Rat liver sinusoidal endothelial cell phenotype is maintained by paracrine and autocrine regulation

Laurie D. DeLeve, Xiandong Wang, Liping Hu, Margaret K. McCuskey, and Robert S. McCuskey. Rat liver sinusoidal endothelial cell phenotype is maintained by paracrine and autocrine regulation. Am J Physiol Gastrointest Liver Physiol 287: G757–G763, 2004. First published June 10, 2004; 10.1152/ajpgi.00017.2004.—The phenotypic features of liver sinusoidal endothelial cells (SEC), open fenestrae in sieve plates and lack of a basement membrane, are lost with capillarization. The current study examines localization of CD31 as a marker for the dedifferentiated, nonfenestrated SEC and examines regulation of SEC phenotype in vitro. CD31 localization in SEC was examined by confocal microscopy and immunogold-scanning electron microscopy. SEC cultured for 1 day express CD31 in the cytoplasm, whereas after 3 days, CD31 is also expressed on cell-cell junctions. Immunogold-scanning electron microscopy confirmed the absence of CD31 surface expression on fenestrated SEC 1 day after isolation and demonstrated the appearance of CD31 surface expression on SEC that had lost fenestration after 3 days in culture. SEC isolated from fibrotic liver do show increased expression of CD31 on the cell surface. Coculture with either hepatocytes or stellate cells prevents CD31 surface expression, and this effect does not require heterotypic contact. The paracrine effect of hepatocytes or stellate cells on SEC phenotype is abolished with anti-VEGF antibody and is reproduced by addition of VEGF to SEC cultured alone. VEGF stimulates SEC production of nitric oxide. Nω-nitro-L-arginine methyl ester blocked the paracrine effect of hepatocytes or stellate cells on SEC phenotype and blocked the ability of VEGF to preserve the phenotype of SEC cultured alone. In conclusion, surface expression of CD31 is a marker of a dedifferentiated, nonfenestrated SEC. The VEGF-mediated paracrine effect of hepatocytes or stellate cells on maintenance of SEC phenotype requires autocrine production of nitric oxide by SEC.

hepatocytes; paracrine communication; endothelial cells; vascular endothelial growth factor; nitric oxide

THE LIVER SINUSOIDAL ENDOTHELIAL CELL (SEC) has a unique phenotype that is well integrated into the special needs of the liver. The open fenestrae and lack of an organized basement membrane enhance hepatocyte exposure to circulating blood. This freer access to blood permits greater oxygenation of hepatocytes and more efficient clearance of drugs and perhaps also of chylomicron remnants. Cross-talk between SEC and hepatocytes may also be critical for recovery of hepatocytes from toxic injury (26).

Capillarization is a change in the phenotype of the SEC to a vascular phenotype with loss of fenestration and formation of an organized basement membrane. Capillarization is a dedifferentiation process that can be reproduced with experimental interventions in vivo (5, 13) and in vitro in SEC (9, 58) but that also occurs in vitro over time with SEC in culture. Capillarization precedes the onset of alcoholic liver disease in humans (21, 22, 47) and mice (42) and has been seen in various rat models of fibrosis and cirrhosis (7, 13, 29, 34). Aging is accompanied by a change in SEC phenotype with loss of fenestration but with less extensive changes in the basement membrane. Given the limited changes in the basement membrane, this change in SEC phenotype has been referred to as pseudocapillarization (23, 30).

Determinants of endothelial cell phenotype include heterotypic contact with pericytes or smooth muscle cells, paracrine effects of epithelial cells, shear stress, and the underlying substratum. Little is known about the determinants of normal SEC phenotype. Proximity to liver tissue can induce the SEC phenotype (31), but the pathways that regulate this have not been established. One of the limitations to performing studies of SEC phenotype has been the lack of a practical marker for the normal, differentiated phenotype. The presence of open fenestration organized in sieve plates and demonstrated by scanning electron microscopy is the only gold standard, but it is not easily amenable to quantitative study.

A defining feature of endothelial cells is the presence and type of adhesion molecules. CD31, or platelet endothelial cell adhesion molecule, is an adhesion molecule that is present on the cell-cell junctions of most endothelial cells and that facilitates leukocyte transmigration. Immunohistochemical studies have demonstrated that differentiated SEC in situ do not express CD31 (48, 49) but that CD31 becomes detectable on SEC in cirrhosis and in focal nodular hyperplasia (12, 50), presumably in areas with capillarization. In contrast, more recent studies (28, 35) have detected CD31 on normal SEC in situ. A recent paper was able to culture human SEC over time and maintain some degree of fenestration and an absence of basement membrane. In these fenestrated SEC, CD31 could only be demonstrated by flow cytometry after permeabilization, suggesting an intracellular localization (58). If this is indeed the case, then the discrepancy in previous studies as to whether CD31 is expressed in normal SEC might be related to differences in permeabilization techniques. SEC are small, flat cells, and the localization of CD31 would not be appreciated on light microscopy of SEC in situ. The current study establishes CD31 localization as a marker of SEC phenotype and uses this marker to examine paracrine and autocrine regulation of the SEC phenotype.
FITC conjugate (1:40). For von Willebrand factor, slides with a goat polyclonal anti-CD31 (1:10) and a rabbit anti-goat IgG新三板 cultured on coverslips were

was plated at a density of 105,000 and 130,000 cells/cm², respectively. For experiments using Transwell inserts in a well of a collagen-coated 24-well plate with or without hepatocytes added, SEC were plated at a density of 105,000 and 130,000 cells/cm², respectively. For experiments in which cells were plated together within the same well, cells were plated at half normal density: SEC plated at a density of 200,000 cells/cm², and stellate cells at a density of 65,000 cells/cm².

CD31, von Willebrand factor, and confocal microscopy. For CD31, SEC cultured on coverslips were fixed in cold methanol and stained with a goat polyclonal anti-CD31 (1:10) and a rabbit anti-goat IgG FITC conjugate (1:40). For von Willebrand factor, slides fixed as above were stained with a rabbit polyclonal anti-human anti-von Willebrand factor (1:100) and a mouse anti-rabbit-Texas red (1:100). Slides were examined using a Nikon PCM-2000 confocal microscope with a 543-nm laser excitation wavelength. Each condition was examined in triplicate, and values were obtained by counting the number of cell-cell interfaces positive for CD31 or von Willebrand factor in 15 randomly selected fields; in the studies comparing both CD31 and von Willebrand factor-positive cells were counted in the same fields. Because both cells need to express CD31 for it to be expressed on cell-cell borders (1), positive expression of CD31 was counted as two cells.

CD31 and immunogold-scanning electron microscopy. Routine methods were used to stain cultured SEC for the surface expression of CD31 with immunogold and then process the cells for examination by scanning electron microscopy. The cells were washed briefly with PBS and then fixed for 15 min in 3% paraformaldehyde at room temperature. After being washed 10 times with PBS, they were permeabilized for <1 min with 0.1% Triton X-100 in PBS and washed again 10 times with PBS. The cells were treated with 0.05 M glycine in PBS for 15 min to inactivate residual aldehyde groups followed by treatment for 30 min with 0.1% gelatine, 5% normal donkey serum, and then a 5-min wash with PBS. Primary antibody
diluted 1:10 in PBS was applied for 2 h at room temperature followed by three 10-min washes in PBS. Then the secondary antibody, 12-nm colloidal gold-Af
nimmune conjugate donkey anti-goat IgG diluted 1:20 in PBS, was applied for 2 h at room temperature followed by ten 5-min washes in PBS. Cells treated as above but with the omission of incubation with the primary antibody served as negative controls. All cells were postfixed in 2% glutaraldehyde in PBS for 2 h before being washed with PBS, dehydrated, critical-point-dried, and coated with carbon. The preparations were then examined using a Phillips XL30 scanning electron microscope (Eindhoven, The Netherlands) equipped with both secondary emission and backscatter detectors.

Nitrite plus nitrate measurements. Nitric oxide (NO) was determined as the sum of nitrite plus nitrate concentrations. Escherichia coli nitrate reductase and NADPH were used to convert nitrate to nitrite in culture medium, and nitrite was quantified according to the Griess reaction (17). One hundred microliter of culture supernatant are mixed with equal volumes of 0.1% N-1-naphthylethanamine hydrochloride and 1% sulfanilamide in 5% H₃PO₄. After 5 min at room temperature, absorbance is measured at 540 nm on a microplate reader (Model 3550; Bio-Rad, Hercules, CA).

In vivo studies. Male Sprague-Dawley rats (250–280 g body wt) were treated with thioacetamide 200 mg/kg ip three times weekly for 6 wk. Experiments were performed in adherence with the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (revised 1985) prepared by the National Academy of Sciences. The experiments followed protocols approved by the Animal Care and Use Committee of the University of Southern California.

Protein assay. Cellular protein was measured by placing a 10-μl aliquot in a 96-well microplate and adding 150 μl of diluted dye reagent (1:5 dilution, Bio-Rad protein assay). After a 15-min incubation on a rotary shaker, absorbance was measured at 595 nm on a Bio-Rad model 3550 microplate reader (Bio-Rad). This method allows rapid quantitation of protein in small aliquots (6).

Statistical analysis. All data that were expressed as means ± SE were from at least three separate experiments. Groups were compared by ANOVA or by Student’s t-test using the Microsoft Excel Analysis ToolPak (Microsoft, Redmond, WA). P < 0.05 was considered significant.

RESULTS

CD31 localization. In vascular endothelial cells, CD31 is expressed on the cell-cell junctions, but it has been unclear whether that is the case for SEC. CD31 expression was examined by confocal microscopy and by immunogold-scanning electron microscopy in SEC that had been kept in homotypic culture for 1 or 3 days (Fig. 1). Localization by confocal microscopy demonstrated CD31 expression in the cytoplasm of SEC in homotypic culture (i.e., culture with a single cell type) for 1 day with 0–1% expression of CD31 on cell-cell junctions. In contrast, in SEC that differentiated after 3 days in homotypic culture, CD31 was expressed on cell-cell junctions of ~20% of cells. Scanning electron microscopy allows careful examination of the cell surface as well as visualization of fenestration. SEC cultured for 1 day exhibited fenestrae in a typical range of sizes (8, 10, 57) organized in characteristic sieve plates and little or no expression of colloidal gold labeling of CD31 on the cell surface (Fig. 2A). In contrast, SEC cultured for 3 days had lost most of their fenestrae and sieve plates and had extensive expression of colloidal gold-labeled CD31 on the cell surface (Fig. 2B). Cell-surface expression of CD31 will be referred to as sCD31. Cells not treated with the
primary antibody exhibited little or no labeling with colloidal gold (Fig. 2C).

Von Willebrand factor is not expressed in normal rat SEC (14, 27) but is expressed in capillarization (33, 34, 55). Figure 3 demonstrates that from days 1 to 3, there is a 4.5- to 5-fold increase in the number of SEC that express sCD31 and von Willebrand factor.

To rule out the possibility that sCD31 was lost by cell isolation, SEC were isolated from the rat thioacetamide model, a fibrotic liver disease model in which SEC lose fenestrae and express von Willebrand factor (33, 34). Isolation of SEC from fibrotic liver requires more extensive digestion by collagenase perfusion, so that the presence of sCD31 despite more extensive digestion would rule out artefactual loss of sCD31 after the less extensive digestion of normal livers. SEC (25.8 ± 1.8%) from rats treated with 6 wk of thioacetamide showed sCD31 expression (n = 3), indicating that sCD31 is increased in capillarized SEC compared with normal liver SEC and that it can be detected on the cell surface of isolated cells despite extensive digestion.

Paracrine regulation of SEC phenotype. SEC were cultured until day 3 in 5 models (Fig. 4): SEC in homotypic culture, SEC in coculture with hepatocytes plated in Transwell inserts, SEC in coculture with stellate cells plated in Transwell inserts, SEC mixed in a well with stellate cells (allowing heterotypic contact), and SEC in coculture with both stellate cells and hepatocytes. In the last model, SEC were mixed in a well with stellate cells and hepatocytes were plated in Transwell inserts. Comparison of coculture with cells in Transwell inserts vs. coculture with heterotypic contact should reveal whether a paracrine effect is mediated by soluble mediators alone or

![Fig. 1. Confocal microscopy images of CD31 in sinusoidal epithelial cells (SEC) in culture. In A, CD31 was present in the cytoplasm but not on cell-cell borders of SEC in culture for 1 day. In B, CD31 was expressed on the cell-cell borders of SEC in culture for 3 days. Arrows indicate CD31 expression.](image1)

![Fig. 2. Immunogold-scanning electron microscopy. SEC cultured for 1 day exhibited fenestrae organized in characteristic sieve plates and little or no expression of colloidal gold labeling of CD31 on the cell surface (A). In contrast, SEC cultured for 3 days lost most of their fenestrae and sieve plates and had extensive expression of colloidal gold-labeled CD31 on the cell surface (B). SEC cultured for 3 days and not treated with the primary antibody exhibited little or no labeling with colloidal gold (C). Note 5-μm size bar.](image2)
whether the paracrine effect requires heterotypic cell contact. The presence of either hepatocytes or stellate cells markedly decreased sCD31 staining on SEC compared with SEC in homotypic culture (Fig. 4). SEC sCD31 expression was comparable in coculture with either hepatocytes or stellate cells. Cell-cell contact with stellate cells (heterotypic contact) or coculture with both hepatocytes and stellate cells was not superior to coculture with either stellate cells or hepatocytes. This suggests that the paracrine effect on SEC phenotype occurs through release of a soluble factor and that either stellate cells or hepatocytes can exert this paracrine effect. Furthermore, the presence of the soluble factor(s) from either cell type is sufficient to suppress SEC sCD31 expression.

**VEGF effect.** Two approaches were used to determine whether VEGF plays a role in the paracrine regulation of SEC phenotype. The effect of neutralizing anti-VEGF antibody was examined on sCD31 expression on SEC in homotypic culture, in coculture with hepatocytes or stellate cells (Table 1). Anti-VEGF antibody abolished the paracrine effect of hepatocytes or stellate cells, whereas an isotype-matched control antibody had no effect. To examine whether supplementation of SEC culture medium could reproduce the effect of coculture, SEC were incubated with 40 ng/ml VEGF for 0, 4, 8, 24, 48, or 72 h. SEC were stained for CD31 on day 3. Incubation with VEGF for 48 or 72 h suppressed sCD31 expression comparably with the effect of coculture with either hepatocytes or stellate cells (Fig. 5).

**Autocrine regulation by NO.** Nitrite plus nitrate in the culture medium were increased from 811 ± 1/11006 30 nmol/mg protein in controls to 1,024 ± 1/11006 34 nmol/mg protein in SEC exposed for 16 h to 40 ng/ml VEGF, which was a 26% increase in nitrite plus nitrate (n = 4; P < 0.005 by ANOVA). Thus, as in other endothelial cells, VEGF stimulates SEC NO production. N\textsuperscript{G}-nitro-\textit{l}-arginine methyl ester (\textit{l}-NAME; 3 mM), an NO synthase inhibitor, was used to determine whether SEC NO production was a downstream mediator of the VEGF-mediated effect on SEC phenotype. Figure 6 demonstrates that the addition of VEGF to SEC in homotypic culture or coculture of SEC with hepatocytes or stellate cells suppresses sCD31 expression. The addition of \textit{l}-NAME blocks the effect of VEGF or of coculture with hepatocytes or stellate cells on suppression of sCD31 expression. This indicates that the paracrine effect induced by VEGF is mediated by NO production by SEC, i.e., downstream autocrine regulation.

![Fig. 3. Comparison of surface expression of CD31 (sCD31) and von Willebrand factor expression. The percentage of SEC that expressed sCD31 and von Willebrand factor (WF) was examined on days 1 and 3 (n = 3). The percentage of SEC that expressed sCD31 increased 4.5-fold from days 1 to 3, and von Willebrand factor expression increased 5-fold.](image1)

![Fig. 4. Paracrine regulation of sCD31 after 3 days in culture. Surface CD31 is suppressed by coculture with hepatocytes or stellate cells in Transwell inserts. Mixed coculture (i.e., with heterotypic contact) with stellate cells in the presence or absence of hepatocytes in Transwell inserts is not superior to coculture with either hepatocytes or stellate cells in Transwell inserts. P < 0.0001 by ANOVA for SEC in homotypic culture vs. coculture with either hepatocytes or stellate cells (n = 8).](image2)

![Fig. 5. VEGF effect on SEC surface expression of CD31. SEC were incubated with 40 ng/ml VEGF for 0, 4, 8, 24, 48, or 72 h. Cells were examined by confocal microscopy on day 3 for surface expression of CD31.](image3)

Table 1. Anti-VEGF antibody effect on percentage of SEC with sCD31 expression

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<tr>
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<th>No Antibody</th>
<th>Anti-VEGF antibody</th>
<th>Nonspecific isotype-matched antibody</th>
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<tr>
<td>SEC alone</td>
<td>22.1 ± 0.5</td>
<td>22.3 ± 1.5</td>
<td></td>
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<tr>
<td>SEC/hepatocyte coculture</td>
<td>5.8 ± 0.2</td>
<td>19.5 ± 1.9*</td>
<td>5.5 ± 0.3</td>
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<tr>
<td>SEC/stellate cell coculture</td>
<td>5.3 ± 0.3</td>
<td>20.4 ± 0.9*</td>
<td>5.6 ± 0.3</td>
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Values are means ± SE (n = 3). Data given as percentage of sinusoidal endothelial cells (SEC) positive for cell-surface expression of CD31 (sCD31). * P < 0.0001 with vs. without anti-VEGF antibody for SEC/hepatocyte or SEC/stellate cell co-culture by ANOVA.
The current study describes localization of CD31 as a measure of SEC phenotype and, more specifically, surface expression of CD31 (sCD31) as a marker of SEC dedifferentiation. SEC that lose fenestration in culture, SEC that become positive for von Willebrand factor in culture, and SEC isolated after long periods of culture with hepatocytes or stellate cells. Inhibition of nitric oxide synthase by 3 mM L-NAME (filled bars) prevented VEGF-induced suppression of sCD31 in SEC in homotypic culture. L-NAME also prevented the paracrine effect of hepatocytes and stellate cells on suppression of sCD31. P < 0.0001 by ANOVA with vs. without L-NAME for SEC/VEGF, SEC/hepatocytes, and SEC/stellate cells (n = 3).

**DISCUSSION**

The current study describes localization of CD31 as a measure of SEC phenotype and, more specifically, surface expression of CD31 (sCD31) as a marker of SEC dedifferentiation. SEC that lose fenestration in culture, SEC that become positive for von Willebrand factor in culture, and SEC isolated from fibrotic liver have increased expression of CD31 on cell-cell borders, whereas fenestrated SEC express CD31 in the cytoplasm but not on the cell surface. These findings are consistent with a flow cytometry study (58) that demonstrated that antibodies to human SEC that induce capillarization shift localization of intracellularly expressed CD31 to the cell surface. This study also demonstrated that mRNA for CD31 was higher in capillarized human SEC and human umbilical vein endothelial cells than in cultured, fenestrated human SEC. Vascular endothelial cells express CD31 on the cell-cell surface in a fashion similar to that seen among the dedifferentiated SEC, further supporting the concept that this relocalization of CD31 is a manifestation of dedifferentiation. CD31 expression on cell-cell borders plays a key role in the acute transendothelial migration of leukocytes (46), suggesting that leukocyte recruitment may differ in the normal and capillarized liver.

Because the goal of the studies was to examine paracrine factors that regulate SEC phenotype, cells were cultured without exogenous growth factors. In the absence of growth factors, SEC morphology deteriorates, and viable cell number declines after day 3. The percentage of SEC that expressed sCD31 over time in culture remained relatively low in these studies, and this may be due to the limited duration of culture to avoid studying deteriorating cells. It seems likely that culture for longer than day 3 would yield a higher percentage of sCD31-positive cells.

The use of scanning electron microscopy as a quantitative measure of SEC differentiation is not feasible. With sCD31 as a marker of SEC dedifferentiation, it becomes much more practical to study changes in SEC phenotype. The coculture studies described here demonstrate that either hepatocytes or stellate cells can suppress sCD31 and maintain the phenotype of SEC. The effect of hepatocytes and stellate cells occurs through paracrine release of a soluble factor or factors, and heterotypic contact does not add to this effect. Either hepatocytes or stellate cells can exert this paracrine effect, and there is no added effect of having both hepatocytes and stellate cells present. VEGF is essential to the paracrine effect seen in the coculture model. VEGF stimulates SEC release of NO, which is then a downstream autocrine determinant of SEC phenotype. Thus both paracrine and autocrine pathways regulate the SEC phenotype. VEGF expression has been demonstrated in both hepatocytes and stellate cells and VEGF protein secretion has been confirmed in hepatocytes and in an immortalized hepatic stellate cell line (2, 11, 32, 52, 53).

These findings are consistent with other studies demonstrating paracrine regulation of the fenestrated endothelial cell type by VEGF. Application of VEGF induces diaphragmed fenestrae in endothelial cells from small venules and capillaries (40). VEGF is highly expressed by cells adjacent to fenestrated endothelium, such as astrocytes adjacent to the choroids plexus and podocytes adjacent to glomerular endothelial cells; VEGF receptor is highly expressed in these fenestrated endothelial cells (39).

The VEGF effect on SEC phenotype required SEC production of NO. It is well established that VEGF stimulates endothelial production of NO, and the current study confirms this in SEC. In vitro studies (3, 4, 37) have shown that NO promotes capillary organization, a manifestation of endothelial cell differentiation. Conversely, SEC in cirrhotic liver, which are presumably capillarized endothelial cells, produce less NO due to decreased endothelial NO synthase (18, 41, 51).

Regulation of SEC phenotype differs from the regulation of phenotype of some vascular endothelial cells by the lack of necessity for heterotypic contact to maintain the phenotype. Pericytes or smooth muscle cells maintain the phenotype of vascular endothelial cells through heterotypic contact (36, 38, 43–45). When vascular endothelial cells and smooth muscle are in contact with each other, plasmin localized on the endothelial cell surface activates latent TGF-β targeted to smooth muscle cells. In the current study, heterotypic contact with stellate cells was not superior to Transwell coculture, indicating that heterotypic contact was not necessary.

SEC phenotype is important for a number of reasons. Fenestration permits chylomicron remnant clearance; enhanced clearance of protein-bound drugs by hepatocytes, and heightenened hepatocyte oxygenation. Loss of fenestration and formation of a basement membrane may lead to reduced chylomicron remnant clearance and age-related atherosclerosis, decreased clearance of macromolecules and xenobiotics, and impaired oxidative drug metabolism (15, 16, 19, 20, 23–25, 29, 30, 57).

In summary, the current study identified sinusoidal endothelial cell-surface expression of CD31 as a marker of dedifferentiation that is inversely correlated with fenestration. Either hepatocytes or stellate cells can prevent sinusoidal endothelial cell dedifferentiation in vitro, as determined by surface expression of CD31, through a paracrine effect that requires VEGF secretion. The VEGF effect on sinusoidal endothelial cell phenotype requires autocrine production of NO by sinusoidal endothelial cells.

**GRANTS**

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REFERENCES


