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Polarized location of SLC and ABC drug transporters in monolayer-cultured human hepatocytes

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ABSTRACT

Human hepatocytes cultured in a monolayer configuration represent a well-established *in vitro* model in liver toxicology, notably used in drug transporter studies. Polarized status of drug transporters, *i.e.*, their coordinated location at sinusoidal or canalicular membranes, remains however incompletely documented in these cultured hepatocytes. The present study was therefore designed to analyze transporter expression and location in such cells. Most of drug transporters were first shown to be present at notable mRNA levels in monolayer-cultured human hepatocytes. Cultured human hepatocytes, which morphologically exhibited bile canaliculi-like structures, were next demonstrated, through immunofluorescence staining, to express the influx transporters organic anion transporting polypeptide (OATP) 1B1, OATP2B1 and organic cation transporter (OCT) 1 and the efflux transporter multidrug resistance-associated protein (MRP) 3 at their sinusoidal pole. In addition, the efflux transporters P-glycoprotein and MRP2 were detected at the canalicular pole of monolayer-cultured human hepatocytes. Moreover, canalicular secretion of reference substrates for the efflux transporters bile salt export pump, MRP2 and P-glycoprotein as well as sinusoidal drug transporter activities were observed. This polarized and functional expression of drug transporters in monolayer-cultured human hepatocytes highlights the interest of using this human *in vitro* cell model in xenobiotic transport studies.

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1. Introduction

Liver transporters, belonging either to the solute carrier (SLC) transporter family or to the ATP-binding cassette (ABC) transporter family, are now well-recognized as major actors of hepatic drug clearance (Giacomini et al., 2010). Indeed, they are expressed either at the sinusoidal pole of hepatocytes, where they mediate uptake of drugs from blood into hepatocytes, *i.e.*, the so-called phase 0 of the hepatic detoxifying system, or at the canalicular pole of hepatocytes, where they secrete drugs or drug metabolites into the bile, *i.e.*, the so-called phase 3a of the hepatic detoxifying system (Funk, 2008). Additionally, some sinusoidal ABC transporters can secrete drug metabolites back into the blood, *i.e.*, the so-called phase 3b, for a secondary renal elimination (Pfeifer et al., 2014).

Activity of hepatic transporters as well as their expression can be regulated by various drugs (Jigorel et al., 2006; Klaassen and Aleksunes, 2010), which can result in clinically-significant drug–drug interactions through altered hepatic elimination of the co-administrated drugs handled by the targeted transporters (Konig et al., 2013). Other xenobiotics such as environmental pollutants and some physiological factors, including hormones and inflammatory cytokines, can also impair hepatic drug transporter expression and activity (Fardel and Le Vee, 2009; Fardel et al., 2001; Klaassen and Slitt, 2005). Identifying putative interactions of drugs, chemical pollutants or endogenous substances with hepatic drug transporters and characterizing the cellular and molecular mechanisms involved in such interactions are therefore likely important issues that have to be addressed, notably during the development of new molecular entities in pharmaceutical companies, as recently recommended by drug regulatory agencies (Giacomini and Huang, 2013; Prueksaritanont et al., 2013).

For performing such studies on hepatic drug transporter activity and expression, primary cultures of hepatocytes represent an *in vitro* choice model (Brouwer et al., 2013; Ramboer et al., 2013). For rodent hepatocytes, such cultures are usually performed under a sandwich configuration, *i.e.*, hepatocytes are plated on collagen-coated dishes and overlaid with collagen or matrigel

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(LeCluyse et al., 1994), which permits to recover a polarized status of hepatocytes and functional bile canaliculi networks (Swift et al., 2010). By contrast, rodent hepatocytes cultivated in a conventional monolayer configuration, *i.e.*, hepatocytes are plated on plastic or collagen-coated dishes, failed to exhibit bile canaliculi-like structures and are therefore not convenient for studying canalicular drug secretion (Luttringer et al., 2002; Noel et al., 2013). In analogy to sandwich-cultured rodent hepatocytes, sandwich-cultured human hepatocytes are thought to represent the gold standard for *in vitro* investigating human hepatic transporters (Bi et al., 2012; De Bruyn et al., 2013). It is however noteworthy that monolayer-cultured human hepatocytes also display notable activity of various drug transporters (Jigorel et al., 2005; Payen et al., 2000) and that absolute quantification of sinusoidal and canalicular transporters through a targeted liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method revealed no major difference between sandwich- and monolayer-cultured human hepatocytes (Schaefer et al., 2012). Moreover, primary human hepatocytes cultivated in a monolayer configuration retain regulatory ways of transporter expression and have been consequently used for analyzing the effects of drugs or physiological effectors such as cytokines or growth factors on drug transporter levels and activities (Jigorel et al., 2006; Le Vee et al., 2008, 2009a; Richert et al., 2009). Whether such monolayer-cultured human hepatocytes exhibit polarized expression of drug transporters, and therefore may closely mimic the *in vivo* situation, remains however yet largely unknown, but is rather important to determine in order to fully assess the relevance of monolayer-cultured human hepatocytes as a valuable *in vitro* model to investigate hepatic drug transport. The present study was therefore designed to get insights about this point, using a combined experimental approach, based on mRNA quantification, immunofluorescence labeling and canalicular transport assays, and very similar to that recently used for characterizing drug transporter location in highly differentiated human hepatoma HepaRG cells (Le Vee et al., 2013).

2. Materials and methods

2.1. Chemicals

Rhodamine 123, verapamil and probenecid were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France), whereas carboxy-2,7-dichlorofluoresceine (CF) diacetate was provided by Invitrogen/Life Technologies (Villebon sur Yvette, France). [^{3}H(G)] taurocholic acid (sp. act. 1.19 Ci/mmol), [6,7-^{3}H(N)] estrone-3-sulfate (E3S) (sp. act. 57.3 Ci/mmol) and [1-^{14}C] tetraethylammonium (TEA) (sp. act. 2.4 mCi/mmol) were from Perkin–Elmer (Boston, MA). All other chemicals were commercial products of the highest purity available.

2.2. Cell culture

Human hepatocytes were obtained from adult donors undergoing hepatic resection for primary and secondary tumors or other pathologies, via the Biological Resource Center (University Hospital, Rennes, France). Cells were prepared by enzymatic dissociation of histologically-normal liver fragments (Fardel et al., 1993). These freshly isolated hepatocytes were either immediately collected for further RNA isolation and analysis or were seeded on standard tissue-culture plastic plates (BD Biosciences, Le Pont de Claix, France) at a density of approximately 2×10^5 cells/cm^2 in Williams' E medium (Invitrogen, Cergy-Pontoise, France), supplemented with 10% fetal calf serum (Perbio Sciences, Brébieres, France), 5 μg/ml bovine insulin (Sigma–Aldrich), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Invitrogen). After 24 h, this seeding medium was discarded, and hepatocytes were cultured in the fetal calf serum-containing Williams' E medium defined above and supplemented with 5×10^{-5} M hydrocortisone hemisuccinate (Upjohn, Paris La Défense, France) and 2% dimethyl sulfoxide (DMSO), as reported previously (Chouteau et al., 2001; Le Vee et al., 2009b), knowing that DMSO is well-known to promote differentiation and survival of hepatocytes (Isom et al., 1985). The culture medium was routinely renewed every 2 days. All experimental procedures complied with French laws and regulations and were approved by the National Ethics Committee. Hepatocytes usually form nearly-confluent monolayers of viable cells and were used for experiments after a 8–10 days culture period.

2.3. RNA isolation and analysis

Total RNA was isolated from freshly isolated and monolayer-cultured human hepatocytes using the TRIzol reagent (Invitrogen/Life Technologies). RNA (20 ng) was then subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR), using the fluorescent dye SYBR Green methodology and an ABI Prism 7300 detector (Applied Biosystem, Foster City, CA, USA), as previously described (Le Vee et al., 2013). The gene primers used in the study were exactly as previously reported (Le Vee et al., 2013; Moreau et al., 2011); their adequate efficiency was routinely checked using serially diluted RNA samples. Amplification curves of the PCR products were analyzed with the ABI Prism SDS software using the comparative cycle threshold (Ct) method. Relative quantification of the steady-state target mRNA levels for transporters was next calculated after normalization of the total amount of cDNA tested to an 18S RNA endogenous reference, knowing that the amount of 18S RNA in freshly isolated human and monolayer-cultured human hepatocytes was found to be constant, *i.e.*, the Ct numbers determined from qPCR assays were similar in these cells (Fig. S1), and was arbitrarily set at 10^6 units in each PCR sample (Moreau et al., 2011). This finally allowed to get a relative value of expression for each transporter comparatively to the 18S RNA reference.

2.4. Light microscopy analysis

Light microscopy analysis of monolayer cultures of human hepatocytes was performed using an Axiovert microscope (Carl Zeiss, Le Pecq, France), as previously described (Le Vee et al., 2013).

2.5. Immunolocalization studies

Immunofluorescence analyses were performed as previously reported (Le Vee et al., 2009b, 2013). Human hepatocytes, cultured on glass coverslips (Millicell EZ slides, Merck Millipore, Billerica, MA) for 8–10 days at the density of approximately 2×10^5 cells/cm^2 in the medium described above, were first fixed in ice-cold acetone for 10 min. Cells were next incubated for 3 h with mouse monoclonal or rabbit polyclonal antibodies diluted to 1:50 (mouse antibodies) or 1:25 (rabbit antibodies) in phosphate-buffered saline supplemented with 4% (weight/weight) bovine serum albumin. The mouse monoclonal antibodies were directed against organic cation transporter 1 (OCT1)/SLC22A1 (Abcam, Cambridge, UK), multidrug resistance gene 1 (MDR1)/ABCB1/P-glycoprotein (Alexis Corporation, Lausen, Switzerland), multidrug drug resistance-associated protein (MRP) 2/ABCC2 or MRP3/ABCC3 (Merck Millipore), whereas the rabbit polyclonal antibodies were raised against organic anion transporting polypeptide (OATP) 1B1/SLCO1B1 or OATP2B1/SLCO2B1 (Huber et al., 2007). Controls were performed in parallel with appropriate mouse or rabbit isotypic Ig

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