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Use of a three-dimensional humanized liver model for the study of viral gene vectors

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ABSTRACT

Reconstituted three-dimensional (3D) liver models obtained by engrafting hepatic cells into an extracellular matrix (ECM) are valuable tools to study tissue regeneration, drug action and toxicology *ex vivo*. The aim of the present study was to establish a system for the functional investigation of a viral vector in a 3D liver model composed of human HepG2 cells on a rat ECM. An adeno-associated viral (AAV) vector expressing the Emerald green fluorescent protein (*EmGFP*) and a short hairpin RNA (shRNA) directed against human cyclophilin b (*hCycB*) was injected into the portal vein of 3D liver models. Application of the vector did not exert toxic effects, as shown by analysis of metabolic parameters. Six days after transduction, fluorescence microscopy analysis of EmGFP production revealed widespread distribution of the AAV vectors. After optimization of the recellularization and transduction conditions, averages of 55 and 90 internalized vector genomes per cell in two replicates of the liver model were achieved, as determined by quantitative PCR analysis. Functionality of the AAV vector was confirmed by efficient shRNA-mediated knockdown of *hCycB* by 70–90%. Our study provides a proof-of-concept that a recellularized biological ECM provides a valuable model to study viral vectors *ex vivo*.

1. Introduction

The liver plays a central role in metabolic processes and in the detoxification of xenobiotics and drugs (Kuntz and Kuntz, 2008). Despite its remarkable regenerative capacity, liver cells placed in culture rapidly lose their typical *in vivo* phenotypic and functional characteristics (Fraslin et al., 1985). Two dimensional (2D) cultivation of hepatic cells does not represent the physiological situation in an adequate manner and results in the loss of expression of hepatospecific genes. These changes are due to the lack of biochemical or mechanical stimuli, which are important for the development and function of real tissues (Gomez-Lechon et al., 1998). Increasing knowledge of the physiological conditions in which hepatic cells grow in living organisms has led to the development of improved culture methods for hepatic cell lines, such as HepG2 cells or primary hepatocytes. To produce structures which more closely reflect physiological conditions, three-dimensional (3D) culture methods involving scaffolds of extracellular matrix (ECM) have been developed (Lang et al., 2011; Zhang et al., 2009).

ECM stores and releases signaling substances like cytokines and growth factors, which regulate cell proliferation and migration as well as cell–cell- and cell–matrix-interactions (Schonherr and Hausser, 2000; Yagi et al., 2013). Tissues can be grown on matrices from allogenic or xenogenic donation materials (Mirmalek-Sani et al., 2013) which may be complete organs, or parts thereof, that still contain a functional vascular system (Yagi et al., 2013). These scaffolds provide sufficient nutrients to maintain physiological conditions. Moreover, it has been demonstrated that tissue-specific gene expression, morphogenesis, and cell migration are promoted by interactions between cells and the surrounding ECM in liver and other organs (Sellaro et al., 2010; Yagi et al., 2013).

To the best of our knowledge, dynamically cultured 3D liver models using native ECM matrices have not yet been applied in studies involving viral vectors or infectious agents. Two applications can be envisaged: (1) The liver is the target organ of various vectors used for gene therapeutic applications such as adenoviral or adeno-associated virus (AAV) vectors (Fechner and Kurreck, 2011; Kay, 2011). Their transduction efficiency, expression of the transgene and toxicity may be investigated in 3D liver models

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prior to initiating pre-clinical *in vivo* studies. In previous studies, spheroids were used to study and modulate cell functions in 3D cell cultures by transduction with adenoviral and AAV vectors (Enger et al., 2002; Thorsen et al., 2006; Tzanakakis et al., 2002). While both adenoviral and AAV vectors transduced 2D cultures of cell lines to a similar degree, AAV vectors penetrated deeper into the spheroids (Enger et al., 2002). However, using three different AAV serotypes, no widespread distribution, particularly to the center of the spheroid, was achieved (Thorsen et al., 2006). (2) Some of the clinically most relevant viruses infect the liver in a species-specific way, impeding the use of animal models. For example, infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) remain a global burden (Guha et al., 2004; MacArthur et al., 2012). Adenovirus is another pathogen which may infect the liver and cause severe medical problems, particular in immunocompromised individuals (Hough et al., 2005). As it is the case for hepatitis viruses, the study of adenovirus biology is hampered by shortcomings of available animal models.

A main challenge in bioengineering of functional whole organs is the choice of cells for recellularization. Depending on the application, cells have to accomplish different features. For toxicity testing, cells have to express all enzymes involved in drug or xenobiotic metabolism. To establish infection models, cells should ensure adequate replication of hepatic viruses. For the production of a transplantable liver graft, cells have to display all typical hepatic functions. Primary cells are thought of as the gold-standard for toxicity studies (Soldatow et al., 2013) and regenerative medicine (Lang et al., 2011; Yagi et al., 2013). However, restricted access to human liver tissue and the very limited ability of adult differentiated hepatocytes to proliferate *in vitro* hinders widespread use of these cells. Therefore, cell lines such as HepG2 or HepaRG have been used, despite their tumorous nature and their differences to primary hepatocytes (Soldatow et al., 2013).

In the present study, we used HepG2 cells to establish a 3D liver model for transduction experiments, as they are among the most widely used human hepatoma cell lines and therefore represent a well characterized model system for first proof-of-principle studies. Like other hepatocyte cell lines, HepG2 cells retain a partially differentiated adult phenotype (Castell et al., 2006). A comparison of HepG2 cells grown in monolayer and 3D culture revealed improved albumin production in 3D culture (Chang and Hughes-Fulford, 2009; Mueller et al., 2011). Additionally, 3D culturing significantly improved formation of bile canaliculi (Bokhari et al., 2007).

The aim of the present study was to use a humanized 3D model whose advantages compared to 2D cultures have been reported previously to investigate liver transduction by AAV vectors. Compared to spheroids, the organ model provides a functional vascular system, which can be expected to improve homogenous distribution of vector transduction over the whole organ. AAV vectors are among the most promising delivery vehicles used in gene therapeutic approaches. The AAV vector employed in the present study expressed *EmGFP* as a reporter to trace transduction efficiency and an shRNA for the knockdown of an endogenous target by means of RNA interference (RNAi).

2. Materials and methods

2.1. Cells and plasmids

HepG2 cells were cultured with RPMI 1640 (BioWest SAS, Nuaillé, France) supplemented with 10% fetal calf serum, 2 mM L-glutamine (BioWest SAS), and 2 mM of penicillin and streptomycin (BioWest SAS), each.

Shuttle plasmids encoding emerald green fluorescent protein (EmGFP) and an shRNA were based on a self complementary (scAAV) shuttle plasmid described previously (Fechner et al., 2008). Plasmids encoded either an shRNA directed against human Cyclophilin B (pAAV-shCycB) or a control shRNA (pAAV-shCtrl.). The following sequences were used:

shCycB: 5′-GGUGGAGAGCACCAAGACAUUCAAGAGAUGUCUU-GGUGCUCUCCACCUU

shCtrl.: 5′-ACUACCGUUGUUAUAGGUGUUCAAGAGACACCU-AUAACAACGGUAGUUU

2.2. Production and purification of scAAV2.6 vectors

Pseudotyped scAAV vectors of serotype 6 were produced and purified as described previously (Wagner et al., 2013). ScAAV vector genomes were titrated by quantitative PCR (qPCR) with the primer sequences described previously (Wagner et al., 2013).

2.3. Liver decellularization

Wister rats were sacrificed by isofluorane inhalation. The inferior vena cava and the portal vein of the liver were cannulated with a 22 G cannula, followed by a wash with PBS containing heparin. Other vessels were clamped. The liver was explanted and placed in a decellularization chamber. The decellularization procedure was adapted from Mertsching et al. (2009, 2005) with minor modifications. Briefly, the portal vein was connected to the circuit and a normal saline solution containing heparin was flushed through the matrix for 0.5 h. Afterwards 1% sodium deoxycholate was perfused for 2.5 h at a flow rate of 10 ml/min. A normal saline wash for 1 h followed. The liver ECM was then placed in a DNase-containing solution overnight. Sterilization was done by gamma irradiation. Organs were obtained from cadavers of animals that were sacrificed for other approved experiments. RL obtained ethical approval for the explantation of organs from the Landesamt für Gesundheit und Soziales (LaGeSo).

2.4. Recellularization with HepG2 cells

Decellularized rat liver scaffolds were placed in a bioreactor system consisting of a growth chamber, media reservoir and perfusion circuit. The circuit comprised a peristaltic pump, a bubble trap and a pressure sensor. The bioreactor maintained standard conditions of 37 °C and 5% CO_2. Before inoculation, the scaffolds were equilibrated with 150 ml medium for 5 d at a flow rate of 1.25 ml/min.

The scaffolds were inoculated with HepG2 cells through the portal vein, followed by an incubation step without pumping (1 h) and a subsequent slow increase of the flow rate. The inoculation procedure was carried out twice for each liver. The total cell number for each of the experiments is given below. During cultivation, the liver matrix was continuously perfused through the portal vein.

2.5. Assessment of recellularization

Vitality of the HepG2 cells was analyzed by measuring lactate dehydrogenase activity (LDH), glucose concentration, and lactate concentration in medium samples according to the manufacturer's recommendation using the LDH Liqui-UV kit (Stanbio Laboratory, Boerne, USA), the Glucose LiquiColor Oxidase kit (Stanbio Laboratory) and the LAC142 kit (Diaglobal, Berlin, Germany), respectively.

2.6. Transduction of HepG2 cells in 2D cultures and the recellularized liver matrix

For the analysis of *hCycB* knockdown in 2D cultures, 5×10^4 HepG2 cells per well were seeded in 24-well plates. At a conflu-