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Liver Sinusoidal Endothelial Fenestrations in Caveolin-1 Knockout Mice

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Abstract

Objective—Fenestrations are pores in the liver sinusoidal endothelium that facilitate the transfer of particulate substrates between the sinusoidal lumen and hepatocytes. Fenestrations express caveolin-1 and have structural similarities to caveolae, therefore might be a form of caveolae and caveolin-1 may be integral to fenestration structure and function. Therefore, fenestrations were studied in the livers of caveolin-1 knockout mice.

Methods—Scanning, transmission and immunogold electron microscopic techniques were used to study the liver sinusoidal endothelium and other tissues in caveolin-1 knockout and wild-type mice.

Results—Comparison of fenestrations in wild-type and knockout mice did not reveal any differences on either scanning or transmission electron microscopy. The diameter of the fenestrations was not significantly different (74 ± 13 nm knockout mice vs 78 ± 12 nm wild-type mice) nor was the fenestration porosity (6.5 ± 2.1 knockout vs $7.3 \pm 2.4\%$ wild-type mice). In contrast, adipocytes and blood vessels in other tissues lacked caveolae in the knockout mice. Caveolin-1 immunogold of livers of wild-type mice indicated sparse expression in sinusoidal endothelial cells.

Conclusions—The normal structure of fenestrations in the liver sinusoidal endothelium is not dependent upon caveolin-1 and fenestrations are not a form of caveolae.

Keywords

fenestrations; fenestrae; sinusoidal endothelial cell; liver; hepatic; caveolin-1; knockout mouse; electron microscopy; immunogold

INTRODUCTION

Liver sinusoidal endothelial cells (LSEC) are highly specialized endothelial cells that line the wall of the hepatic sinusoid. The cytoplasmic extensions of LSECs are very thin and perforated with fenestrations, which are circular and oval pores approximately 50–200 nm in diameter. Fenestrations are complete gaps in the endothelium, lacking either a diaphragm or underlying basal lamina. There are approximately 3–20 fenestrations per μm^2 of endothelial surface and between 2 and 20% of the surface of the LSEC is covered by fenestrations [1,5,19,31]. Sometimes dumb-bell shaped fenestrations are seen occurring as a result of fusion of two adjacent fenestrations. Fenestrations are also found in meshlike structures similar in appearance to vesiculo-vacuolar organelles [8] and pored domes [23]. Fenestrations can be either scattered individually across the endothelial surface or clustered into groups of tens to hundreds called liver sieve plates. Between 60 and 75% of fenestrations are found within sieve plates in rats [29]. There are dozens of sieve plates present in the cytoplasmic extension of a single cell, representing many hundreds or even thousands of fenestrations per cell [6,12]. Fenestrations may not be single and independent pores but instead interconnected labyrinthine structures, which have been seen on transmission electron microscopy of thicker cell sections and isolated cells [36].

Understanding of the mechanisms for the regulation and formation of fenestrations has remained elusive. The greatest evidence currently available for agents that influence fenestrations is for vascular endothelial growth factor (VEGF) and actin disruptors, both which increase fenestrations and the porosity of the LSEC. However, various other agents have been found to influence the number and diameter of fenestrations including calcium, serotonin, ethanol, prostaglandins and endothelin-1 [1,5,13]. It is known that fenestrations and their sieve plates are supported by the F-actin cytoskeleton. A number of studies have attempted to identify proteins surrounding fenestrations or in the cell membranes that line the walls of the fenestrations. Immunogold electron microscopy has been used to identify caveolin-1, endothelial nitric oxide synthase and calcium ATPase in or around fenestrations of LSECs [22,32,34,35]. Caveolin-1 expression has also been found in fenestrations of SK Hep1 cells which are a liver tumor cell line with many characteristics of endothelial cells [4]. On the basis of the expression of caveolin-1 in fenestrations and the similarity in their dimensions and appearances, it has been proposed that fenestrations are in fact a form of caveolae or that caveolin-1 is important for the structure of fenestrations [21,25,32,35].

Caveolae are flask-shaped invaginations in cell membranes that measure about 50–100 nm in diameter [7,11,24]. Amongst the increasingly recognized number of physiological roles of caveolae, they are responsible for transcytotic transport of solutes and across the endothelium. Caveolin-1 is a fundamental component of caveolae and is generally considered to be a marker for caveolae. Caveolin-1 knockout mice lack caveolae which proves the critical role that caveolin-1 has in the formation of caveolae. Although these knockout mice are viable and fertile they do have a number of phenotypic changes including dyslipidemia, cardiac and pulmonary disease, impaired angiogenic responses and deregulation of the NO pathways [7,11,18].

In this study, to determine the role of caveolin-1 in the formation of fenestrations and whether fenestrations are a form of caveolae, the ultrastructure of fenestrations and caveolae were studied in caveolin-1 knockout mice. In addition, to confirm the distribution of caveolin-1 in fenestrations, LSECs from intact livers were investigated using immunogold electron microscopy.

METHODS

Animals and Preparation of Liver Samples

Young C57BL/6 mice were purchased from the Animal Resources Centre, Perth, WA, and Cav-1^{-/-} mice were acquired from the Institute for Molecular Bioscience, University of Queensland as a kind gift (Professor Rob Parton) [9]. The presence of the knockout genotype was confirmed by PCR of toenail clippings and the phenotype was confirmed by transmission electron microscopy of capillaries and adipocytes. Eight wild-type and 8 Cav-1^{-/-} mice (4 males and 4 females) age 9–10 months were used for this study. After anesthesia, livers were perfusion-fixed via a 25 G needle inserted into the portal vein. Tissue was fixed with 3% glutaraldehyde /2% paraformaldehyde in 0.1 M sodium cacodylate buffer (0.1 M sucrose, 2 mM CaCl₂). The study was approved by the Sydney South West Area Health Welfare Committee.

Transmission Electron Microscopy and Immunogold

Fixed tissue was automatically processed and embedded in Spurr's resin. Two blocks per liver were qualitatively studied and for each animal. Tissue for immunogold was fixed with 4% freshly prepared paraformaldehyde in PBS (0.1% sucrose) and infiltrated in 2.3 M sucrose at 4°C. Small liver fragments were mounted on metal pins and frozen in liquid nitrogen. Cryosections were obtained and immunogold labeling was performed on ultra-thin sections collected on carbonized formvar-coated nickel grids. Grids were incubated with 1% cold fish skin gelatin in PBS and then with primary antibody caveolin-1 (N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) [1:10] 2 hours at room temperature. After extensive washes with PBS, grids were incubated with protein-A gold-6 nm [1:10] for 15 min. Grids were washed as above and contrasted with 1% uranyl acetate in methylcellulose and viewed with a JEOL JEM-1010 transmission electron microscope (Frenchs Forest, NSW, Australia).

Scanning Electron Microscopy

Perfusion-fixed livers were post-fixed with osmium, dehydrated in ethanol gradients and incubated for 5 min in hexamethyl-disilazane (Sigma, St Louis, MO, USA). Tissue was mounted on stubs, gold sputter-coated and observed with a JEOL JSM-6380LV scanning electron microscope. Between 6 and 10 micrographs from each liver were taken randomly and studied under the same conditions (magnification $\times 20000$, working distance 10 mm and 15 kV). For each photograph, a representative area was chosen for fenestration diameter measurements. Approximately 1000 fenestrations were analyzed for each group of mice using ImageJ software (<http://rsb.info.nih.gov/ij/>). When fenestrations appeared elliptical the maximum diameter was measured. Porosity, which represents the percentage of the surface area of sinusoidal endothelium perforated with fenestrations, was calculated. In wild-type

mice, 47 micrographs were analyzed from eight livers and in the knockout mice, 39 micrographs were analyzed from eight mice.

Statistics

Results are presented as mean $\pm$ SD. Comparisons were performed using Student's *t*-test and ANOVA and considered significant when $P < 0.05$.

RESULTS

Caveolae Ultrastructure in Other Tissues

Figure 1 shows the presence of numerous caveolae in the adipose tissue and the blood vessels of lung and heart tissue of wild mice while there is an almost complete absence of caveolae in the knockout mouse tissues.

Fenestrations in the LSECs

Figure 2 shows transmission and scanning electron micrographs of the LSEC in wild-type and caveolin-1 knockout mice. There were no obvious differences in the appearances of the fenestrations in terms of size, frequency, shape or their clustering into liver sieve plates.

The diameter, frequency and porosity of the fenestrations determined by scanning electron microscopy were quantified. There were no statistically significant differences in fenestration diameter (74 ± 13 knockout mice vs 78 ± 12 nm wild-type mice), fenestration frequency (13.4 ± 4.4 knockout vs 13.5 ± 3.6 fenestrations/ μm^2 wild-type mice) or total porosity (6.5 ± 2.1 knockout vs $7.3 \pm 2.4\%$ wild-type mice). Gender had no influence on these results. The fenestration diameter frequencies are compared in Figure 3.

Caveolin-1 Immunogold Electron Microscopy

Figure 4 shows the results of immunogold electron microscopy for caveolin-1 in the livers harvested from wild-type mice. Caveolin-1 was rarely expressed in LSECs (Figure 4A) and many, if not all, fenestrations did not demonstrate caveolin-1 expression. Caveolin-1 was also seen in caveolae of other tissue (4B).

DISCUSSION

Fenestrations in LSECs are of physiological significance because they permit the transfer of substrates between the sinusoidal blood and hepatocytes. This includes substrates in solution via ultrafiltration as well as particulate substrates such as lipoproteins and albumin-bound ligands which appear to directly traverse the fenestration lumen [5,16,17]. When fenestrations are decreased, such as in some liver diseases and old age, there is impaired hepatic disposition and clearance of such substrates [14,17,28]. In addition, fenestrations are involved in the process of liver-induced immunotolerance by providing a portal for communication between T cells and hepatocytes [30]. Despite the importance of fenestrations, little is known about their structure and regulation, apart from the role of the cytoskeleton. Liver sieve plates, and possibly fenestrations themselves are surrounded by F-actin cytoskeleton. Various actin disruptors have potent effects on the structure of LSECs

with an increase in fenestration size and frequency associated with a loss of liver sieve plates [2,3,27].

Recent studies have identified a number of proteins present in the cell membranes lining the fenestrations. It has been shown that fenestrations are associated with caveolin-1, endothelial nitric oxide and calcium ATPases [22,32,33,35]. It is well established using immunohistochemistry that caveolin-1 is expressed in the perisinusoidal cells [15,22,32]. With regards to fenestrations, the presence of caveolin-1 was detected by immunogold in labyrinthine fenestral structures seen on tangential sections of liver tissue and in similar structures in isolated LSECs [32]. In isolated rat LSECs, caveolin-1 was detected by immunogold in labyrinthine structures consistent with fenestrations [22,35]. In SK Hep1 cells, which have some characteristics of an immortal LSEC cell line, caveolin-1 was also found in fenestrations [4]. Furthermore, caveolae and fenestrations have similar dimensions, both have lumens approximately 100 nm in diameter. Together such findings suggest that caveolin-1 may have a role in the morphology of fenestrations and, as has been suggested previously, that fenestrations may be a form of caveolae [20,22,32,33,35].

To determine whether caveolin-1 is required for the formation of fenestrations, we studied caveolin-1 knockout mice [9]. As well described elsewhere [7,18], we noted a marked reduction of caveolae in the adipocytes and blood vessels in lungs and heart. However, fenestrations in the LSECs appeared to be quite normal both on scanning and electron microscopy. Quantification of the scanning electron microscopy confirmed that there was not any statistically significant effect of the knockout of the caveolin-1 gene on the diameter, frequency or overall porosity of the fenestrations. It is of interest that the morphology of glomerular endothelial cell fenestrations were also unaffected by knockout of the caveolin-1 gene [26]. However, unlike LSEC fenestrations, caveolin-1 was completely absent from the fenestrated sections of the glomerular endothelium in wild-type mice [26]. Previously we have shown using immunohistochemistry that caveolin-1 is expressed in LSECs [15]. In this current transmission electron microscopic study of intact mouse liver tissue, immunogold staining for caveolin-1 revealed some expression in LSECs although we were unable to confirm the presence of caveolin-1 in fenestrations. Yokomori et al. [34] found some expression of caveolin-1 in the membranes around fenestrations in isolated LSECs, but most of the expression was cytoplasmic, especially after stimulation of LSECs with VEGF. It is also important to note that Frank et al. [10] found that LDL uptake by the liver was unexpectedly increased in caveolin-1 knockout mice. Overall, it can be concluded that caveolin-1 is not required for the formation of fenestrations including those in the LSEC. The question then arises as to the role of caveolin-1 in LSECs. A recent study suggests that caveolin-1 might have an important role in LSEC proliferation, for example after partial hepatectomy [35].

In conclusion, the cell membranes around fenestrations of LSECs show some expression of caveolin-1. However, knockout of the caveolin-1 gene was not associated with any change in the structure of fenestrations, despite ablation of caveolae in other tissues. Therefore, fenestrations are not a form of caveolae and do not require caveolin-1 for normal morphology.

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Figure 1. Transmission electron micrographs of adipose tissue (**A**, **B**), and capillaries in lung (**C**, **D**) and heart (**E**, **F**) of wild-type mouse (left panel, **A**, **C**, **E**) and caveolin-1 knockout mouse (right panel, **B**, **D**, **F**). Arrows show presence of numerous caveolae in the wild-type mouse tissues.

Figure 2. Transmission electron micrographs of liver sinusoidal endothelial cell in wild-type mouse (A) and caveolin-1 knockout mouse (B). Scanning electron micrographs of the luminal surface of the liver sinusoidal endothelial cell in wild-type mouse (C) and caveolin-1 knockout mouse (D). Arrows show fenestrations clustered in liver sieve plates.

Figure 3.
Fenestration diameter frequency of wild-type and caveolin-1 knockout mice.

Figure 4. Immunogold electron micrographs for caveolin-1 in a liver sinusoidal endothelial cell (A) and fat tissue (B) from a wild-type mice.