Role of Differentiation of Liver Sinusoidal Endothelial Cells in Progression and Regression of Hepatic Fibrosis in Rats

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BACKGROUND & AIMS: Capillarization, characterized by loss of differentiation of liver sinusoidal endothelial cells (LSECs), precedes the onset of hepatic fibrosis. We investigated whether restoration of LSEC differentiation would normalize crosstalk with activated hepatic stellate cells (HSC) and thereby promote quiescence of HSC and regression of fibrosis. **METHODS:** Rat LSECs were cultured with inhibitors and/or agonists and examined by scanning electron microscopy for fenestrae in sieve plates. Cirrhosis was induced in rats using thioacetamide, followed by administration of BAY 60-2770, an activator of soluble guanylate cyclase (sGC). Fibrosis was assessed by Sirius red staining; expression of α-smooth muscle actin was measured by immunoblot analysis. **RESULTS:** Maintenance of LSEC differentiation requires vascular endothelial growth factor-A stimulation of nitric oxide–dependent signaling (via sGC and cyclic guanosine monophosphate) and nitric oxide–independent signaling. In rats with thioacetamide-induced cirrhosis, BAY 60-2770 accelerated the complete reversal of capillarization (restored differentiation of LSECs) without directly affecting activation of HSCs or fibrosis. Restoration of differentiation to LSECs led to quiescence of HSCs and regression of fibrosis in the absence of further exposure to BAY 60-2770. Activation of sGC with BAY 60-2770 prevented progression of cirrhosis, despite continued administration of thioacetamide. **CONCLUSIONS: The state of LSEC differentiation plays a pivotal role in HSC activation and the fibrotic process.**

Keywords: VEGF; Rat Model; Chronic Liver Disease; Fenestration.

BASIC AND TRANSLATIONAL LIVER

The liver sinusoidal endothelial cell (LSEC) undergoes loss of its highly specialized phenotype before activation of the hepatic stellate cell (HSC) and fibrosis.[1,2] If changes in LSEC phenotype not only precede HSC activation, but also were causally linked to it, this would enhance our understanding of the development of chronic liver disease significantly.

There is evidence that LSECs and HSCs maintain each other's differentiated phenotype. Vascular endothelial growth factor-A (VEGF-A; henceforth referred to as VEGF) production by either HSCs or hepatocytes maintains LSEC differentiation.[3] Conversely, in vitro studies have shown that differentiated LSECs (ie, fenestrated LSECs) prevent HSC activation and promote reversal of activated HSCs to quiescence, but that LSECs lose this effect when they are de-differentiated or "capillarized."[4] Capillarization is defined as the in vivo loss of LSEC fenestration with development of an organized basement membrane.[5] Capillarization occurs in fibrotic liver in human beings[5–7] and experimental animal models[8–10] and precedes onset of fibrosis in alcoholic liver injury,[1] in nonalcoholic fatty liver disease,[2] and in experimental liver disease models induced by either intragastric alcohol infusion or thioacetamide (DeLeve et al, unpublished data). Taken together, these findings suggest that capillarization not only precedes fibrosis, but also may be permissive for it and that changes in LSEC differentiation might be an integral part of the development of fibrosis.

LSEC fenestration is maintained by paracrine secretion of VEGF by hepatocytes or HSCs and downstream autocrine production of nitric oxide (NO) by VEGF-stimulated endothelial NO synthase (eNOS).[3,11] NO could act through either the soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (cGMP)/protein kinase G pathway[12] or through protein S-nitrosylation.[13] To examine the role of capillarization in the fibrotic process, we examined which of these signaling pathways controls LSEC differentiation in vitro, using the presence of fenestration as a surrogate marker for LSEC differentiation and defenestration as a surrogate marker for capillarization. In vivo studies examined whether reversal of capillarization by activation of the relevant signaling pathway promotes HSC quiescence and regression of fibrosis, and prevents progression of cirrhosis.

Materials and Methods

Reagents

A list of reagents is provided in the Supplementary Material and Methods.

Animal studies. Male Sprague–Dawley rats (body weight, 220–250 g) were obtained from Bantin and Kingman Laboratories (Fremont, CA). Thioacetamide, 200 mg/kg intraperitoneally, was given twice weekly. BAY 60–2770,[14] 0.3 mg/kg

Abbreviations used in this paper: α-SMA, α-smooth muscle actin; cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; HSC, hepatic stellate cell; LSEC, liver sinusoidal endothelial cell; L-NAME, NG-nitro-L-arginine methyl ester; NO, nitric oxide; TAA, thioacetamide; sGC, soluble guanylate cyclase; VEGF, vascular endothelial growth factor.

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intragastric, was administered daily. VEGF antisense oligonucleotides, 20 mg/kg intraperitoneally, were given twice weekly for 4 weeks.

All protocols were reviewed and approved by the Animal Care and Use Committee at the University of Southern California to ensure ethical and humane treatment of animals. This study followed the guidelines outlined in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (publication 86–23, revised 1985).

Cell isolation and culture. LSECs were isolated by collagenase perfusion, iodixanol density gradient centrifugation, and centrifugal elutriation as previously described and modified.[15,16] Yields from normal rat liver averaged 100 million cells with more than 95% viability and 99% purity as determined by uptake of formaldehyde-treated serum albumin, a specific marker of LSECs.[17–19]

HSCs were provided by the USC Nonparenchymal Liver Cell subcore. HSCs were isolated by collagenase/pronase digestion and Stractan density gradient centrifugation. Co-culture of HSCs and LSECs was performed as previously described[4] (Supplementary material).

Scanning electron microscopy and quantitative imaging. Sample preparation for scanning electron microscopy and porosity (percentage of LSEC surface occupied by fenestrae) measurements of cells and liver tissue were performed as previously described[20] (details in Supplemental material).

Evaluation of fibrosis and cirrhosis. Assessment of fibrosis and cirrhosis in Sirius red–stained sections was performed blindly by G.C.K. Whole-section–scanning morphometric image analysis was performed blindly by R.D.A. as previously described.[2]

Statistics. All data, expressed as mean ± standard error of the mean, were from at least 3 separate experiments. Groups were compared by analysis of variance with a posteriori contrast by least significant difference; or by Student *t* test using the Microsoft Excel Analysis ToolPak (Microsoft, Redmond, WA). Results with a *P* value of less than .05 were considered significant.

Results

VEGF-Stimulated NO/cGMP Pathway Is Required to Maintain LSEC Differentiation

The key morphologic features of differentiated LSECs, presence of fenestrae grouped into sieve plates, are rapidly lost in vitro.[3,21,22] LSECs cultured for 2 days lost fenestration, but fenestration was well maintained in the presence of 40 ng/mL VEGF (Figure 1*A*). Conversely, knockdown of hepatic VEGF protein by antisense oligonucleotides (Figure 1*B*) led to marked sinusoidal defenestration (Figure 1*C*), confirming that VEGF maintains LSEC fenestration in vivo. VEGF acts through eNOS-catalyzed NO production,[3] and NO signaling can occur through the sGC/cGMP/protein kinase G pathway[12] and/or through protein S-nitrosylation.[13]

Inhibition of sGC activity by 10 μmol/L ODQ (Supplementary Figure 1*A*) or protein kinase G activity by 10 μmol/L Rp-8-pCPT-PET-cGMPS (Supplementary Figure 1*B*) completely blocked VEGF maintenance of LSEC fenestration (Figure 2*A* and *B*), confirming that the VEGF/cGMP pathway is necessary to maintain LSEC fenestration.

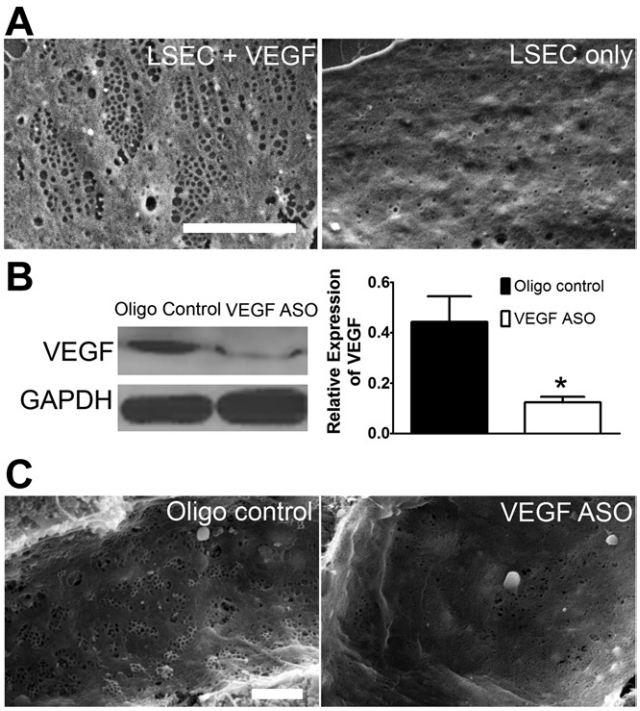

Figure 1. VEGF is required to maintain LSEC phenotype both in vitro and in vivo. (*A*) Representative scanning electron microscopy of LSECs cultured with (*left panel*) and without (*right panel*) VEGF for 2 days show loss of fenestrae in sieve plates in vitro in the absence of VEGF. *Scale bar*, 5 μm. (*B*) Hepatic expression of VEGF on immunoblot with densitometry and (*C*) representative scanning electron microscopy of hepatic sinusoids from rats treated with VEGF anti-sense oligonucleotides (ASO) or control oligonucleotides. *$P < .05$. *Scale bar*, 2 μm. All figures represent n ≥ 3. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Maintenance of LSEC Differentiation Requires Both VEGF-Stimulated NO via the cGMP Pathway Plus VEGF via a NO-Independent Pathway

Primary LSECs were cultured with YC-1 (a NO-independent sGC activator) or 8-pCPT-cGMP (a cGMP analog) in the absence of VEGF to determine whether rescuing the cGMP pathway alone is sufficient to maintain LSEC differentiation. Neither 30 μmol/L YC-1 nor 100 μmol/L 8-pCPT-cGMP was able to maintain normal LSEC fenestration and porosity in the absence of VEGF (Figure 2*C* and *D*), although 30 μmol/L YC-1 led to cellular cGMP levels that were twice as high as in VEGF-stimulated LSECs. These data show that maintaining the cGMP pathway by itself fails to maintain normal LSEC fenestration. Thus, the cGMP pathway is necessary, but not sufficient, to maintain LSEC differentiation.

In addition to the classic cGMP pathway, NO also can induce protein S-nitrosylation. To determine whether protein S-nitrosylation is required, LSECs were treated with VEGF, 3 mmol/L NG-nitro-L-arginine methyl ester (L-NAME) to inhibit VEGF-stimulated NO production, and