Vascular Endothelial Cadherin Modulates Renal Interstitial Fibrosis

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Key Words

Vascular endothelial cadherin • Renal microcirculation • Renal fibrosis • Unilateral ureteral obstruction

Abstract

Background/Aims: Renal interstitial fibrosis is a final common pathway of all chronic, progressive kidney diseases. Peritubular capillary rarefaction is strongly correlated with fibrosis. The adherens junction protein vascular endothelial cadherin (VE-cadherin) is thought to play a critical role in vascular integrity. We hypothesized that VE-cadherin modulates the renal microcirculation during fibrogenesis and ultimately affects renal fibrosis. **Methods:** Unilateral ureteral obstruction (UUO) was used as a renal fibrosis model in VE-cadherin heterozygote (VE+/-) and wild-type (WT) mice, and the kidneys were harvested at days 3, 7, and 14. Peritubular capillary changes and fibrogenesis were investigated. **Results:** VE+/- mice had lower levels of VE-cadherin protein than WT mice at 3 and 7, but not 14 days after UUO. Vascular permeability was significantly greater in VE+/- mice 7 days after UUO, while peritubular capillary density was not significantly different in VE+/- and WT mice. Interstitial myofibroblast numbers and collagen I and III mRNA levels were significantly higher in VE+/- mice, consistent with a stronger early fibrogenic response. Expression of the pericyte marker

neuron-glia antigen 2 was upregulated after UUO, but was not greater in VE+/- mice compared to the WT mice. **Conclusion:** Our data suggest that VE-cadherin controls vascular permeability and limits fibrogenesis after UUO.

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Introduction

Chronic kidney disease (CKD) is a major health care problem that ultimately progresses to renal failure and the need for dialysis and/or renal transplantation [1]. While transplantation offers the best chance for long-term rehabilitation, most renal allografts are ultimately destroyed by chronic rejection [1–3]. In both native and transplanted kidneys, progressive fibrosis of the renal interstitium is the predominant final common pathway of renal destruction, regardless of the etiology of the original kidney disease [4–6].

During fibrogenesis, renal peritubular capillaries undergo major structural and functional changes, ultimately leading to capillary loss. Peritubular capillary rarefaction is correlated with the severity of fibrosis in rodent

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CKD models such as 5/6 nephrectomy [7–9] and unilateral ureteral obstruction (UUO) [10, 11] and in human patients with chronic allograft rejection [12] and CKD [13, 14]. The extent of capillary loss predicts interstitial damage and the decline in glomerular filtration rate in human subjects [13, 14]. Capillary loss may propagate kidney damage due to hypoxia [15, 16] and/or decreasing nutrient supply to local regions.

Functional changes to capillaries also contribute to interstitial inflammation and subsequent fibrosis. Capillary endothelial cells promote inflammation by upregulating their expression of luminal adhesion molecules responsible for monocyte adhesion and transmigration [17]. Increased microvascular permeability may also contribute to interstitial inflammation by allowing chemottractant plasma proteins such as oxidized albumin and fibrinogen to enter the interstitium. Enhanced permeability also increases fluid efflux, leading to tissue edema. Capillaries may also regulate fibrogenic responses via interactions with fibroblast precursors. Recently, Lin et al. [18] and Humphreys et al. [19] reported that pericytes and perivascular fibroblasts are a major source of renal interstitial myofibroblasts. As these cells are closely associated with the endothelium, it is likely that their differentiation is triggered in part by endothelial cell signals and/or endothelial cell death. In addition, evidence from lineage tracing studies performed by Zeisberg et al. [20] in mouse models of renal fibrosis suggests that endothelial cells may even transform into interstitial fibroblasts, a phenomenon called endothelial-to-mesenchymal transition.

An important molecular component of the capillary endothelial barrier that may regulate many of these structural and functional changes is the adherens junction protein vascular endothelial cadherin (VE-cadherin). VE-cadherin forms homophilic links with partners on the adjacent cell membrane [21] and is essential for vascular remodeling and the maintenance of vascular integrity [21–25]. Studies in cultured endothelial cells have shown that VE-cadherin limits the permeability of the endothelial monolayer [26]. In addition, VE-cadherin anchors a molecular complex that contains several important signaling molecules as well as cytoskeletal attachments. Thus, VE-cadherin can activate intracellular signals (e.g. kinase activation stimulated by VE growth factor) that are critical for endothelial cell proliferation and survival [21, 25]. Based on these previous findings, we hypothesized that VE-cadherin has a critical role in the maintenance of capillary structure and function during CKD.

Because the homozygous deletion of VE-cadherin is embryonically lethal in mice, the present studies used VE-cadherin heterozygote (VE+/-) mice in the UUO model of CKD to investigate how the level of VE-cadherin expression affects peritubular capillary structure and function and the severity of renal interstitial fibrosis. Our results indicate that VE-cadherin was increased after UUO in wild-type (WT) mice; this upregulation was delayed in VE+/- mice, resulting in expression levels below WT on days 3–7 but similar to WT on day 14. Studies comparing VE+/- and WT mice suggest that upregulation of VE-cadherin controls renal vascular permeability and may limit fibrosis.

Animals and Methods

Animals

The experiments were performed on WT and VE-cadherin heterozygote (VE+/-) littermate mice on a mixed Swiss background, which were generously provided by Dr. Elisabetta Dejana (The FIRC Institute of Molecular Oncology, Milan, Italy). The genotype was confirmed by polymerase chain reaction (PCR). VE-/- mice could not be used for these studies because the homozygous deletion is embryonically lethal.

Renal Interstitial Fibrosis Model

UUO or sham surgery was performed on 9- to 11-week-old male mice as described previously [27–29]. Kidney tissue was harvested 3, 7, or 14 days after UUO or sham surgery. Because previous studies showed no differences between these time points in sham-operated mice, a single time point (day 3 or 7) was used for sham controls in each experiment. All procedures were performed in accordance with the guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Seattle Children's Institute.

Histological Examination and Analysis

Immunohistochemical staining was performed on kidney tissues using procedures established in our laboratory [27, 28, 30]. Endothelial cells were stained using biotinylated or non-biotinylated rat anti-mouse CD31 (platelet endothelial cell adhesion molecule-1), monoclonal antibody (BD Pharmingen, San Diego, Calif., USA; 1:1,000 or 1:500). VE-cadherin staining was obtained using rat anti-mouse CD144 (BD Pharmingen; 1:100). The macrophage marker CD68 was stained using rat anti-mouse CD68 (Serotec Ltd., Oxford, UK; 1:100). The myofibroblast marker α -smooth muscle actin (α -SMA) was stained using alkaline phosphatase-conjugated mouse anti-human α -SMA 1A4 monoclonal antibody (Sigma-Aldrich, St. Louis, Mo., USA; 1:10). Pericytes were investigated by staining with rabbit anti-NG2 (anti-neuronal antigen 2) chondroitin sulfate proteoglycan (Millipore, Billerica, Mass., USA; 1:500). The antibodies were visualized using the VECTASTAIN Elite ABC kit and VIP or Fast Red substrate (Sigma-Aldrich, St. Louis, Mo., USA). A Tyramide Signal Amplification Kit (Invitrogen, Carlsbad, Calif., USA) was used for im-

munofluorescent staining of VE-cadherin, CD31, and NG2 according to the manufacturer's instructions. Briefly, cryosections were labeled with rat anti-mouse CD144, rat anti-mouse CD31, and/or rabbit anti-NG2 antibodies. The CD31 and CD144 fluorescence was amplified using horseradish peroxidase (HRP) conjugated anti-rat secondary antibody (Jackson ImmunoResearch) and Alexa Fluor® 488-conjugated tyramide. NG2 was visualized using HRP-conjugated anti-rabbit and Alexa Fluor 594-conjugated tyramide. All sections were stained with DAPI or methyl green for nuclear visualization and mounted with VectaShield Fluorescence Mounting Medium or Vectamount (Vector Labs).

For analysis, the images ($\times 400$) of 8–10 random, non-overlapping cortical fields per slide were captured using a SPOT digital camera (Diagnostic Instruction, Inc., Sterling Heights, Mich., USA). The CD68 or α -SMA-stained tubulointerstitial area was quantified using Image-Pro Plus software (Media Cybernetics, Silver Spring, Md., USA) as described previously [27]. The results were expressed as the percentage of total tubulointerstitial area stained. An index of peritubular capillary density was determined by a grid method modified from previous studies [31, 32]. Each image (total area = 0.0625 mm^2) was divided into 10×10 rectangular boxes (each $22 \times 28.5 \mu\text{m}$), and boxes intersected by at least one CD31-positive peritubular capillary were counted. The maximal possible capillary staining index is 100 (i.e. every square in the grid contains at least one capillary), and the minimal index is 0 (no capillaries). Observers were blinded to the animal group at the time of analysis.

Measurement of mRNA Expression

Total RNA was extracted from kidney tissue using TRIzol reagent (Invitrogen). The first-strand cDNA was generated by reverse transcription (iScript cDNA Synthesis kit, Bio-Rad Laboratories, Hercules, Calif., USA). Semiquantitative real-time PCR was performed with the iQ SYBR Green Supermix kit (Bio-Rad Laboratories) using the iCycler (Bio-Rad) standard protocol. The primers used for qRT-PCR were as follows: pro-collagen type I α_1 forward 5'-CTTCACCTACAGCACCTTGTG-3' and reverse 5'-GATGACTGTCTTGCCCCAAGTT-3', pro-collagen type III α_1 forward 5'-TGAATGGTGGTTTTCAGTTCAG-3' and reverse 5'-GGTCACTTGCCTGGTTGATAA-3', fibronectin forward 5'-ACACGGTTTCCCATACGCCAT-3' and reverse 5'-AATG-ACCACTGCCAAAGCCCA-3', GAPDH 5'-AACTTTGTGAGCTCATTTCTCTGGTAT-3' and reverse 5'-CCTTGCTGGGCTGGGTGGT-3'. Standard curves were obtained for each gene. Reactions were run in triplicate, and the starting quantity of the mRNA of interest was measured and quantified relative to GAPDH.

Immunoblotting

Pieces of frozen kidney were homogenized in RIPA buffer supplemented with PMSF, protease inhibitor cocktail, sodium orthovanadate, and NaF (Santa Cruz Biotechnology, Inc.). The protein concentration was determined using the BCA protein assay (Pierce Biotechnology, Rockford, Ill., USA). Protein samples (20 μg) were separated by electrophoresis in 4–15% pre-cast polyacrylamide gels (Bio-Rad Laboratories, Hercules, Calif., USA) and transferred to nitrocellulose membranes. The primary antibodies used were goat anti-mouse VE-cadherin (R&D Systems, Inc.; 1:2,000), β -actin (1:2,000), monoclonal anti- α -SMA (clone 1A4; Sigma-Aldrich; 1:2,500), and rabbit anti-NG2 chondroitin

sulfate proteoglycan (Millipore; 1:1,000). The secondary antibodies were IRDye™ 800 infrared dye-labeled antibodies (Rockland, Gilbertsville, Pa., USA), and Alexa Fluor 680-labeled antibodies (Molecular Probes, Eugene, Oreg., USA). Protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Nebr., USA) for fluorescent Western blots. All Western blot results were normalized by dividing each fluorescence measurement by that of β -actin in the same sample. Lanes in some representative blots shown in the figures have been rearranged for correspondence with the text; vertical lines indicate where data from different blots or non-adjacent lanes have been juxtaposed.

Qualitative Evaluation of Vascular Permeability by Injection of Fluorescein-Conjugated Dextran

Fluorescein-conjugated dextran (FITC-dextran; 500 kDa, anionic, lysine fixable) (Invitrogen) was dissolved in normal saline at 7.5 mg/ml, and 150 μl was injected into the tail vein [33]. Kidneys were harvested after 10 min of incubation and fixed in 10% formalin overnight. Sections of paraffin-embedded tissue were examined, and images were captured using a fluorescence microscope equipped with a SPOT camera.

Measurement of Vascular Permeability using Evans Blue Dye

Vascular permeability was determined by intravascular injection of Evans blue dye (Sigma-Aldrich). Evans blue (20 $\mu\text{g/g}$ mouse body weight, 2 mg/ml in 0.9% NaCl) was injected into the mouse tail vein. After 30 min, dye remaining in the vessels was washed out by perfusing the mouse with 0.9% NaCl via a needle inserted into the left ventricle of the heart. The mouse was then euthanized immediately, and the kidneys were removed. Each kidney was cut into two pieces along the horizontal plane, and both pieces were weighed. One piece was used for Evans blue extraction with 100% formamide (4 ml/g wet kidney weight) at room temperature for 24 h. The other piece was dried for 24 h at 55°C and weighed in order to obtain the ratio of dry weight to wet weight. The extracted dye was measured by spectrophotometry (absorbance at 620 nm) using a 96-well plate reader. The results were calculated from a standard curve of Evans blue dye (0.05–50 $\mu\text{g/ml}$) and expressed as $\mu\text{g/g}$ dry kidney weight [34, 35].

Data Analysis and Statistics

Data are expressed as mean $\pm$ standard deviation unless indicated otherwise in the figure legends. Statistical significance was evaluated by t test and one-way or two-way ANOVA using StatView version 5.0 software. p values <0.05 were considered statistically significant.

Results

Changes in VE-Cadherin Expression after UO

To investigate the role of VE-cadherin in the UUO model, VE-cadherin mRNA and protein expression were measured in the WT mouse kidneys after sham and UUO surgery. qRT-PCR and Western blotting showed that VE-cadherin mRNA and protein were significantly elevated

Fig. 1. VE-cadherin expression after UUO in the WT mouse. **a** Immunostaining for VE-cadherin in frozen kidney sections prepared 7 days after sham surgery (top) or UUO (bottom). $\times 200$. **b** Top, representative Western blot showing VE-cadherin (~120-kDa), shed VE-cadherin (~100–110 kDa), and β -actin (42 kDa). Bottom right, VE-cadherin protein (120-kDa band) measured by

Western blotting, normalized by β -actin. All values are expressed relative to the mean sham level. * $p < 0.05$ (n = 5–7). Bottom left, relative expression of VE-cadherin mRNA 3, 7, and 14 days after sham surgery and UUO, measured by qRT-PCR and normalized by GAPDH. All values are expressed relative to the mean sham. * $p < 0.05$ (n = 4–6).

Fig. 2. Relative VE-cadherin expression in VE+/- and WT mice. Top, representative Western blot. Bottom, VE-cadherin protein expression. VE+/- mice showed lower VE-cadherin protein expression at days 3 and 7 but not at day 14 after UUO. All values are expressed relative to the mean WT sham. * $p < 0.05$ (n = 5–7).

3, 7 and 14 days after UVO (fig. 1). Immunofluorescent staining confirmed stronger staining 7 days after UVO (fig. 1). Thus, any loss of vascular integrity in the UVO model of CKD (see results below) cannot be explained by a decrease in total VE-cadherin protein. Instead, the observed *increase* in VE-cadherin may represent a compensatory mechanism that *limits* vascular damage and/or hyperpermeability (see below).

VE-Cadherin Expression in Heterozygote Mice

To investigate whether the level of VE-cadherin expression modulates interstitial fibrosis, we first determined whether deletion of one copy of the VE-cadherin gene affects the level of VE-cadherin protein. After sham surgery, VE-cadherin levels did not differ significantly between WT and VE+/- mice. After UVO, VE-cadherin expression increased in both WT and VE+/- mice. However, VE+/- mice showed a delayed response, with significantly lower expression at days 3 and 7 (fig. 2). VE-cadherin expression at day 14 did not differ between WT and VE+/- mice. Thus, the VE+/- mice provided a good system to investigate the early effects of the VE-cadherin expression level during chronic renal injury days 3–7 after UVO, but were not well suited for investigating the effects of VE-cadherin at later time points.

Peritubular Capillary Loss

Peritubular capillaries were identified by CD31 immunofluorescent staining of frozen kidney sections from WT mice. In sham-operated mice, a large number of peritubular capillary cross sections were positive. After UVO, the density of positive capillaries decreased progressively (fig. 3a). Although VE+/- mice showed a trend toward lower peritubular capillary density at day 7, the differences were not statistically significant between VE+/- and WT mice after sham or UVO (fig. 3b).

Renal Vascular Permeability

As an initial method to assess the changes in vascular permeability after UVO, a qualitative visualization using dextran (500 kDa) conjugated to fluorescein isothiocyanate (FITC) was employed. Thirty minutes after intravenous injection of FITC-dextran, the fluorescent label was detected within the interstitium in UVO kidneys (day 7), whereas labeled dextran appeared largely confined to intravascular areas in sham-operated kidneys (fig. 4a). These results suggest that capillary permeability is increased in response to UVO, allowing large macromolecules to enter the interstitium. However, the presence of the intravascular fluorescent label and the

autofluorescence of kidney tissue limited our ability to quantify the change using this method. Therefore, we quantified the permeability in WT and VE+/- mice 7 days after UVO using a different method based on intravascular injection of Evans Blue dye. After 30 min following the injection, dye remaining in the vessels was washed out by perfusing the mouse with 0.9% NaCl before harvesting the kidney. On visual inspection, the obstructed left kidney showed strong staining, whereas the contralateral kidney retained much less dye (fig. 4b). The measured tissue Evans Blue levels were greater in VE+/- mice than in WT mice (fig. 4c). These data support the hypothesis that rapid upregulation of VE-cadherin expression in WT mice limits the increase in renal vascular permeability after UVO.

Macrophage Accumulation

Interstitial inflammation during CKD is thought to depend in large part on the transmigration of circulating monocytes and their differentiation into macrophages [4, 36]. Therefore, we investigated whether VE+/- and WT mice show a difference in interstitial macrophage accumulation after UVO. CD68+ interstitial macrophages were present in the interstitium after UVO, whereas few cells were detected after sham surgery. The percentage of tubulointerstitial area with positive CD68 staining increased rapidly and peaked at day 7 after UVO. VE+/- and WT mice showed no difference in CD68+ macrophage infiltration in the interstitium (fig. 5). An additional study using F4/80 immunostaining confirmed that there was no difference in interstitial macrophage density between VE+/- and WT mice (data not shown). These data suggest that differences in the fibrogenic response between VE+/- and WT mice (see below) do not result from an alteration of total macrophage accumulation.

Activated Fibroblasts

Activated fibroblasts, or myofibroblasts, are thought to be the major source of extracellular matrix production that leads to interstitial fibrosis. To measure myofibroblast accumulation, the marker α -SMA was quantified by Western blotting and morphometry. As shown in numerous previous studies, α -SMA immunoreactivity was seen in the interstitium after UVO (fig. 6a), and increased α -SMA protein expression was detected by Western blotting (fig. 6b). The α -SMA-positive tubulointerstitial area was increased significantly after UVO, and was significantly higher in VE+/- mice than in WT mice at day 7 after UVO (fig. 6a). α -SMA protein levels were also sig-

Color version available online

3a

3b

Fig. 3. Peritubular capillary rarefaction. **a.** Representative CD31 immunostaining of renal tissue from WT mice 7 days after sham surgery (top) and UUO (bottom). $\times 200$. **b.** Change in peritubular capillary density quantified by grid analysis ($n = 4-8$).

Fig. 4. Vascular permeability after UUO. **a** Permeability study performed by injection of fluorescent-labeled dextran (500 kDa) 7 days after surgery. Top, WT sham; bottom, WT UUO. $\times 400$. **b** Permeability study performed by injection of Evans blue dye. Obstructed left kidney (7 days after UUO) in WT mice shows Evans blue accumulation after saline washout, whereas the unobstructed right kidney retained less dye. **c** Concentration of retained Evans blue at day 7 after UUO. The obstructed kidneys retained much more dye than the contralateral kidneys, showing that vascular permeability was greater. The asterisk indicates significantly greater Evans blue concentration in VE+/- mice compared to WT ($p < 0.05$, $n = 6$).

Color version available online

4a

4b

4c

nificantly higher in VE+/- mice than in WT mice at days 3 and 7 after UUO (fig. 6b), suggesting that myofibroblast accumulation was greater in VE+/- mice.

Upregulation of Extracellular Matrix Gene Expression

Collagen I and III and fibronectin are major extracellular matrix molecules that accumulate in the interstitium during renal fibrosis. In order to investigate how the level of VE-cadherin affects matrix protein expression, the levels of pro-collagen I, pro-collagen III, and fibronectin mRNA were quantified by qRT-PCR. The results show that VE+/- mice had significantly higher collagen I and III mRNA levels at day 7, but not at day 14 after UUO. Fibronectin transcripts showed a tendency toward higher levels in VE+/- mice than in WT mice at days 7 and 14 after UUO, but these differences were not significant (fig. 7).

The accumulation of interstitial collagen reflects the net effect of differences in collagen synthesis and degradation. Total kidney collagen levels were calculated from the hydroxyproline concentration in hydrolyzed protein extracts from sham and UUO kidneys. Total collagen was significantly increased at days 7 and 14 after UUO compared to sham surgery in both VE+/- and WT mice; however, there were no significant differences between VE+/- and WT (data not shown). Differences were also absent 7 days after UUO when interstitial collagen-positive areas were measured by collagen III immunohistochemistry (data not shown). The discrepancy between collagen gene expression and total collagen may be due to the narrow time window (days 3–7) when VE-cadherin expression differs between VE+/- and WT mice after UUO.

Studies of NG2+ Pericyte Expansion

Recent studies have suggested that pericytes associated with capillary endothelial cells are precursors of myofibroblasts [18, 19]. Thus, we hypothesized that a greater expansion of the pericyte population contributed to the increased myofibroblast accumulation seen in VE+/- mice. To test this hypothesis, we measured the expression of the chondroitin sulfate proteoglycan NG2, which is thought to identify a subset of activated pericytes during development and in pathological conditions.

The distribution of NG2+ pericytes at day 7 after UUO was investigated by double immunofluorescence staining for CD31 and NG2 (fig. 8a). In the sham-operated kidney, NG2 was restricted to a few glomerular mesangial cells and larger vessels. NG2 staining in the tubulointerstitial area was markedly increased at days 3, 7, and 14 after UUO, consistent with an expansion of the pericyte popu-

Fig. 5. Expression of the macrophage marker CD68. **a** CD68 immunohistochemistry in frozen kidney sections prepared 7 days after sham surgery (top) or UUO (bottom) in WT mice. $\times 400$. **b** The percent positive area was measured in VE+/- and WT mice after sham surgery and 3, 7, and 14 days after UUO. CD68 expression in the tubulointerstitial area was significantly increased relative to sham at days 3, 7, and 14. However, there was no significant difference between VE+/- and WT mice. * $p < 0.05$ ($n = 5-6$).

lation. To quantify the total level of NG2 expression, NG2 protein levels were measured by Western blotting (fig. 8b). The level of NG2 was significantly increased at days 3, 7, and 14 after UUO, peaking at day 7. However, contrary to our hypothesis, VE+/- mice showed less NG2 expression than WT mice at day 7 and no significant difference at

Fig. 6. Expression of the myofibroblast marker α -SMA. **a** Left, immunohistochemistry for α -SMA in frozen kidney sections prepared from VE+/- and WT mice 7 days after sham surgery or UUO. $\times 400$. Right, percent of tubulointerstitial area positive for α -SMA was measured. α -SMA expression was significantly higher in VE+/- mice than in WT at day 7 after UUO. * $p < 0.05$ ($n = 5-8$). **b** Representative Western blot bands for α -SMA (quantified using a secondary antibody with an infrared fluorophore) and β -actin (quantified using an FITC-labeled primary antibody) from VE+/- and WT mice after sham surgery or UUO. α -SMA expression was significantly higher in VE+/- than WT mice at days 3 and 7 after UUO. * $p < 0.05$ ($n = 5-7$).

Fig. 7. Relative expression of collagen I, collagen III, and fibronectin mRNA 7 days and 14 days after sham surgery and UUO measured by qRT-PCR and normalized by GAPDH. All values are expressed relative to the mean sham. * $p < 0.05$ ($n = 4-8$).

Fig. 8. Expression of the pericyte marker NG2. **a** Immunostaining of CD31 (green) and NG2 (red) in frozen kidney sections prepared 7 days after sham surgery or UUO from VE+/- and WT mice. $\times 600$. **b** Top, representative Western blot bands for NG2 and β -actin from VE+/- and WT mice after sham surgery and UUO. Bar graph compares NG2 protein expression relative to mean sham in VE+/- and WT mice at days 3, 7, and 14 after UUO. VE+/- mice had significantly lower NG2 expression than WT mice at day 7 after UUO. * $p < 0.05$ ($n = 4-7$).

days 3 and 14. These data suggest that the greater myofibroblast accumulation in VE+/- mice may not be associated with an expansion of the NG2+ pericyte population. However, further studies are required to better understand the roles of pericytes in the UUO model of CKD, and the possible effects of VE-cadherin and other endothelial cell molecules on pericyte differentiation and interstitial migration.

Discussion

The results of this study demonstrate that peritubular capillaries undergo a significant, progressive rarefaction and show increased permeability in the mouse UUO model of CKD. Unexpectedly, these changes were associated with a significant increase in VE-cadherin protein expression. Based on our immunostaining data and pre-

vious reports showing that VE-cadherin expression is relatively selective for endothelial cells once embryonic development is complete, VE-cadherin expression after UUO appears to be restricted to the endothelium. Given published evidence that VE-cadherin normally functions to maintain vascular integrity and limit permeability, the current data suggest that VE-cadherin upregulation in endothelial cells represents a compensatory response that serves to reduce the permeability associated with renal damage.

To test this hypothesis, studies using the UUO model of CKD compared outcomes between VE+/- and WT mice. We found that VE+/- and WT mice had similar VE-cadherin expression at baseline, in sham-operated kidneys. However, VE+/- mice showed a delayed increase in VE-cadherin expression from 3 to 7 days after UUO. This short time window likely limited our ability to detect longer-term effects of VE-cadherin deficiency that might have developed if the expression of this gene were suppressed more completely or for a longer period of time. However, we observed several differences in fibrogenic responses between VE+/- and WT mice. In particular, the kidneys of VE+/- mice showed a greater increase in vascular permeability, higher levels of the myofibroblast marker α -SMA, and higher kidney collagen I and III mRNA levels. These findings are consistent with the hypothesis that the upregulation of VE-cadherin is protective in CKD.

Changes in VE-Cadherin Expression in Renal Diseases

Data on the role of VE-cadherin in renal disease have been limited to studies of acute kidney injury and acute allograft rejection. In an acute kidney injury model (ischemia-reperfusion of bilateral renal arteries in mice and rats), Sutton et al. [33] and Horbelt et al. [37] demonstrated increased renal microvascular permeability together with changes in morphology of the renal microvascular endothelium. These investigators also reported a loss of endothelial cell integrity, alterations in the actin cytoskeleton, and loss of VE-cadherin expression by endothelial cells [33]. VE-cadherin expression was also reduced in acute rejection after renal transplantation, and the downregulation of VE-cadherin was thought to contribute to the acute rejection of allografts [38]. These observations suggest that the mechanisms of capillary injury may differ in acute and chronic renal disease.

In order to determine why VE-cadherin is upregulated after UUO, it will be necessary to better understand the signaling pathways that regulate VE-cadherin transcrip-

tion and translation. Several transcription factors have been shown to induce VE-cadherin gene expression, including Ets family members such as Ets1 [39], Erg [40], and TAL-1/SCL [41]. Twist, Snail, and Slug, which are known to regulate epithelial-mesenchymal transition, may also modulate the activity of the VE-cadherin promoter [42]. Some of these transcription factors are regulated by inflammatory and angiogenic signaling molecules released during CKD. For example, Ets-1 can be stimulated by tumor necrosis factor- α , fibroblast growth factor-2, TGF- β , and VEGF [43]. Future studies are needed to understand how differences in extracellular signals and transcription factor activation influence the up- or downregulation of VE-cadherin expression in specific disease models.

Implications for the Endothelial Role in Fibrogenesis

Our data indicate that reduced VE-cadherin expression in heterozygote mice, compared to WT, was associated with more myofibroblast accumulation and higher levels of collagen gene expression. These changes may represent an adaptive response to increased vascular permeability and/or other endothelial cell signals that are triggered in response to renal damage. Our data appear to exclude two possible cellular mechanisms that could connect permeability changes with fibrogenesis. First, we found that macrophage accumulation was not altered in VE+/- mice. Second, our data suggest that the population of NG2+ pericytes was not increased in VE+/- mice, relative to WT; in fact, they were significantly lower on day 7. These data are of interest because recent cell lineage tracing studies support the view that pericytes are the major precursors of renal interstitial myofibroblasts in chronically injured kidneys [18, 19]. In this case, a possible explanation for the findings in the present study is that in VE+/- mice, NG2+ pericytes differentiated more rapidly into myofibroblasts, under the influence of factors derived from plasma transudate and/or hyperactivated endothelial cells. This scenario could account for lower expression of NG2 as well as greater myofibroblast accumulation on day 7. A more aggressive pace of pericyte loss in VE+/- mice might also contribute to greater vascular permeability, as previous studies have shown that pericytes act to maintain vascular structural integrity and induce endothelial cell quiescence [44, 45]. An alternative possibility is that the pericytes are a heterogeneous population, with only a subset re-expressing NG2 as they transition to myofibroblasts. The possibility that these subpopulations are differently regulated by VE-cadherin deserves further consideration.

While much remains unknown about the endothelial changes in CKD, our findings suggest that changes in endothelial cell-cell interactions might play a role in fibrogenesis. Further studies are needed to better understand how capillary hyperpermeability, profibrotic signaling interactions, and ultimately capillary loss regulate the severity and reversibility of CKD.

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Disclosure Statement

None.

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