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A microfluidically perfused three dimensional human liver model

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ABSTRACT

Within the liver, non-parenchymal cells (NPCs) are critically involved in the regulation of hepatocyte polarization and maintenance of metabolic function. We here report the establishment of a liver organoid that integrates NPCs in a vascular layer composed of endothelial cells and tissue macrophages and a hepatic layer comprising stellate cells co-cultured with hepatocytes. The three-dimensional liver organoid is embedded in a microfluidically perfused biochip that enables sufficient nutrition supply and resembles morphological aspects of the human liver sinusoid. It utilizes a suspended membrane as a cell substrate mimicking the space of Disse. Luminescence-based sensor spots were integrated into the chip to allow online measurement of cellular oxygen consumption. Application of microfluidic flow induces defined expression of ZO-1, transferrin, ASGPR-1 along with an increased expression of MRP-2 transporter protein within the liver organoids. Moreover, perfusion was accompanied by an increased hepatobiliary secretion of 5(6)-carboxy-2′,7′-dichlorofluorescein and an enhanced formation of hepatocyte microvilli. From this we conclude that the perfused liver organoid shares relevant morphological and functional characteristics with the human liver and represents a new *in vitro* research tool to study human hepatocellular physiology at the cellular level under conditions close to the physiological situation.

1. Introduction

The liver plays a central role in metabolism, biotransformation of endogenous and exogenous substrates, and detoxification of xenobiotica. Non-parenchymal cells (NPCs) are essential for the physiological function of the liver. NPCs including Kupffer cells, stellate cells and endothelial cells account for about 40% of total liver cells. *Ex vivo* it has been shown that the presence of NPCs is a requirement for hepatocyte function [1]. Kupffer cells are specialized tissue macrophages that represent 15% of total liver cells and almost 80–90% of all tissue macrophages in the body [2]. Macrophages are key regulators of inflammatory response during infection and the major source of inflammatory cytokines such as interleukin (IL)-6 driving acute phase protein production in hepatocytes [3,4]. However, macrophages as well as hepatic stellate cells (HSCs) also mediate tissue regeneration in response to drug-induced liver damage [5], regulate the complex balance of inflammation and tissue regeneration [6,7], and facilitate cell–cell

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communication between hepatocytes and endothelial cells (ECs) [8]. HSCs and ECs represent 6% and 19% of total liver cells, respectively [9]. ECs do not simply form a barrier to restrict access of blood-borne compounds to the parenchyma, but also mediate clearance of i.e. endotoxins and bacteria, and regulate migration of leukocytes into the liver. The integrity of the liver microvasculature is thus fundamental for maintaining liver perfusion and cell viability [10].

In vitro monolayer cultures of hepatocytes are well-established in research but are accompanied by a reduction of major hepatic functions such as secretion of plasma proteins or detoxification due to down-regulation of several phase-I, -II and phase-III enzymes [11–13]. Co-culture approaches with NPCs have been shown to prevent hepatocyte dedifferentiation. Hepatocytes show improved urea production and a stable up-regulation of CYP1B1, CYP2C9, CYP2E1, and CYP3A4 during long-term co-culture with ECs [14–16]. Similarly, co-culture of hepatocytes and HSCs was reported to increase albumin secretion and CYP2B1/2 expression [17]. In addition to simple co-culture, three-dimensional liver tissue culture involving NPCs in contact with hepatocytes is required for improved maintenance of hepatocyte function [18,19]. In two-dimensional hepatocyte cultures a loss of hepatocyte cell polarization is frequently observed and associated with a diminished expression of distinct transporter proteins at the sinusoidal, basolateral and canalicular membranes [20].

So far, no *in vitro* model of the human liver is available that integrates ECs, macrophages and HSCs in co-culture with hepatocytes and also mimics the specific three-dimensional morphology of the human liver sinusoid, including the endothelial cell layer. While cells in the body are embedded and oriented in a complex three-dimensional network, *in vitro* tissue models need optimized perfusion strategies for a continuous supply of oxygen and nutrients. In addition, the removal of waste products is critical for culture of complex three-dimensional tissues with a high cellular density as diffusion of endogenous catabolites within tissues is impeded in static conditions. We recently introduced the Multi-Organ-Tissue-Flow (MOTiF) biochip design that features a suspended and freely perfusable membrane acting as a cell culture substrate [21]. Here, we report the establishment of a three-dimensional liver organoid embedded in microfluidically-supported biochips, which is structurally inspired by the morphology of the liver. HepaRG cells were used for the assembly of the liver organoids since preparation of primary liver cells is time- and cost-consuming. Moreover, availability of primary liver tissue specimens is often limited to donors suffering from pre-existing liver disorders and receiving extended medication. This likely affects liver cell function and contributes to experimental bias. To overcome these limitations, we used freshly isolated human umbilical vein endothelial cells (HUVEC) instead of liver sinusoidal endothelial cells (LSEC), since LSEC rapidly tend to de-differentiate *in vitro*, which is associated with a loss of fenestrae and re-organization of the cytoskeleton [22]. This de-differentiation process is difficult to monitor or control, and potentially adds an additional bias in day-to-day experimentation. Monocyte-derived macrophages were used to mimic Kupffer cell function and the immortalized human stellate cell line LX-2 as primary stellate cell surrogate. Immortalized cell lines have the advantage of continuous growth, unlimited availability and their clonal origin usually guarantees a constant phenotype allowing reproducible experimentation [23,24]. During culture in the biochip HepaRG cells consistently differentiate into cells exhibiting a hepatocyte phenotype and into cells with biliary epithelial cell phenotype that self-organize into a hepatocyte layer with functional bile ducts between hepatocyte-like cells [25] essential for liver function [26].

2. Material & methods

2.1. Cell culture

HepaRG: HepaRG cells were obtained from Biopredic International (Rennes, France). They were seeded at a density of 2.7×10^4 cells/cm^2 and cultured in William's Medium E (Biochrom, Berlin, Germany) containing 10% (v/v) FCS (Life Technologies, Darmstadt, Germany), 5 μg/ml insulin (Sigma–Aldrich, Steinheim, Germany), 2 mM glutamine (GIBCO, Darmstadt, Germany), 50 mM hydrocortisone-hemisuccinate (Sigma-Aldrich) and 100 U/ml Penicillin/100 μg/ml Streptomycin mixture (Pen/Strep) (GIBCO). The cells were cultured in a humidified cell incubator at 5% CO_2 and 37 °C for 14 days before differentiation. Medium was renewed every 3–4 days. Cell differentiation was induced as described [27] and cells were used up to 4 weeks. Endothelial cells: Human umbilical cord vein endothelial cells (HUVECs) were isolated from human umbilical cord veins as described [28]. Donors were informed about the aim of the study and gave written consent. HUVEC cells were seeded at a density of 2.5×10^4 cells/cm^2 and cultured in Endothelial Cell Medium (ECM) (Promocell, Heidelberg, Germany) up to passage 4. LX-2 stellate cells (kindly provided by Scott L. Friedman, Division of Liver Diseases, Mount Sinai School of Medicine, New York City, NY, USA) were seeded at a density of 2.0×10^4 cells/cm^2 and cultured in Dulbecco's Minimum Essential Medium (DMEM) (Biochrom) supplemented with 10% (v/v) FCS, 1 mM sodium pyruvate (GIBCO) and Pen/Strep. Peripheral Blood Mononuclear Cells (PBMCs) were isolated by Ficoll density gradient centrifugation as described previously [29] and seeded at a density of 1.0×10^6 cells/cm^2 in X-VIVO 15 medium (Lonza, Cologne, Germany) supplemented with 10% (v/v) autologous human serum, 10 ng/ml human granulocyte macrophage colony-stimulating factor (GM-CSF) (PeproTech, Hamburg, Germany) and Pen/Strep. After 3 h incubation in a humidified cell incubator at 5% CO_2 and 37 °C the cells were washed twice with X-VIVO 15 medium. Adherent monocytes were cultivated for 24 h in X-VIVO 15 medium and seeded into the liver organoid.

2.2. Liver organoid assembly

Liver organoids were assembled by staggered seeding of vascular and hepatic cell layers. In each sterilized biochip 2.7×10^5 HUVEC's/cm^2 (in total 3.0×10^5 cells) and 0.9×10^5/cm^2 monocytes (in total 1×10^5 cells) were mixed and seeded on top of the membrane in the upper chamber. HUVEC/monocytes were co-cultured for at least 5 days with a daily medium exchange in endothelial cell culture medium (ECM) supplemented with 10 ng/ml epidermal growth factor, 90 μg/ml heparin, 2.8 μM hydrocortisone, endothelial cell growth supplement, 10 ng/ml GM-CSF to induce macrophage differentiation, 100 U/ml penicillin/100 μg/ml streptomycin and 10% (v/v) autologous human serum (Life Technologies, Karlsruhe, Germany). M-CSF was not supplemented to the medium as human serum contains sufficient amounts for the differentiation of the macrophages [30–33]. Subsequently, 2.7×10^5/cm^2 differentiated HepaRG (in total 3×10^5 cells) and 3.6×10^5/cm^2 LX-2 (in total 4×10^4 cells) were seeded on the membrane at the opposite side of HUVEC cells and cultured for 24 h in DMSO-free William's Medium E (Biochrom, Berlin, Germany) hepatocyte growth medium containing 50 μM hydrocortisone, 10% (v/v) FBS containing, 5 μg/ml insulin, 2 mM glutamine and 100 U/ml penicillin/100 μg/ml streptomycin prior to experimental use.

2.3. Biochips

MOTiF biochips were made from cyclic olefin copolymers (COC) – TOPAS® and obtained from microfluidic ChipShop GmbH (Jena,