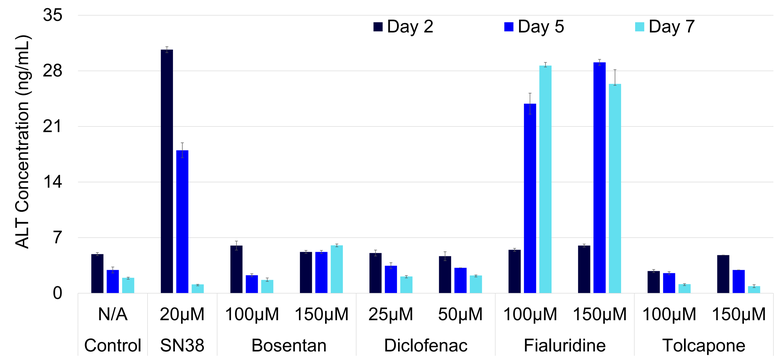

Figure 4: Exposure of EpiLiver to drugs known to induce liver toxicity resulted in increases in ALT (shown above) and AST (not shown) release, two biomarkers used clinically to screen for liver injury and functionality.

Summary

- EpiLiver is a novel 3D human liver tissue model produced using primary hepatocytes which can be successfully cultured with no changes to phenotype and CYP450 enzyme expression levels for up to 3 weeks (**Figure 1 & 2** and **Table 2**).
- Histology and immunohistochemical analysis showed a well-polarized tissue structure that produces albumin (**Figure 2**).
- The function of the EpiLiver tissue to metabolize drugs was demonstrated by exposing the tissue to Midazolam which is a substrate for CYP3A4. (**Table 1**).
- The reconstructed liver tissue model expresses cytochrome P450 (CYP) enzymes that are known to be involved in phase I liver drug metabolism and Phase II detoxification process (**Table 2**).
- Drug induced liver injury was demonstrated following chronic exposure of EpiLiver to drugs known to cause liver toxicity. Chronic exposure to these model drugs resulted in increases in alanine aminotransferase (ALT) release and reduction in albumin production (**Figures 3 & 4**).
- The development of EpiLiver presents a novel in vitro human liver tissue model which is reproducible, cost effective, and closely mimics in vivo human tissue.
- The EpiLiver model will provide drug development scientists with a valuable tool for screening compounds for potential liver toxicity and will significantly decrease the number of animals used for drug induced liver injury/toxicity studies.

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