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EDITORIAL

Human hepatic sinusoidal endothelial cells can be distinguished by expression of phenotypic markers related to their specialised functions *in vivo*

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Abstract

The hepatic sinusoids are lined by a unique population of hepatic sinusoidal endothelial cells (HSEC), which is one of the first hepatic cell populations to come into contact with blood components. However, HSEC are not simply barrier cells that restrict the access of blood-borne compounds to the parenchyma. They are functionally specialised endothelial cells that have complex roles, including not only receptor-mediated clearance of endotoxin, bacteria and other compounds, but also the regulation of inflammation, leukocyte recruitment and host immune responses to pathogens. Thus understanding the differentiation and function of HSEC is critical for the elucidation of liver biology and pathophysiology. This article reviews methods for isolating and studying human hepatic endothelial cell populations using *in vitro* models. We also discuss the expression and functions of phenotypic markers, such as the presence of fenestrations and expression of VAP-1, Stabilin-1, L-SIGN, which can be used to identify sinusoidal endothelium and to permit discrimination from vascular and lymphatic endothelial cells.

Key words: Human; Liver; Endothelium; Sinusoid; Phenotype

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INTRODUCTION

The liver has a unique dual blood supply receiving blood from both the portal vein and the hepatic artery. Unlike other organs, which are supplied by arterial blood through arterioles, the liver receives venous blood at low pressures through the portal vein as well as arterial blood *via* the hepatic artery. The intrahepatic portal venous system consists of conducting and distributing systems that ensure blood is carried throughout the parenchyma and evenly delivered to individual hepatocytes *via* the sinusoidal network[1]. A uniform and strict branching pattern appears at the level of the terminal vessels and is maintained throughout the distributing system. The first order branches of the distributing system arise from the terminal branches of the conducting system and give rise to second order vessels of approximately 70 μm diameter that correspond to the terminal portal vein branches seen in portal tracts. Third order vessels arise from these and correspond to the septal or interlobular branches. These exhibit a classical sinusoidal appearance, lack a connective tissue sheath and have no basement membrane. The distributing system then branches into the hexagonal lobule before draining into the hepatic vein. The hepatic artery supplies 25% of hepatic blood flow and provides blood to both the parenchyma and the portal tracts. Axial hepatic arterial branches run parallel to the conducting portal veins and terminate in inlet venules, terminal portal veins and the sinusoids, thereby supplying blood to the parenchyma. The axial arteries also give rise to peribiliary branches that supply the accompanying bile ducts and portal interstitium. These arteries form the peribiliary plexus consisting of efferent and afferent capillaries that wrap around the surface of bile ducts. Small channels from the peribiliary plexus drain into the sinusoids or portal vein branches. Thus, complex anastomoses exist between the axial arteries and peribiliary arteries[2].

ISOLATION AND CULTURE OF HEPATIC SINUSOIDAL ENDOTHELIAL CELLS

Isolated cultures of hepatic sinusoidal endothelial cells present a valuable tool for the study of liver physiology and pathophysiology. Most published studies used cells isolated from rodent livers and have defined the role of fenestrations and specific receptors in uptake and

processing of circulating factors including pathogens (reviewed in[3]). Most investigators use a combination of enzymatic digestion and density gradient centrifugation to isolate HSEC from liver tissue although there is considerable variation in the protocols used by different groups and for cells from different species. HSEC from rodents are commonly isolated by enzymatic digestion, either by perfusion of an intact organ with an enzymatic cocktail or by mechanical disruption followed by enzymatic digestion[4-7]. The cell suspension generated by such methods is fractionated using differential centrifugation techniques, including counterflow elutriation or density gradient centrifugation. Endothelial cells are then grown on matrix coated flasks in selective growth media containing growth factors.

Similar methods can be used to isolate HSEC from human liver samples[8-10] and between 10^3-10^6 cells have been isolated from whole livers unsuitable for transplantation[9]. However, the limited access to intact human liver means that most groups use diseased tissue removed at transplantation or surgical resection or biopsy specimens that generate low numbers of cells per isolation. Nevertheless, successful isolation is aided by the large numbers of HSEC relative to other vascular endothelial cells present in the liver. Some researchers further reduce the potential of contaminating vascular endothelium by excising visible large vascular structures from the liver tissue prior to enzymatic digestion. Although several different isolation procedures have been described these are not all equally effective in our hands. Our experience, and that of other groups, suggests that counterflow elutriation alone is not useful for selection of human HSEC from a mixed non-parenchymal cell preparation as most of the cells have similar centrifugal densities[6,11]. This led us to routinely include a step of immunomagnetic depletion to remove common contaminating cell populations, such as biliary epithelial cells, followed by positive selection of endothelial cells using antibody against CD31 (see later)[10]. Similar methods using magnetic beads coated with Evonymus europaeus agglutinin have been used to isolate HSEC from the liver of other primates[12] and there are now commercial antibody-based magnetic kits for isolation of rodent HSEC. Despite these refinements there is considerable variability in the yield and viability of cells obtained from diseased human liver tissue. Thus, although there are compelling reasons to study human HSEC *in vitro* low cell yields mean that it is difficult to use primary cells without passage.

PROBLEMS WITH EXISTING PHENOTYPIC MARKERS AND USE OF HSEC IN MONOCULTURE

As scientific technology advances it is possible to carry out increasingly complex genetic, proteomic and functional analyses on cells grown in culture. However such techniques require highly purified populations of cells of defined phenotype. Some of the methods previously used to confirm HSEC purity and phenotype, such as AcLDL uptake[8] and binding of Ulex lectin[8], are not specific. For example, other hepatic cell populations, including dendritic cells, take up AcLDL and Ulex lectin binds to fucosylated receptors on both Kupffer cells and HSEC[13]. Similarly, endothelial cells share many cell surface receptors with leukocytes, including CD31, CD4[14], CD11b, and CD11c[11], which may contaminate endothelial cell preparations in culture. Care also needs to be taken when using antibody staining to define HSEC phenotype because HSEC express high levels of FcγR[15] allowing them to bind antibodies non-specifically. In light of such problems, it has been suggested that the presence of open fenestrations arranged in sieve plates is the only true marker of hepatic sinusoidal endothelial cells[16]. These pores are indeed classic features of liver sinusoidal endothelial cells *in vivo* but present problems when used to identify cells *in vitro* (see below).

All of these problems are compounded by the fact that HSEC are most commonly cultured as a monolayer of cells on matrix-coated tissue culture plates *in vitro*. This perturbs the normal morphology of the cells and they become flattened and rapidly lose fenestrations. Part of this effect may be the loss of paracrine signals from other cells of the sinusoid that maintain the phenotype and differentiation of HSEC *in vivo*. For example, crosstalk between hepatic sinusoidal endothelial cells and closely juxtaposed hepatocytes is essential for the maintenance of sinusoidal endothelial cell growth and differentiation. This is demonstrated by studies where implantation of foetal liver fragments into quail chorioallantoid membrane resulted in the acquisition of a sinusoidal phenotype by the chorioallantoid microvessels[17] and also *in vitro* studies where co-culture of HSEC with other liver cells resulted in a more stable endothelial phenotype and function[18]. Thus, markers used to determine phenotypes of HSEC must take into account alterations in phenotype as a consequence of culturing cells in isolation in the absence of local paracrine signals.

FENESTRATIONS

Endothelial cells throughout the adult organism are derived from common early embryological precursors and have broadly similar functions and histological appearance. However, there is important organ and tissue-specific heterogeneity that results in phenotypic and functional variations (reviewed in[19]). For example, high endothelial venules in lymph nodes are lined by morphologically and phenotypically distinct endothelial cells that have the unique ability to promote the recruitment of naïve lymphocytes whereas lymphatic endothelium express several receptors that allow uptake of macromolecules found in lymph[20]. Sinusoidal endothelial cells are found in the spleen and bone marrow, as well as in the liver, and in all these sites they have a minimal basement membrane and lack classical tight junctions. Hepatic sinusoidal endothelium differs from sinusoidal endothelium in these other beds by its discontinuous nature, being interspersed with kupffer cells and by the presence of open fenestrations arranged in sieve plates[16].

The vascular architecture in the human liver develops by 17-25 wk of gestation, and the sinusoids acquire their

Table 1 The expression of classical markers of endothelial phenotype by human sinusoidal endothelial cells

Marker	Extra-hepatic endothelial expression	Sinusoidal endothelial cells *in vivo*	Sinusoidal endothelial cells *in vitro*	Problems with use in phenotyping?
CD31	Vascular and lymphatic endothelial cells	Yes, but at low levels	Yes	Widely expressed on all EC thus not specific for HSEC
vWF	Vascular endothelial cells	Controversial, Yes	Yes	Widely expressed on all EC thus not specific for HSEC
Ulex lectin binding	Endothelial cells	Yes	Yes	Widely expressed on all EC thus not specific for HSEC
Uptake of AcLDL	Endothelial cells	Yes	Yes	Also taken up by macrophages in liver and other EC
CD34	Vascular and lymphatic endothelial cells	No	Absent or low	May be upregulated during capillarisation or with passage *in vitro*
E-Selectin	Vascular endothelial cells	Low or absent under normal conditions	Low, can be upregulated by cytokines	Widely expressed on activated vascular EC, not specific for HSEC
Pal-E antigen	Vascular endothelial cells	No	No?	May be upregulated during capillarisation
CD105/ endoglin	May be upregulated during capillarisation	Yes	Yes	Widely expressed on all EC, also by stellate cells and fibroblasts in liver

While many of the above markers are indeed expressed on hepatic sinusoidal endothelial cells and provide a means of confirming "sinusoidal endothelial identity", none is specific to sinusoidal endothelial cells.

distinctive fenestrated phenotype by wk 20 (reviewed in[21]). The fenestrations act as a 'dynamic filter'[3] allowing macromolecules in blood controlled access to parenchymal cells[22]. Evidence from animal studies suggests that fenestrations constitute up to 40% of the cell and that the size, distribution and clustering of the pores in sieve plates varies with the zonal distribution of the endothelium[23] and across the endothelial surface. Although normal hepatic sinusoidal cells in most mammals are characterised by the presence of fenestrations (reviewed in[3]), caution must be exercised when translating these observations to human cells. Studies of fenestrations in human liver samples are rare, and suggest that the number and size of fenestrations differs from that observed in other mammals[3]. Furthermore, the number of fenestrations per endothelial cell decreases in disease[24,25], following viral infection[22] or with ageing[26]. During cirrhosis and chronic hepatitis, HSEC develop a more vascular morphology and produce a basement membrane in a process known as 'capillarisation' (reviewed in[27]). This is associated with increased expression of CD31 and VCAM-1 and loss of fenestrations[27]. These changes may impede the transfer of materials to and from the parenchyma and contribute towards regional hepatocyte hypoxia. Fenestrations are not unique to hepatic EC but are found in endothelium in endocrine glands, kidney, gastrointestinal tract, choroid plexus, lymphatic organs such as the spleen and are sometimes observed in tumour vasculature. Many studies have implicated VEGF as an essential factor for regulation of fenestrations in these organs (reviewed in[28]).

Thus considerable variations in the number, size and localisation of fenestrations are seen among species and also in health and disease. The situation becomes more complex when cells are removed from the hepatic microenvironment and cultured *in vitro*. The fenestrations documented in freshly isolated rat HSEC begin to disappear within 48 h of cell culture[29] and are almost gone within a week[30]. We have made similar observations with human cells from normal livers and also find very few fenestrated cells when HSEC are isolated from cirrhotic livers (Lalor and Adams unpublished observations). However, the number of fenestrations on rat HSEC can be maintained *in vitro* by the addition of VEGF and by culturing cells on extracellular matrix constituents, such as collagen, that are secreted by endothelial cells[29,31]. Both human and rodent HSEC need growth factors and attachment to appropriate extracellular matrix molecules to survive and will rapidly undergo apoptosis in the absence of these. Thus in order to maintain cell survival, cultured HSEC must be grown in the presence of VEGF which induces and maintains expression of fenestrations as well as promoting HSEC proliferation. VEGF is also a growth factor for vascular endothelial cells which induces the production of matrix molecules essential for survival and proliferation[32]. At higher concentrations, VEGF can induce the formation of fenestrations in vascular endothelial cells[28,32,33] and, although these pores are not organised into sieve plates, they can be very difficult to distinguish from the fenestrations that characterise HSEC. This inducibility of fenestrations in vascular endothelial cells together with the impracticality of using electron microscopy for routine phenotyping means that the presence of fenestrations alone cannot be used to define HSEC in most experimental situations.

NORMAL 'ENDOTHELIAL' CELL PHENOTYPE AND FUNCTIONS

HSEC form a single cell barrier between the hepatocytes and the bloodstream and are strategically situated to interact with leukocytes and other blood constituents. The cells produce a minimal basement membrane[34], which is mostly composed of type IV collagen in normal liver[35], and have a high endocytotic capacity[3]. They express some markers that are common to all endothelial cells and these provide a useful means to positively identify a cell of 'endothelial' lineage (Table 1).

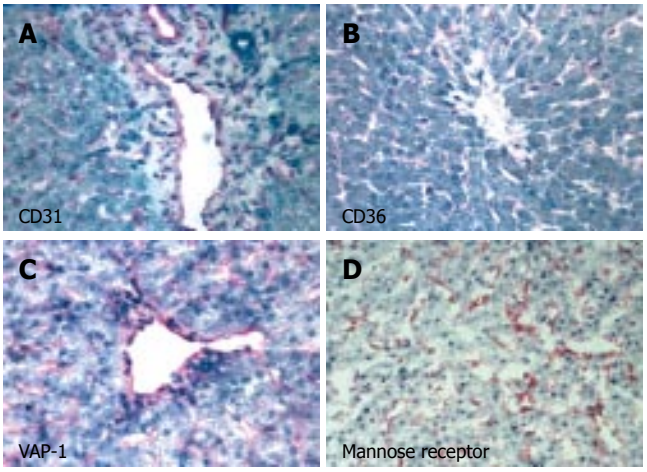

Figure 1 Human hepatic sinusoidal endothelial cells *in situ* express the classical endothelial phenotypic markers CD31 (**A**) and CD36 (**B**) as well as more recently identified markers such as VAP-1 (**C**) and Mannose receptor (**D**). Images represent immunohistochemical staining of human liver sections using specific primary antibodies in an indirect immunoperoxidase protocol with haematoxylin counterstain. Positive staining of sinusoidal endothelial cells is indicated by pink pigmentation. CD31, CD36 and VAP-1 are observed on both portal vessel endothelial cells and sinusoidal endothelium, whilst the mannose receptor is localised on sinusoidal endothelial cells and Kupffer cells.

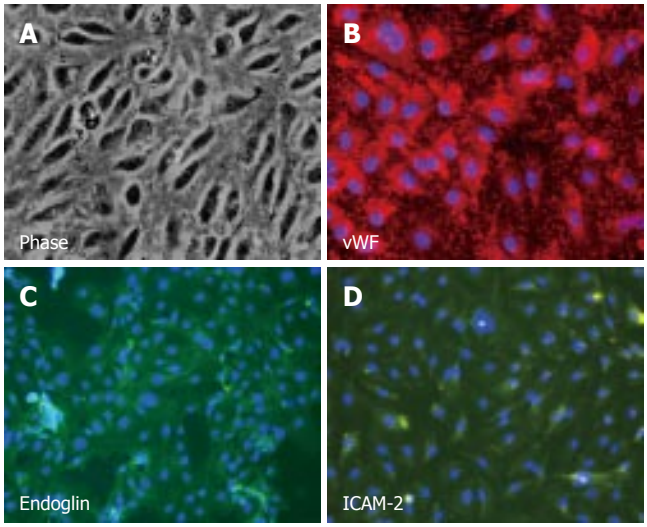

Figure 2 Isolated, cultured human hepatic sinusoidal endothelial cells exhibit classical morphology under phase contrast microscopy (**A**), and stain positively with antibody directed against endothelial phenotypic markers. Images represent immunofluorescent staining of cultured cells using specific primary antibodies in an indirect fluorescent protocol with DAPI nuclear counterstain (blue). Positive staining for vWF (**B**) is visualised using a Texas Red-labelled secondary antibody, whilst expression of endoglin (CD105, **C**) and ICAM-2 (**D**) are visualised with a FITC-conjugated secondary antibody (green).

CD31

CD31 or PECAM-1 is an abundantly expressed membrane glycoprotein member of the immunoglobulin superfamily (reviewed in[36]). It is constitutively expressed on endothelial cells and some haematopoetic cells and has functions in cell adhesion and signalling. Expression of CD31 is used widely as a marker of 'continuous' or classical vascular endothelium, but expression of CD31 by hepatic sinusoidal endothelial cells remains controversial. In rodent studies, CD31 has been localised to normal sinusoidal endothelial cells using a variety of techniques[4,37] and has been shown to be downregulated following CCl_4 or TNFα mediated liver injury[37]. However, other studies suggest that the protein is absent under normal conditions[34] and indeed a lack of CD31 has been used to characterise rodent HSEC[7].

The situation is similarly complex in human HSEC. During human embryonic development CD31 is absent from HSEC until wk 25. In the adult, CD31 has been reported as present on HSEC by FACS and immunohistochemistry on sections from cirrhotic liver but is minimally present on normal liver[8] or alternately present on normal liver and enhanced on cirrhotic liver[38]. Our own data show that CD31 is expressed by both normal and diseased liver HSEC (Figures 1 and 2). In general, it seems that HSEC express lower levels of CD31 than vascular EC ([16] and our observations). However, one needs to interpret immunohistochemical analysis with caution because CD31 is present on kupffer cells in sinusoids. Studies on isolated HSEC using both PCR and antibody-based assessment of characteristic endothelial markers, such as CD31 and vWF (see below) in parallel, show varying results[39]. Some studies suggest that subcellular localisation of CD31 can be used as a marker of HSEC phenotype to indicate whether cells have dedifferentiated in culture[16]. Thus cells with cytoplasmic CD31 are 'normal' whereas dedifferentiated/capillarised EC demonstrate increased membranous expression.

Von Willebrand Factor (vWF)

vWF is a multimeric glycoprotein that binds and stabilises the coagulation factor FVIII as well as supports the adhesion of platelets to subendothelial structures during vascular damage. It is expressed by both platelets and endothelial cells and is often used as a marker to identify endothelium. In most vascular endothelial cells, von Willebrand Factor is stored in cytoplasmic vesicles called Weibel Palade bodies. Expression of vWF varies between different vascular beds *in vivo*[40] and particularly low levels are observed in the liver, most of which is detected in vascular rather than sinusoidal endothelial cells. The low levels of vWF detected in HSEC are consistent with the reported lack of Weibel Palade bodies. However, definitive evidence supporting the presence or absence of these structures is also lacking. Studies in mice suggest that HSEC contain Weibel-Palade bodies[41] and produce VWF at the mRNA and protein level[4,11,41]. Other groups working with rat HSEC report that vWF is not expressed in normal rat cells[42] and these findings are supported by porcine and rat studies showing absence of Weibel-Palade bodies in HSEC[9,43]. In human cells, vWF expression has been reported in both normal[8,44] and diseased samples[45] and we and others[8] have demonstrated expression on passaged, cultured HSEC *in vitro* (Figure 2).

E-Selectin

E-Selectin is a member of the selectin family of adhesion molecules that supports leukocyte binding. Expression of E-Selectin is restricted to cells of endothelial lineage[46]

and is induced by inflammation *in vivo* and exposure of endothelial cells to proinflammatory cytokines and LPS *in vitro*. The ability to express E-selectin can thus be used to define endothelial cells in culture. However, expression of E-selectin is restricted to vascular endothelial cells in the normal liver[47] , although it may be upregulated on sinusoidal endothelium in disease and during metastatic processes[48] and animal studies demonstrate a minimal role for E-selectin in leukocyte recruitment to liver tissue[49]. However, we have reported expression of functional E-Selectin on cultured cytokine-stimulated human HSEC suggesting that HSEC can express E-Selectin under restricted circumstances *in vivo* and *in vitro*[18]. Recent studies demonstrating that E-Selectin expression by HUVEC in response to TNFα is reduced by pretreatment with HGF suggest that paracrine factors from adjacent hepatocytes may suppress E-selectin in the sinusoids *in vivo*[50].

Binding of lectins and Acetylated LDL uptake

The ability to bind Ulex lectin and take up acetylated LDL is often used to define HSEC. Ulex lectin, from the gorse family of plants, binds alpha-L fucose containing receptors and is commonly used as a histological marker for endothelial cells, although in some tissues it also binds epithelial structures[51]. In the liver, different lectins bind differentially within the vasculature. Concanavalin A binds with equal affinity for all segments of the microvasculature whereas wheat germ agglutinins show preferential binding to the sinusoidal vasculature as a consequence of differences in distribution of glycosylated ligands throughout the acinus. In most studies Ulex lectins do not bind preferentially to sinusoidal endothelium as a consequence of the relative paucity of alpha-L fucose motifs. The ability of wheat germ lectins to bind sinusoidal endothelium has led to their use in selectively purifying sinusoidal EC and their preference for periportal HSEC has even led to the suggestion that they can be used to differentially purify periportal verses perivenous HSEC[5]. Staining with Ulex lectin is increased in disease and is particularly pronounced during capillarisation of the sinusoids[52]. Thus although binding of Ulex lectin is indicative of an endothelial phenotype, it is not restricted to 'sinusoidal' endothelial cells[52] and is not a good marker of HSEC.

The liver is the major site for the scavenger receptor-mediated clearance of lipoproteins from the circulation. Acetylated LDL is mainly cleared by hepatic endothelial cells[53] by binding to scavenger receptors including scavenger receptor class A (SR-AI/Ⅱ). However, these receptors are expressed by both HSEC and macrophages[54] and the ability to take up acetylated LDL is common to many extrahepatic endothelial cell populations, again reducing the specificity of acLDL uptake as a characteristic property of HSEC.

CD34

CD34 is a type 1 transmembrane sialomucin expressed by haematopoetic stem cells, capillary and lymphatic endothelial cells. CD34 is absent from most sinusoidal endothelial cells in normal liver but expression increases[8,27,55] during capillarisation in chronic inflammatory disease and in the sinusoidal-type vasculature within hepatocellular carcinomas[56,57]. CD34 expression has also been shown to increase in other tissues including the rheumatoid joint and at sites of neolymphoid development during chronic inflammation. We have described CD34 positive lymphatic-like vessels in portal associated lymphatic tissue in chronic inflammatory liver diseases including PSC[58] and hepatitis C (Heydtmann 2006 *J Immunol* in press). However, CD34 is not expressed on most HSEC in non-inflamed tissue *in vivo* and is absent from low passage human HSEC *in vitro*.

Pal-E Antigen

The antigen recognised by the Pal-E antibody is a widely used marker of vascular endothelial cells[59-61] The identity of the protein has been proposed as either a secreted form of vimentin produced by endothelial cells[61] or a protein designated PV-1/FELS (plasmalemmal vesicle-1/fenestrated endothelial-linked structural protein)[60]. PV-1 is particularly interesting in the context of HSEC. As its name implies, PV-1/FELS is expressed by fenestrated endothelial cells in the kidney and pancreas[62] but it is absent from fenestrated hepatic sinusoidal endothelial cells[60,62] and is restricted to vascular endothelium and neovessels in areas of capilliarisation in chronic liver disease and hepatocellular carcinoma[52,58].

Vascular Endothelial-Cadherin (VE-Cadherin)

Cadherins are a family of adhesion molecules that demonstrate cation- dependent homophilic and heterophilic binding. Endothelial cells express at least three cadherins: N-, P-, and VE-cadherin. VE-cadherin is localised to the inter-endothelial cell junction where it is an essential part of the adherens junctions that maintains endothelial permeability, monolayer integrity, morphogenesis and angiogenic reponses. Most studies suggest that sinusoidal endothelium in normal liver lacks VE-cadherin or expresses it at low levels, although it can be detected in chronic inflammation[38,63]. It seems likely that the relative lack of VE-cadherin on HSEC is a consequence of the absence of classical adherens junctions between HSEC and this is consistent with a lack of other junctional proteins, including vascular endothelial junctional adhesion molecule (VE-JAM/JAM-2), which is a member of the immunoglobulin superfamily structurally similar to JAM-1, which is absent from foetal and adult liver[64].

CD105/endoglin

CD105 (endoglin) is a hypoxia-inducible protein that is widely expressed on endothelial cells and is upregulated during angiogenesis. It is a receptor for transforming growth factor (TGF) -β1 and -β3 and modulates TGF-β signalling by interacting with TGF-β receptors I and/or Ⅱ (for review see[65]). CD105 has been used as a marker of angiogenesis, particularly in tumour tissue[66], and because it is a transmembrane molecule, it has been used in antibody-mediated positive selection strategies for endothelial cell isolation. However, expression of the

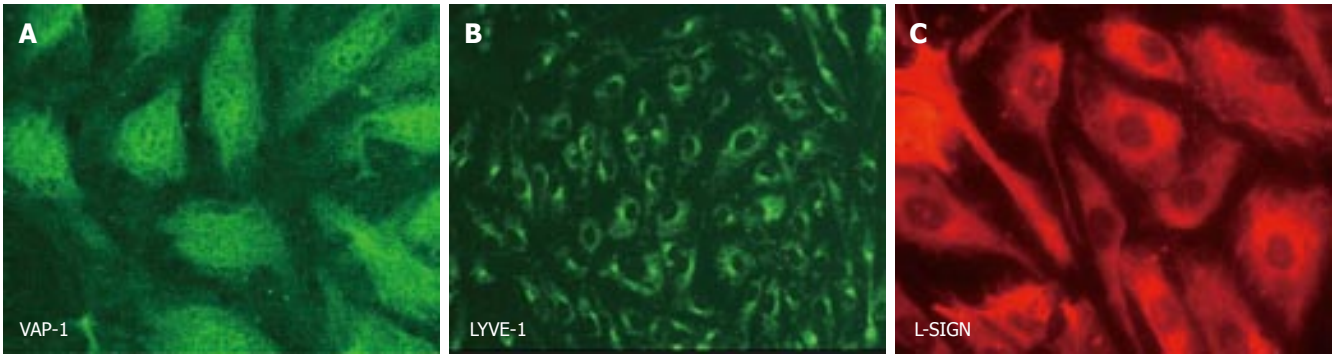

Figure 3 Cultured human hepatic sinusoidal endothelial cells stain positively with antibody directed against 'non-classical endothelial' phenotypic markers. Images represent immunofluorescent staining of cultured cells using specific primary antibodies in an indirect fluorescent protocol. Expression of VAP-1 (**A**) and LYVE-1 (**B**) are visualised with a FITC-conjugated secondary antibody (green), whilst positive staining for L-SIGN (**C**) is visualised using a Texas Red-labelled secondary antibody.

molecule is not restricted to endothelial cells[65], and in the liver, expression has been reported in both stellate cells and myofibroblasts[67]. We have demonstrated that CD105 is expressed on hepatic sinusoidal endothelial cells (Figure 2) but again emphasise that this is not a tissue- or cell lineage-specific marker and urge caution when using it as a phenotypic identifier.

NEWER MARKERS OF ENDOTHELIAL PHENOTYPE RELATE TO THE FUNCTIONS OF HSEC WITHIN THE LIVER MICRO-ENVIRONMENT

As well as exhibiting features characteristic of all endothelial cells, hepatic sinusoidal endothelial cells fulfil many specific features within the liver environment. These include providing a barrier to minimise access of blood-borne material into the parenchyma and specific protein/antigen uptake and presentation. The embryonic origins of these cells and their similarity with lymphatic endothelial cells (see later) mean that they express several markers that are not present on vascular endothelium and these can be used to distinguish them from other endothelial cells. Thus, it is possible to use protein markers related to these specific origins and functions to confirm the phenotype of HSEC *in vitro*.

Scavenger functions/lipid uptake functions of EC

The exposure of sinusoidal endothelial cells to blood originating from both the systemic circulation and the gut means that HSEC are strategically situated to remove and recycle blood-borne proteins and lipids. In combination with Kupffer cells, HSEC constitute the most powerful scavenger system in the body[68]. The uptake of solutes is facilitated by the presence of fenestrae, the lack of a classical basement membrane and the expression of multiple scavenger receptors that allow them to bind and take up specific classes of molecules. These properties facilitate bidirectional transfer of materials to the parenchyma. Many of the scavenger receptor proteins can be used to determine the phenotype of HSEC.

The link family of proteins has recently been described as scavenger receptors responsible for clearance of a variety of proteins, including advanced glycation end products, modified LDL and bacteria[69]. Two members of this protein family, Stabilin-1 and -2, are constitutively expressed by hepatic sinusoidal endothelial cells[69,70]. Stabilin-2 is the major lymph node and liver hyaluronan and glycosaminoglycan scavenger receptor whilst Stabilin-1 (also called Feel-1 or CLEVER-1[71-73] is a more promiscuous scavenger receptor. In common with many other scavenger-type receptors, these proteins are present on sinusoidal endothelial cells in spleen and lymph node as well as the liver. Most of the scavenger functions assigned to this molecule relate to endocytosis of hyaluronic acid, acetylated LDL and glycation end products, but there is also evidence to support roles for Stabilin-1 in leukocyte adhesion and tumour metastasis[69,74]. Another member of the link protein family is LYVE-1, an endothelial hyaluronan receptor predominantly restricted to lymphatic endothelial cells (reviewed in[75]). Putative functions for LYVE-1 include the uptake of hyaluronic acid and regulation of leukocyte adhesion or migration within the lymphatic circulation. Interestingly, hepatic sinusoidal endothelial cells also express LYVE-1 constitutively[76] (Figure 3) with evidence of a zonal distribution, the highest levels being detected in acinar zone 2. This hyaluronan receptor is present on both normal and diseased human HSEC, although lower levels are observed in cirrhosis and the protein is absent in HCC[76]. Expression is also seen on portal-associated lymphatics in chronic liver disease[58,76].

The liver is the major site for synthesis and metabolism of cholesterol and scavenger receptors of class A (SR-A) on both KC and HSEC[77] are responsible for the uptake of oxidised/acetylated LDL, which is subsequently passed on to hepatocytes. Another HDL/LDL receptor, CD36[78], also known as GPIV, is expressed at high levels on platelets, monocyte/macrophages and vascular endothelial cells. In the liver, CD36 is strongly expressed on sinusoidal endothelial cells[79] where it fulfills multiple functions including acting as a scavenger receptor for oxidised lipid[80] and as an adhesion receptor for red blood cells infected with malarial parasite[81]. There are two alternatively spliced members of the scavenger receptor B family (SR-BI and -BⅡ). Scavenger receptor-B1 is expressed by HSEC and

is responsible for the uptake of HDL cholesterol esters to liver parenchymal cells and also acts as a coreceptor for HCV infection[82].

The calcium-dependent C-type lectins, Dendritic Cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) or CD209 and the related molecule Liver/lymph node-specific intercellular adhesion molecule-3-grabbing nonintegrin (L-SIGN) or CD209L (Figure 3), are constitutively expressed on hepatic endothelial cells[83]. DC-SIGN is expressed at high levels on myeloid dendritic cells in tissues where it interacts with ICAM-3 on T cells as part of the immunological synapse as well as being an attachment factor for HCV, ebola virus, CMV, HIV and other lentiviruses (reviewed in[84,85]). We have recently reported that DC-SIGN is present on hepatic sinusoidal endothelial cells and that expression is increased in response to treatment with cytokines including IL-4[86]. DC-SIGN on endothelial cells acts as an attachment factor for HCV but does not mediate HCV entry directly but rather enhances infection of hepatocytes in trans. DC-SIGN is absent from most other vascular beds, although it has been reported on a brain microvascular cell line[87].

Liver/lymph node-specific ICAM-3-grabbing integrin (L-SIGN), otherwise known as DC-SIGN-related (DC SIGNR) (CD209L), shares 77% amino acid homology with DC-SIGN. Like DC-SIGN it can also bind ICAM-3, HIV and HCV. L-SIGN is strongly and constitutively expressed on sinusoidal endothelial cells in the liver and on endothelium in lymph nodes but not on DCs or on endothelium in other tissues. L-SIGN is thus an excellent marker of liver endothelium[44,88]. A related molecule, Liver and Lymph node Sinusoidal Endothelial C-type lectin (LSECtin) is also expressed on sinusoidal endothelial cells and has recently been demonstrated to mediate attachment of filovirus and coronavirus particles[89]. Thus sinusoidal endothelial cells[90] can bind a wide variety of pathogens after which they pass hepatotropic viruses on to adherent hepatocytes in trans[91] thereby concentrating viral pathogens within the liver.

Antigen presentation by HSEC

As well as being equipped with scavenger receptors that facilitate efficient uptake of viruses and potential antigens, HSEC also have the ability to phagocytose particles and to present antigen to lymphocytes(reviewed in [92-94]). There is evidence that such interactions are important for generating immunological tolerance to gut-derived antigens although recent work suggests that local antigen presentation cannot explain liver tolerance and that, on the contrary, the liver may be an excellent priming site for naive CD8+ T cells[95]. Antigen presentation is facilitated by the expression of MHC class I and Ⅱ[96,97] molecules together with co-stimulatory molecules such as CD40 and more contentiously CD80 and CD86[98]. These receptors are upregulated on HSEC in fulminant liver failure[99] and may contribute to disease pathogenesis by allowing ongoing presentation of stimulatory antigen. HSEC also express the mannose receptor (Figure 1), a 175 kDa transmembrane glycosylated protein involved in uptake of Ag by both DCs and HSEC[97]. Competitive inhibition of this receptor by mannan reduces antigen-specific T cell activation by murine HSEC[97].

Similarities between lymphatic endothelial cells and sinusoidal endothelium

Both the liver and pancreas develop from buds of the embryonic endoderm[100], however, the vasculature components of the liver have distinct origins. The portal vessels are derived from viteine veins whereas the sinusoids develop from the capillary vessels of the septum transversum and acquire their distinctive fenestrated phenotype by wk 20 of gestation (reviewed in[21]). From this point onward, sinusoidal endothelial cells remain functionally and phenotypically distinct from the other vascular endothelial cells in the liver and express several receptors that are otherwise confined to lymphatic endothelial cells that are derived from buds from the cardinal vein. Hence, both lymphatic and sinusoidal endothelial cells have minimal basement membranes, loosely organised cell junctions and constitutively express LYVE-1 and VAP-1 (SSAO/AOC3) but lack CD34 (reviewed in[101]). VAP-1 is a type Ⅱ transmembrane protein that can support leukocyte adhesion *via* interactions with sialic acid rich side chains. It is also an amine oxidase and enzyme activity is also involved in regulating leukocyte adhesion and transmigration[101]. VAP-1 is expressed on all vascular compartments within the liver (Figures 1 and 2) where it supports the adhesion and transmigration of leukocytes[10,102]. The only extrahepatic site where VAP-1 is constitutively expressed at high levels is endothelial cells in high endothelial venules within lymph nodes[103] where again it is proposed to have a role directing the adhesion of lymphocyte populations[104]. Similarly both lymphatic endothelium and HSEC express the Reeler gene product Reelin[105]. Reelin is a secreted glycoprotein with roles in embryonic development and organisation. Expression is restricted during embryogenesis but in the adult organism high levels are detected on lymphatic endothelial cells and within the sinusoids localised either to stellate cells[106] or HSEC[105]. This has led to the hypothesis that reelin may be involved in the regulation of lymphoangiogenesis or regulation of lymphatic endothelial phenotype and thus may have similar roles within the liver sinusoids.

Thus there are many similarities between HSEC and lymphatic endothelial cells and some antigens originally defined on lymphatic endothelium can also be used to differentiate between HSEC and vascular endothelial cells in the liver. It is possible to exclude contamination with lymphatic EC in HSEC cultures on the basis that LYVE-1 positive HSEC do not express PROX -1, a transcription factor found exclusively in lymphatic EC[107].

CONCLUSIONS

Cultures of endothelial cells are valuable tools to investigate mechanisms of liver physiology and pathophysiology *in vitro*. However, the study of endothelial cells *in vitro* is complicated by the marked heterogeneity of endothelial cells between and within different organs and the tendency for cells to lose tissue-specific markers

when cultured *in vitro*. Although all endothelial cells share some characteristic features (as described in the first part of this review), there is a need for specific markers or combinations of markers that define distinct populations of endothelial cells. To date, the study of HSEC *in vitro* has been hampered by the lack of specific markers that can conclusively identify these cells and discriminate them from vascular or lymphatic endothelial cells. To some extent this remains the case, since many classical endothelial markers are widely expressed (Table 1) and there is considerable reported variability in detection of phenotypic markers between animal and human systems (eg CD31 and vWF). To date there is no known single molecule that is only expressed on hepatic sinusoidal and no other type of endothelia. However, the increasing knowledge of endothelial receptors is providing us with a larger and better defined set of phenotypic makers. In addition to their use in phenotyping or sorting/selecting specific endothelial cells for culture, receptors that show tissue-specific expression provide clues to specific functions of the cells being studied. Examples of this are the large number of scavenger receptors expressed by HSEC. Cultured HSEC do exhibit some useful identifying features, however. Very low passage cells retain fenestrations *in vitro* for a short time but these rapidly disappear within a passage or two in culture[8,16], as does expression of VAP-1[10]. Apart from these changes, however, the cells remain relatively phenotypically stable for 7-8 passages[8] and can be identified by expression of CD31, LYVE-1, DC-SIGNR(L-SIGN), Stabilin-1 and lack of CD34 and PROX-1[107]. These markers confirm endothelial identity, while excluding vascular and lymphatic endothelial contamination and in conjunction with markers to exclude cells of leukocyte origin can be used to confirm the sinusoidal nature of the cells.

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