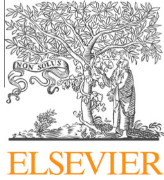
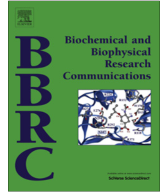
Biochemical and Biophysical Research Communications xxx (2014) xxx–xxx



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc



An immortalized human liver endothelial sinusoidal cell line for the study of the pathobiology of the liver endothelium

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ARTICLE INFO

Article history:
Received 5 May 2014
Available online xxxx

Keywords:
Liver sinusoidal endothelial cells
Inflammation
Differentiation
Tubulogenesis
Adhesion molecules
Uptake

ABSTRACT

Background: The endothelium lines blood and lymph vessels and protects underlying tissues against external agents such as viruses, bacteria and parasites. Yet, microbes and particularly viruses have developed sophisticated ways to bypass the endothelium in order to gain access to inner organs. De novo infection of the liver parenchyma by many viruses and notably hepatitis viruses, is thought to occur through recruitment of virions on the sinusoidal endothelial surface and subsequent transfer to the epithelium. Furthermore, the liver endothelium undergoes profound changes with age and in inflammation or infection. However, primary human liver sinusoidal endothelial cells (LSECs) are difficult to obtain due to scarcity of liver resections. Relevant derived cell lines are needed in order to analyze in a standardized fashion the transfer of pathogens across the liver endothelium. By lentiviral transduction with hTERT only, we have immortalized human LSECs isolated from a hereditary hemorrhagic telangiectasia (HHT) patient and established the non-transformed cell line TRP3. TRP3 express mesenchymal, endothelial and liver sinusoidal markers. Functional assessment of TRP3 cells demonstrated a high capacity of endocytosis, tube formation and reactivity to immune stimulation. However, TRP3 displayed few fenestrae and expressed C-type lectins intracellularly. All these findings were confirmed in the original primary LSECs from which TRP3 were derived suggesting that these features were already present in the liver donor. We consider TRP3 as a model to investigate the functionality of the liver endothelium in hepatic inflammation in infection.

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1. Introduction

Liver sinusoidal endothelial cells (LSECs) form the interface between the microvascular sinusoidal compartment of the liver and the liver parenchyma. LSECs thus form a protective barrier that senses pathogens and has a predominant role in receptor-mediated clearance of infectious agents and toxins from the blood [1]. Among the different types of endothelia, the liver endothelium in particular is thought to serve as an important sink for elimination and degradation of pathogens and digestive products, and also to regulate inflammation, leukocyte recruitment and immune responses [2,3]. However, hepatotropic viruses have developed means to overcome the protective filter formed by LSECs in order to gain access to the underlying liver parenchyma. Indeed, Duck Hepatitis B virus is thought to cross the liver endothelium via transcytosis [4], and a number of other hepatotropic viruses were shown to specifically bind C type lectins expressed on endothelial cells [5–7]. Finally, LSECs are a reservoir for mCMV latency [8]. Thus several viruses can cross the protective liver endothelium in order to gain access to and infect the liver parenchyma. However, the limited access to human liver samples renders the work with primary human LSECs difficult and it remains technically challenging to explore the interaction between LSECs and hepatotropic viruses in more detail. Indeed, with the exception of one approach which describes the reversible immortalization of primary human

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http://dx.doi.org/10.1016/j.bbrc.2014.05.038
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LSECs, which was however based on the use of hTERT as well as SV40T [9], no adequate culture systems and non-transformed, tissue culture adopted human LSECs are available, and this lack has hampered the elucidation of the functional relevance of LSECs in the infection process of the liver [2]. In an attempt to develop endothelial cell lines of intrasinusoidal origin for liver physiopathology research, immortalized but untransformed hepatic endothelial foci were isolated from the liver of a hereditary hemorrhagic telangiectasia (HHT) patient. Phenotypic and functional characterization allowed the establishment of the cell line TRP3. These cells are of mesenchymal and endothelial origin, display a rapid endocytic capacity, responsiveness to mediators of inflammation and differentiation, and express a number of liver sinusoidal endothelium-specific markers.

2. Materials and methods

2.1. Patient of origin

The patient from whom TRP3 originate is a 65 year-old female with a history of hepatic arteriovenous malformations related to HHT. Clinical diagnosis of HHT was made according to international consensus criteria [10]. The patient suffered from nosebleed and telangiectasia and displayed an aneurysm of the hepatic artery and shunting from the hepatic artery to hepatic veins on MRI, CT-scan and ultrasound/Doppler study. The patient underwent a liver transplantation at the Lyon University Medical Center. Serum and liver samples were harvested soon after transplantation with informed consent from the patient according to French bioethics laws.

2.2. Liver histology and immunostaining

A minimum of 10 representative tissue blocks were systematically taken from the right and the left lobes of the patient liver and serially sectioned at 0.5 cm intervals. Tissues were formalin-fixed, paraffin-embedded, cut in 5 μm sections, stained with standard hematoxylin–eosin–safranin or immunostained with CD31 and CK8 (Dako) or L-SIGN antibody CD209L (R&D Systems) followed by a biotinylated secondary antibody bound to a streptavidin peroxidase conjugate (Lsab + Kit, Dako). Peroxidase activity was revealed using DAB as a chromogen.

For immunofluorescence cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin/PBS and immunostained with antibodies against vimentin (Immunotech, 1/20), von Willebrand factor (Dako, 1/400), CD34 (clone QBEND10, Novocastra, 1/50), L-SIGN (MAB162, R&D systems, 1/50) followed by detection with alexa-488 conjugated goat anti-mouse (Molecular Probes). Counterstaining was performed with 0.25 μg/ml DAPI (Sigma).

2.3. Mutation screening

Germ line mutation screening was performed on DNA isolated from peripheral blood lymphocytes as previously reported [11,12]. The research for somatic mutations on the second allele was performed by dHPLC on DNA extracted from cultured TRP3 cells, followed by automated sequencing. Quantitative Multiplex PCR of Fluorescent Short Fragments was used to search for a loss of heterozygosity [12].

2.4. Isolation and culturing of endothelial cells and cell lines

Endothelial cells (EC) were isolated from the freshly explanted liver according to a modified version of the protocol described by Daneker et al. [13]. Large abnormal intrahepatic vessels and liver parenchyma were separated and treated separately using the same protocol. HTT is characterized by a very strong capillary enrichment of sinusoids in the liver, and this impedes proper hydrodynamics of the perfusion. Therefore, livers were cut into 3–5 mm cubes and incubated with 0.1% collagenase (Sigma–Aldrich) overnight at 4 °C and 1 h at 37 °C. Cubes were then disaggregated and filtered through a 60 mesh filter. Cell suspensions were centrifuged at 4 °C for 10 min at 1800 rpm and washed 3x with Hepes buffered HBSS. Cells were placed on a density gradient of 18%, 13% and 8% metrizamide and centrifuged for 20 min at 2400 rpm at 20 °C. Cells present between the 13% and 18% layers were collected, centrifuged, washed, resuspended in MCDB131 culture medium (Invitrogen), supplemented with 20% fetal calf serum (Fetalclone I, Hyclone), 10 mmol/L L-glutamine, 250 μg/ml AMPc, 50 μg/ml endothelial cell growth supplement (ECGS, Beckton Dickinson), 1 μg/ml of hydrocortisone and penicillin–streptomycin, seeded in collagen-coated Petri dishes (Beckton Dickinson) and incubated at 37 °C in a 5% CO2 atmosphere. TRP3 cells were selected by successive limited trypsination. Immortalization was achieved by lentiviral transduction with a bicistronic vector encoding hTERT and a puromycin selection marker. Throughout passages 4–11 post-immortalization, the most stably growing puromycin-resistant foci were amplified and screened using endothelial markers.

TRP3 cells were routinely cultured in the endothelial selective medium MCDB131, as mentioned above. HUVEC, Huh7 [14] and HepaRG [15,16] cells were cultured as previously described.

2.5. Flow cytometry and Immunoblotting

Q4 Flow cytometry was performed using PBFA (PBS, 5% FCS, 0.2% sodium azide) for surface staining and PBFA containing 0.1% saponin for total cellular staining. Antibodies used were anti-SRB1 (1/200 BD Biosciences #610883) and anti-Stabilin2 (1/200 abcam #ab12 1893), anti-LDLr (1/25 Calbiochem # LP02), anti-CD81 (1/500 BD Biosciences #JS-81), anti-DC-SIGNR # MAB162 and anti-DC-SIGN # MAB161 (1/25 R&D Systems), anti-LYVE1 (1/50 abcam #ab14 917) and anti-CD32B (abcam #ab151497), and anti-mouse/rabbit Alexa Fluor 647 # A21237 (1/200 Invitrogen). Immunoblotting was performed under denaturing conditions using 50 μg cell lysate the indicated primary antibodies and HRP-conjugated secondary antibodies prior to revelation with in-house made chemiluminescent reagent. Quantification was done using ImageQuant software.

2.6. L-SIGN RT-PCR and Southern blotting

1 or 5 μg of total RNA from TRP3 and HepaRG were harvested using Extract-all reagent (Eurobio) and reverse transcribed using One Step RT-PCR kit (Qiagen). RNA integrity was verified by agarose electrophoresis and 28S and 18S RNA pattern visualization. L-SIGN specific primers (5′-CAGTGGCATCAGACTTTTTC-3′; 5′-CCTGGTAGA TCTCCTGCA-3′) were used as previously described [17]. After migration of the 418 base pair amplicons on a 1.5% agarose gel, transfer onto Hybond N+ membrane (GE-Healthcare) and prehybridation, hybridization was performed o/n with ^{32}P radiolabelled oligoprobe (1.10^6 cpm/ml) using Terminal deoxynucleotide Transferase (Roche). The probe sequence was 5′-CCAGAACCTGACCCAGCT TAAAGCTGCAGT-3′.

2.7. Tube formation and endocytosis assays

TRP3 cells and myofibroblasts (MF) were seeded (3×10^4/well) in 24-well plates coated with 0.3 ml of growth factor-containing Matrigel™ (Becton Dickinson) and incubated at 37 °C with complete MCDB131 medium. Images were taken at ×100 magnification (Nikon TE2000E) at 6 h post-seeding. For endocytic uptake, HepaRG and endothelial cells were incubated with 5 or 15 μg/ml of alexa-labeled acetylated LDL (Life Technologies) for the indicated time

Please cite this article in press as: R. Parent et al., An immortalized human liver endothelial sinusoidal cell line for the study of the pathobiology of the liver endothelium, Biochem. Biophys. Res. Commun. (2014), http://dx.doi.org/10.1016/j.bbrc.2014.05.038

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