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Functionality based method for simultaneous isolation of rodent hepatic sinusoidal cells

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ABSTRACT

Chronic liver disease is the result of long term exposure to viruses or toxins such as alcohol, fat and drugs, and forms the basis for the development of liver fibrosis and primary liver cancer. *In vitro* and *in vivo* models are key to study the pathways involved in chronic liver disease and for the development of therapeutics. 3D co-culture systems are becoming the *in vitro* standard, which requires freshly isolated primary hepatic cells. We developed a novel isolation method to simultaneously isolate liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs) and hepatic stellate cells (HSCs). The method exploits the scavenging activity of LSECs, the phagocytic capacity of KCs and the retinoid content of HSCs *in vivo* to enable direct processing by fluorescence-activated cell sorting without additional antibody binding and washing steps. UFACS3, for UV-FACS-based isolation of 3 non-parenchymal liver cell types, yields functional and pure LSECs (98 ± 1%), KCs (98 ± 1%) and HSCs (97 ± 3%), with less hands-on time from healthy and diseased rodent livers. This novel approach allows a fast and effective combined isolation of sinusoidal cells for further analysis.

1. Introduction

Chronic liver injury caused by for example chronic drug or alcohol abuse, obesity or hepatitis infections leads to excessive scar formation by myofibroblasts, resulting in tissue fibrosis. This fibrotic environment promotes progression to hepatocellular carcinoma. It is estimated that 29 million EU citizens suffer from a chronic liver disease and this represents a growing concern in Europe [1]. Although liver cancer is the second most common cause of cancer-related death, little is known about the contribution of different liver cell types to the evolution of the disease. This is partially due to lack of effective isolation and culture techniques of non-parenchymal cells for *in vitro* studies.

Animal testing has become the golden standard to study the pathways involved in the development, progression and resolution of this disease and consequently to improve therapeutics. Nevertheless, ethical issues and the need for international standards regarding *in vivo* experimentation have led to an animal welfare legislation which was based on the 3R principles, which were firstly proposed by Russel and Burch in 1959 [2–5]. With the eye on refinement, reduction and replacement of animals for experimental use, progress has been made during the last two decades to develop *in vitro* alternatives which mimic the *in vivo* situation [6]. These systems vary from simple mono-cultures to co-cultures with two different cell types or the use of chip technology with four different cell types and a flowing current, using either primary cells or immortalized cell lines [7–9]. Establishing these *in vitro* models is not an easy task, since the liver is a complex organ, composed of different cell populations that perform a variety of functions to maintain a healthy homeostasis. The parenchymal cells, or hepatocytes (~70–85% of liver cells), are responsible for many liver functions, such as protein and bile acid synthesis, biotransformation of xenobiotics and energy metabolism. The sinusoidal non-parenchymal fraction (NPF) mainly consists of liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs) and hepatic stellate cells (HSCs). LSECs (~20% of liver cells) form the fenestrated "sieve" endothelium, lining the sinusoidal walls, located at the interface between luminal blood and hepatocytes, and represent the most

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effective scavengers of blood-borne waste macromolecules (<200 nm) in the body. LSECs have the capacity to clear the blood at a high-rate mainly thanks to three receptor types: mannose receptor, Stabilin-1 and-2 receptors and Fcγ receptor IIb2. Loss of LSEC fenestration precedes the activation of HSCs and development of liver fibrosis [10,11]. KCs (~15% of liver cells) are the resident macrophages, found in the luminal space, involved in phagocytosis of pathogens and cellular debris (>200 nm) and initiation and propagation of inflammation in response to injury [12]. The HSCs (~5–8% of liver cells), found in the perisinusoidal space, are the major scar tissue forming cell type involved in liver fibrosis. Upon injury these different cell types try collectively to cope with the altered situation to restore homeostasis [13–15]. Co-culture systems where two or more cell types are being cultured together, have demonstrated to be very promising tools for liver toxicology and to study disease mechanisms [7,16,17]. On the other hand, omics analyses of freshly isolated (uncultured) NPF cells from rodent models of liver disease are increasingly used to improve our understanding of the disease progression mechanisms [18,19]. The major hurdle for these techniques is obtaining pure cells in a reproducible manner.

Hepatocytes can be relatively easily isolated with a percoll gradient [20]. The isolation procedures of HSCs, KCs and LSECs are more tedious and often result in a lower number of cells with lower purity. Due to their lipid droplets, HSCs can either be isolated by a nycodenz gradient or sorted with a fluorescence-activated cell sorter (FACS), based on the autofluorescence of retinyl esters stored in these droplets [21–24]. Previously, KC and LSEC isolations were also based on density gradients, centrifugal elutriation, selective adhesion or a combination thereof [25–29]. Currently, KCs and LSECs are commonly isolated using antibody-based (conventional) methods that include incubation of the NPF's with antibodies that recognize cell type-specific proteins located on the cell membrane followed by FACS or magnetic cells sorting (MACS) [22,30,31]. Sometimes, even a selection step by density gradient or by selective adhesion after sorting is carried out to increase cell purity [32,33]. These methods demand long procedures and a strong expertise to obtain reproducible results.

We report the development and validation of a novel method for the simultaneous isolation of HSCs, KCs and LSECs by using their scavenging and phagocytic activity rather than marker expression that characterizes KCs and LSECs. The novel method is based on the uptake of tail-vain injected Alexa-labelled IgG's by LSECs and KCs, which can thereby be distinguished from each other and from UV-positive HSCs by FACS. We refer to our isolation method as UFACS3, for UV-FACS-based isolation of 3 non-parenchymal liver cell types.

2. Material and methods

2.1. Animals

All methods, experimental protocols and animal experimentation ethics were carried out in accordance with the approved guidelines of the Vrije Universiteit Brussel (VUB, Belgium) and according to European Guidelines for the Care and Use of Laboratory Animals. All animal experimentation protocols were approved by the Ethical Committee of Animal Experimentation of the Vrije Universiteit Brussel (VUB, Belgium) (LA 123 02 12, projects 13-212-3; 15-212-4;16-212-1). The animals were maintained in controlled temperature, humidity and a light-dark cycle from 07.00 a.m. to 07.00 p.m. and allowed food and water *ad libitum*. Rodents were housed in conventional cages with up to 6 mice or 4 rats per cage. The age of the mice ranged from 8 to 48 weeks and rats had an average weight of 350 g. To avoid bias due to age, mice of different ages were randomly selected for all experiments except for the injury models, where mice of 8 weeks old underwent a bile duct ligation and mice of 10 weeks old were injected with CCl_4. The animals were routinely tested for background pathogens (QM Diagnostics, Nederland's). All animals were supplied by Charles River Laboratories International, Inc.

2.2. Liver cell isolation methods

Two isolation protocols were performed. For the UFACS3 procedure, BALB/c mice were injected with 10 μg donkey anti-rabbit IgG Alexa Fluor® 488 or Alexa Fluor®647, which was diluted in 100 μl 50 mM hepes solution (H4034, Sigma-Aldrich, Belgium), pH 7.0, 2 h before sacrifice, unless mentioned otherwise. Mice were anaesthetized with 150 μl Nembutal (Ceva santé animale®) and the liver perfused via the portal vein 3 times for 5 min with the following solutions: (A) SC-1 solution consisting of 8000 mg/L NaCl, 400 mg/L KCl, 75.5 mg/L NaH_2PO_4, 120.45 mg/L Na_2HPO_4, 2380 mg/L HEPES, 350 mg/L $NaHCO_3$, 190 mg/L EGTA, 900 mg/L glucose, pH 7.3, (B) 0.04% pronase E (Merck, Darmstadt, Germany) and (C) 0.02% collagenase P (Boeringer Mannheim, Germany). Both enzymes were dissolved in SC-2 solution consisting of 8000 mg/L NaCl, 400 mg/L KCl, 75.5 mg/L NaH_2PO_4 H_2O, 120.45 mg/L Na_2HPO_4, 2380 mg/L HEPES, 350 mg/L $NaHCO_3$, 560 mg/L $CaCl_2 \cdot 2H_2O$, pH 7.3. The digested liver was excised and incubated at 37 °C for 15 min in a solution containing 0.03% collagenase, 0.03% pronase E and 0.001% DNase (Grade II, Boeringer Mannheim, Germany). The resulting suspension was filtered through a 100 μm grid and centrifuged 2 times for 2 min at 50 g to remove the hepatocytes. The resulting NPF was centrifuged for 8 min at 640 g. Red blood cells were removed by incubating the pelleted NPF with a lysis solution (Miltenyi Biotec, Germany) for 3 min, followed by a wash in GBSS-B, consisting of 370 mg/L KCl, 210 mg/L $MgCl_2 \bullet 6$ H_2O, 70 mg/L $MgSO_4 \bullet 7$ H_2O, 120 mg/L Na_2HPO_4, 30 mg/L KH_2PO_4, 990 mg/L Glucose, 227 mg/L $NaHCO_3$, 225 mg/L $CaCl_2 \bullet 2$ H_2O, pH 7.3. The resulting cell pellet was resuspended in a mix of SC1 and DNase (3:1) and filtered through a 40 μm mesh before FACS. The NPF is analyzed by FACS (FACS Aria II, Becton-Dickinson, Belgium), on an Alexa 488-UV or Alexa 647-UV dot-plot. Liver cells were also isolated from healthy C57BL/6 mice (35 weeks) and Sprague Dawley rats (±350 g), using the UFACS3 method (for rats, 70 μg IgG Alexa647 in 400 μl Hepes solution).

For the membrane protein based cell type-specific isolation, mice livers were perfused as mentioned above. After the red blood cell lysis step, NPF was washed twice with PBS +0,1% BSA, cells counted and washed again. The pellet was resuspended in 900 μl PBS+ 5% BSA +2 mM EDTA (BP-E), and 1 μl rat anti-mouse Fc block™ (Becton-Dickinson, Belgium) added to 10^7 cells, followed by incubation at 4 °C for 10 min with shaking. After one wash, the cell pellet was incubated with 200 μl of BP-E, to which 1 μl rat anti-F4/80-APC (MF8021, Thermo Scientific, USA) and 0,5 μl mouse anti-CD32-PE (ab30357, Abcam, UK) were added to 10^6 cells. Following an incubation of 15 min at 4 °C on a shaker, the cells were washed with SC1 and dissolved in a mix of SC1 and DNase (3:1) before FACS.

2.3. FITC-beads and FSA-FITC in vivo

In order to identify KCs and LSECs based on their functionality, mice were injected with FITC-labelled 1 μm latex beads (L4655, Sigma-Aldrich, Belgium) [34] and FITC-labelled FSA [35]. KCs were identified by injecting mice 24 h before sacrifice with 10 μg Alexa Fluor®647 antibodies and 2 h before sacrifice with 4,4 μg of 1 μm FITC-beads. LSECs were identified by injecting mice 24 h before sacrifice with 10 μg Alexa Fluor®647 antibodies and 30 min before sacrifice with 70 μg FSA-FITC.