DEVELOPMENT OF A NOVEL HUMAN 3D IN VITRO MODEL FOR EVALUATING NEW ANTI-FIBROTIC DRUGS

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Abstract

The discovery and development of anti-fibrotic therapies remains reliant on animal testing. There is an urgent need to develop robust and relevant in vitro models to support the identification and preclinical evaluation of potential new anti-fibrotic drugs. Here, we describe the development of a 3D human in vitro liver culture model using an proprietary ORGANDOT® platform. This liver ORGANDOT cultures (human hepatocytes, Kupffer cells and stellates) not only maintain viability and functionality up to four weeks in culture, but can also be treated with TGFβ1 to induce a fibrotic phenotype. The TGFβ1-treated ORGANDOT cultures show a decrease in hepatic function and a concomitant increase in fibrogenic gene expression (COL1A2, TIMP2, ACTA2, ORC1, CCL2, ITGB1, SPARC). Furthermore, co-administration of the AK5 inhibitor was able to completely prevent these fibrogenic changes and rescue the functionality of the cultures. These data demonstrate the potential utility of this in vitro model for testing the efficacy of new anti-fibrotic drug candidates and for research into the mechanism of bile acid cell activation and fibroblast induction.

Methods

Isolation of Human Hepatocyte Cells

Macropinocytically necrotized human liver tissue was used for the isolation of the cells. All samples were provided with informed consent. Hepatocytes were isolated in-house according to established protocols. In brief, the tissue was minced and digested by incubating with 0.03% PronaseE and 0.05% collagenase 8 (for 40 minutes at 37°C in an incubating incubator at 100rpm). The crude cell suspension was then filtered, washed and the adherent cells purified using a discontinuous gradient (sediment). The freshly isolated cells were then expanded in culture in D-MEM+high glucose supplemented with 10% EMEM medium supplemented with FCS and (Crock'. A). Hepatocytes were harvested with 0.07% trypsin. Hepatocytes were seeded at 400000 cells per 24well plate and cultured in complete D-MEM. The cell suspensions were then combined and ORGANDOT cultures were created using 5µl (5K hepatocytes, 3K stellates and 1.5K Kupffers) onto 12mm MBS/Cell-VA inserts (PIERART®) with 5µl hepatocyte maintenance medium in basolateral well plus 7ml D-MEM in apical well, both at 37°C, 5% CO2, with renewal of the basolateral medium every week.

The ORGANDOT platform

Preparation of human ORGANDOT cultures was achieved by setting up in a 3D culture apparatus. The Hepatocytes were cultured with 0.07% trypsin. Hepatocytes were seeded at 400000 cells per 24well plate and cultured in a 5µl D-MEM+high glucose supplemented with FCS and (Crock'. A). Hepatocytes were harvested with 0.07% trypsin. Hepatocytes were seeded at 400000 cells per 24well plate and cultured in complete D-MEM. The cell suspensions were then combined and ORGANDOT cultures were created using 5µl (5K hepatocytes, 3K stellates and 1.5K Kupffers) onto 12mm MBS/Cell-VA inserts (PIERART®) with 5µl hepatocyte maintenance medium in basolateral well plus 7ml D-MEM in apical well, both at 37°C, 5% CO2, with renewal of the basolateral medium every week.

Compound treatment

ORGANDOT cultures were maintained for 3 days before being treated with 0.5µg/mL transforming Growth Factor (TGFβ) (R&D Systems, 250-5) for 4 days. After renewal of medium (hepatocyte+hepatoprotein (complete D-MEM), the cultures were then expanded in culture in D-MEM+high glucose supplemented with 10% EMEM medium supplemented with FCS and (Crock'. A). Hepatocytes were harvested with 0.07% trypsin. Hepatocytes were seeded at 400000 cells per 24well plate and cultured in complete D-MEM. The cell suspensions were then combined and ORGANDOT cultures were created using 5µl (5K hepatocytes, 3K stellates and 1.5K Kupffers) onto 12mm MBS/Cell-VA inserts (PIERART®) with 5µl hepatocyte maintenance medium in basolateral well plus 7ml D-MEM in apical well, both at 37°C, 5% CO2, with renewal of the basolateral medium every week.

Cytochrome C oxidase assay

Cytochrome C oxidase activity in the ORGANDOT cultures was measured using Promega’s CytoC-Test® Cytochrome C Assay (V9710). Basolateral culture medium was replaced with fresh medium containing 3µl luciferin (30µg/ml basolateral and 10µg/ml apical) and the cultures incubated for 40 minutes at 37°C. After that, the cultures were then treated with 20µM ferricytochrome and the number of luciferase loading was read at 30 minutes post exposure to TGFβ1. Luciferase activity was measured in ORGANDOT cultures treated with 5µg/ml lipopolysaccharide (3µl, Sigma L6415) for 24 hours.

APF assay

APF assay was performed using Promega’s CellTiter-Glo® 3D Cell Viability Assay Kit (MTG20). The basolateral medium was removed from each dish and 100µl of CellTiter-Glo reagent added optically. The culture plates were then placed for 10 minutes to induce cell lysis and an equal volume of medium was added per culture. Each sample was briefly pipetted mixed and 100µl transferred to a luminometer. The plate was left to incubate at room temperature for 10 minutes and then the luminescent signal was read on a Wallac Victor plate reader. The ORGANDOT cultures were then assayed for APFcontent.

EUSA

Conditioned supernatants were assayed for Albumin, E4 and Hyaluronic acid using BIO-RAD’s Anti-human Albumin (Bio-RAD A0081) and Hyaluronic acid (Bio-RAD A0181) and EUSA Systems’ Hyaluronan and E4-Dextran EUSA (DY14 and DY24), all EUSA were performed as described in the manufacturers’ protocols.

bDNA Assay and qFIBR

bDNA Assay was performed with Promega’s bDNA LYS/FT Assay buffer and then stored at 4°C pending bDNA extraction. Total RNA was then extracted using Promega’s Trizol (Life Technologies) at 5µl 17°C. bDNA assay (Promega’s Hybridization Buffer) was performed according to the bDNA and qFIBR. bDNA’s global standard (10 pmol) was used as a reference point to determine the copy number of target genes for normalisation to GAPDH.

Immunostaining

The ORGANDOT cultures were used in the assays. The sections were deparaffinised, antigen retrieved and rehydrated in a Dako PT Link apparatus (Dako A0081) for 5 minutes at 39°C in a solution of 0.3M Na citrate pH 6.0, followed by a 1 minute 3% H2O2 rinse in 1:1 antigen retrieval buffer. Immunostaining was performed on a Dako Autostainer plus using the Dako® REAL IHC kit. The slides were sectioned at 3µm, rehydrated and blocked with 1:400 diluted 1:400 Blocking Reagent Plus and 5µl/cm2 goat anti-rabbit (Vector Laboratories) for 30 minutes. The slides were rinsed with Dako wash buffer and followed by 5 minutes with Dako poly-HRP secondary antibody followed by 5 minutes with Dako DAB-20 for 10 minutes. The sections were then washed for 10 minutes. The sections were then washed in Dako wash buffer and followed by 5 minutes with Dako Poly-HRP secondary antibody followed by 5 minutes with Dako DAB-20 for 10 minutes. The sections were then counterstained with haematoxylin, dehydrated in an ascending series of ethanol solutions (70-100%), cleared in xylene and cover-slipped under DePeX.

Conclusions

Liver ORGANDOT cultures (human hepatocytes, Kupffer cells and stellates) maintain viability and functionality up to 28 days in culture.

Exposure to TGFβ1, a central regulator in liver fibrosis, induced a fibrotic phenotype with an increase in fibrogenic gene expression, hyaluronic acid secretion and collagen I deposition.

Co-administration of the AK5 inhibitor 3825334, was able to prevent these fibrotic changes and rescue the functionality of the ORGANDOT cultures.

These data demonstrate the potential utility of this novel 3D in vitro model for evaluating new anti-fibrotic drugs or for studying the mechanism of fibrosis induction and hepatic stellate cell activation.

These data also extend the use of the ORGANDOT platform to additional diseases models, which now include diabetes, oncology, and as demonstrated here, liver fibrosis.

References