PECAM-1 isoform-specific regulation of kidney endothelial cell migration and capillary morphogenesis

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Kondo S, Scheef EA, Sheibani N, Sorenson CM. PECAM-1 isoform-specific regulation of kidney endothelial cell migration and capillary morphogenesis. Am J Physiol Cell Physiol 292: C2070–C2083, 2007; doi:10.1152/ajpcell.00489.2006.—Platelet endothelial cell adhesion molecule-1 (PECAM-1) has been implicated in angiogenesis through its involvement in endothelial cell-cell and cell-matrix interactions and signal transduction. Recent studies indicate that the cytoplasmic domain of PECAM-1 plays an important role in its cell adhesive and signaling properties. However, the role PECAM-1 isoforms play during angiogenic events such as cell adhesion and migration requires further delineation. To gain insight into the role PECAM-1 plays during vascular development and angiogenesis, we examined the expression pattern of PECAM-1 isoforms during kidney vascularization. We show that multiple isoforms of PECAM-1 are expressed during renal vascular development with different frequencies. The PECAM-1 that lacks exons 14 and 15 (Δ14&15) was the predominant isoform detected in the renal vasculature. To further study PECAM-1 isoform-specific functions we isolated kidney endothelial cells (EC) from wild-type and PECAM-1-deficient (PECAM-1+/−) mice with B4-lectin-coated magnetic beads. PECAM-1+/− kidney EC showed reduced migration, inability to undergo capillary morphogenesis in Matrigel, dense peripheral focal adhesions, and peripheral cortical actin distribution compared with wild-type cells. PECAM-1+/− kidney EC secreted increased amounts of fibronectin and decreased amounts of tenasin-C and thrombospondin-1. Reexpression of Δ14&15, but not full-length, PECAM-1 in PECAM-1+/− kidney EC restored cell migration and capillary morphogenesis defects. Thus PECAM-1 may regulate the adhesive and migratory properties of kidney EC in an isoform-specific fashion through modulation of integrin activity and extracellular matrix protein expression. Our results indicate that regulated expression of specific PECAM-1 isoforms may enable EC to accommodate the different stages of angiogenesis.

CD31; alternative splicing; angiogenesis; integrins; extracellular matrix

ENDOTHELIAL CELLS (EC) organize, forming vascular structures that are a central component of organogenesis. Blood vessels are formed by either vasculogenesis, in which endothelial progenitor cells assemble vessels, de novo or angiogenesis, in which existing vessels send out new sprouts. In either process, EC play a pivotal role in which they migrate, reorganize, and assemble into tubes with tight cell-cell connections (5). The kidney vasculature is formed by a combination of vasculogenesis and angiogenesis. Signaling between endothelium and tissue during development may form the molecular basis for physiological and physical interrelationships that extend through organ maturation. Tissues can regulate their vascular architecture by signaling EC via potent angiogenic and angiostatic agents.

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a 130-kDa glycoprotein consisting of a single-chain molecule of six Ig-like extracellular domains, a transmembrane portion, and a relatively long cytoplasmic domain. PECAM-1 cytoplasmic domain is encoded by multiple exons (exons 10–16) that undergo alternative splicing generating eight isoforms in mouse endothelium (35, 39). Each of the alternatively spliced exons contains a tyrosine residue in a highly conserved motif such that when phosphorylated it provides a docking site for signaling molecules with Src homology domain 2 (SH2). Thus the inclusion or exclusion of these exons from PECAM-1 cytoplasmic domain impacts on its potential interactions with intracellular signaling molecules and signaling capacity.

PECAM-1 generally concentrates at sites of EC cell-cell contacts and is expressed at a lower level diffusely on the surface of platelets and leukocytes (19, 25). PECAM-1 is required for endothelial cell-cell interactions and monolayer formation in culture. It mediates these interactions through homophilic binding with PECAM-1 molecules on neighboring cells. PECAM-1 also interacts with αvβ3-integrin and CD38 (19, 25). Antibodies to PECAM-1 block EC migration and capillary morphogenesis in Matrigel (33) and angiogenesis in vivo (7). PECAM-1-deficient mice are viable but exhibit defects in angiogenesis and inflammation in a number of assays (9, 15). PECAM-1 is expressed in renal glomerular and peritubular EC, and its expression is lost in sclerotic or fibrotic areas (18, 36, 43). However, the expression pattern of PECAM-1 isoforms, the role they play in the development of kidney vasculature, and how their altered expression may contribute to various renal pathologies require further investigation.

Nephrogenesis in the mouse begins during embryonic day (E)11 when the ureteric bud encounters the metanephric blastema. The metanephric blastema supports ureteric bud growth and branching, giving rise to the collecting system (30). After induction of the metanephros and condensation of the metanephric blastema, tubulogenesis proceeds through a histologically defined sequence of stages during which developing nephrons assume comma and S shapes (30). The cells on the terminal side of the comma- and S-shaped bodies develop and form the glomerular visceral epithelial cells and the parietal epithelial cells that line the Bowman capsule (1, 30). The cleft of the S-shaped body becomes occupied by microvascular cells.

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that will develop into the glomerular capillary tufts. In the metanephric stroma at E12 capillaries are observed, with the first vascular glomeruli forming at E14. The rodent is born with immature kidneys in which nephrons continue to develop from the nephrogenic zone located at the periphery of the kidney. This period of time is termed renal maturation and is complete by postnatal day (P)16 (22). The vasa recta continue to mature alongside the loops of Henle, growing into the medulla even after nephron development has ceased (14).

We previously showed (35) that multiple isoforms of PECAM-1 are present in vascular beds of different tissues of adult mice including kidney. To better understand the role of PECAM-1 in renal vascular development, we determined the expression pattern of PECAM-1 isoforms in embryonic and postnatal mouse kidneys. Here we demonstrate that the identity of the PECAM-1 isoforms expressed in the kidney vasculature does not change during embryonic and postnatal kidney development, although their frequency of detection varies. PECAM-1 lacking exons 14&15 (Δ14&15) was the most frequently detected isoform in the renal vasculature. We next isolated kidney EC from wild-type (PECAM-1+/+) and PECAM-1-deficient (PECAM-1−/−) mice to examine PECAM-1 isoform-specific functions in EC. PECAM-1−/− kidney EC were less migratory and failed to undergo capillary morphogenesis in Matrigel compared with PECAM-1+/+ kidney EC. PECAM-1−/− kidney EC also exhibited altered expression of extracellular matrix (ECM) proteins and adhesive properties. Furthermore, we showed that expression of Δ14&15, but not full-length, PECAM-1 restores migratory, adhesive, and capillary morphogenesis defects of PECAM-1−/− kidney EC. Thus PECAM-1-specific functions during vascular development and angiogenesis may be modulated by alternative splicing of its cytoplasmic domain.

**MATERIALS AND METHODS**

*Experimental animals.* All experiments were carried out in accordance with the American Physiological Society’s “Guiding Principles in the Care and Use of Animals” and approved by the Institutional Animal Care Committee of the University of Wisconsin School of Medicine and Public Health. The University of Wisconsin is accredited by the American Association for Accreditation of Laboratory Animal Care. Immortomice expressing a temperature-sensitive SV40 large T antigen were obtained from Charles River Laboratories Animal Care. Immortomice and the immorto/PECAM-1−/− mice were crossed with PECAM-1+/+ mice to examine PECAM-1−/− kidney EC. PECAM-1−/− kidney EC also exhibited altered expression of extracellular matrix (ECM) proteins and adhesive properties. Furthermore, we showed that expression of Δ14&15, but not full-length, PECAM-1 restores migratory, adhesive, and capillary morphogenesis defects of PECAM-1−/− kidney EC. Thus PECAM-1-specific functions during vascular development and angiogenesis may be modulated by alternative splicing of its cytoplasmic domain.

**Table 1. Distribution of PECAM-1 isoforms during embryonic and postnatal murine kidney development**

<table>
<thead>
<tr>
<th>Stages</th>
<th>Full</th>
<th>Δ12</th>
<th>Δ12&amp;14</th>
<th>Δ12,14&amp;15</th>
<th>Δ14</th>
<th>Δ14&amp;15</th>
<th>Δ15</th>
</tr>
</thead>
<tbody>
<tr>
<td>E13 (26)</td>
<td>ND</td>
<td>ND</td>
<td>4±0.2</td>
<td>77±6</td>
<td>ND</td>
<td>19±2</td>
<td>ND</td>
</tr>
<tr>
<td>E15 (24)</td>
<td>ND</td>
<td>ND</td>
<td>46±3</td>
<td>4±0.3</td>
<td>50±3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>E16 (34)</td>
<td>ND</td>
<td>ND</td>
<td>9±1</td>
<td>24±2</td>
<td>9±0.4</td>
<td>58±3</td>
<td>ND</td>
</tr>
<tr>
<td>E18 (22)</td>
<td>5±0.4</td>
<td>5±0.4</td>
<td>ND</td>
<td>10±1</td>
<td>5±0.3</td>
<td>73±5</td>
<td>5±0.2</td>
</tr>
<tr>
<td>P0 (37)</td>
<td>8±1</td>
<td>ND</td>
<td>3±0.2</td>
<td>14±1</td>
<td>19±2</td>
<td>51±3</td>
<td>5±0.3</td>
</tr>
<tr>
<td>P7 (23)</td>
<td>ND</td>
<td>ND</td>
<td>4±0.3</td>
<td>9±2</td>
<td>87±6</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>P10 (32)</td>
<td>3±0.2</td>
<td>ND</td>
<td>3±0.2</td>
<td>9±1</td>
<td>6±0.3</td>
<td>68±5</td>
<td>9±0.4</td>
</tr>
<tr>
<td>P15 (22)</td>
<td>5±0.3</td>
<td>5±0.3</td>
<td>ND</td>
<td>18±2</td>
<td>ND</td>
<td>73±5</td>
<td>ND</td>
</tr>
<tr>
<td>P20 (31)</td>
<td>ND</td>
<td>ND</td>
<td>29±2</td>
<td>10±1</td>
<td>58±5</td>
<td>3±0.2</td>
<td></td>
</tr>
</tbody>
</table>

Values (%) are mean ± SD frequency at which each platelet endothelial cell adhesion molecule-1 (PECAM-1) isoform was detected for the number of clones examined per experiment (in parentheses). Isoforms of PECAM-1 were identified by cloning and sequencing RT-PCR products from RNA isolated from embryonic and postnatal kidneys of PECAM-1+/+ mice as described in MATERIALS AND METHODS. Experiments were performed 3 times. The differences among the isoforms were significant (P < 0.01–0.05). Δ12&15 PECAM-1 isoform was not detected at any of the time points. Full, full-length PECAM-1; ND, not detected; E, embryonic day; P, postnatal day.

**Fig. 1. Morphology of mouse kidney endothelial cells (EC) cultured in gelatin-coated plates.** Platelet endothelial cell adhesion molecule-1 (PECAM-1+/+) (A and C) and PECAM-1−/− (B and D) kidney EC were cultured on gelatin-coated plates. Cells were photographed with a phase microscope in digital format (A and B, ×40; C and D, ×100). Note that kidney EC from PECAM-1+/+ and PECAM-1−/− mice share a similar morphology. These experiments were repeated with 3 different isolations of EC, with similar results.
Fig. 2. Expression of EC markers in kidney EC isolated with lectin-coated magnetic beads. Kidney EC prepared from PECAM-1+/+ (A, C, and E) and PECAM-1−/− (B, D, and F) mice were examined for expression of PECAM-1, vascular endothelial (VE)-cadherin, and B4-lectin by FACScan analysis. Shaded areas show staining in the presence of control IgG. Note similar expression of VE-cadherin and B4-lectin in both cell strains. These experiments were repeated with 3 different isolations of EC, with similar results.

TAA ATC TCT GTA GGT AG-3'; Neo forward: 5'-TGA CTG GGC ACA ACA GAC AAT CGG C-3', Neo reverse: 5'-TGA GCC AAC GCT ATG TCC TGA TAG C-3'; PECAM-1 forward: 5'-TGC TCT CGA AGC CCA GTA TT-3', PECAM-1 reverse: 5'-CGC TGA ACA CCG CGG GGT GGG AAT GGC-3'.

RNA isolation. Kidneys were dissected and washed with diethyl pyrocarbonate-treated phosphate-buffered saline (PBS). Total RNA isolation was performed with single-step RNA isolation as described by the supplier (RNAwizTM, Ambion, Austin, TX). Poly(A)/RNA was prepared from kidney EC as described previously (32).

RT-PCR analysis and DNA sequencing. Total RNA (1 μg) or poly(A)+ RNA (0.1 μg) was utilized as a template for RT-PCR (Superscript One-Step RT-PCR, Invitrogen, Carlsbad, CA) to amplify the cytoplasmic domain of all possible PECAM-1 isoforms. The sense primer was designed as 5-atggatcc1941AGG AAA GCC AAG GCC AAA1958-3, which spans the border of exons 9 and 10 within the intracellular domain. The antisense primer was designed as 5-cggaattc2291TTG ACT GTC TTA AGT TCC2274 –3, which spans the border of exon 16 and 3'-untranslated region. The primers carry a Bam HI and an Eco RI recognition sequence (lowercase letters) to facilitate subsequent cloning. The PCR products were examined on 2.4% agarose gels to assess their integrity and expected size. For cloning, PCR products were purified with Spin Q columns (Qiagen, Valencia, CA), digested with Bam HI and Eco RI, ligated into the pGEX-2T vector (Amersham, Piscataway, NJ) cut with the same enzymes, and transformed into Escherichia coli DH5α (Invitrogen). Bacterial colonies were screened by Bam HI and Eco RI digestion of minipreps, and those with inserts were sequenced with the Big Dye reagent (Perkin Elmer, Wellesley, MA) as described previously (35). All PCR reactions were performed in an Eppendorf Gradient Cycler (Westbury, NY). For DNA sequencing the following PCR parameters were used: 96°C for 5 min, followed by 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. The DNA samples were ethanol precipitated and prepared for analysis by the DNA sequencing facility at the University of Wisconsin Biotechnology Center.

Identification of PECAM-1 isoforms. The exonic mutation sites of PECAM-1 cDNA molecules were identified by comparison of the mutant sequences with that of the wild type (35, 44, 45). The isof orm
Δ12 with a new junction in the cDNA sequence that lacks exon 12 was identified at G2135–A2190 (loss of 55 bp); isoform Δ14, which lacks exon 14, at G2252–A2310 (loss of 58 bp); isoform Δ15, which lacks exons 15, at G2309–A2333 (loss of 24 bp); isoform Δ12&14, which lacks exons 12 and 14, at G2135–A2190 and G2252–A2310 (loss of 113 bp); isoform Δ12&15, which lacks exons 12 and 15, at G2135–A2190 and G2309–A2333 (loss of 79 bp); isoform Δ14&15, which lacks exons 14 and 15, at G2252–A2333 (loss of 81 bp); and isoform Δ12,14&15, which lacks exons 12, 14, and 15, at G2135–A2190 and G2252–A2333 (loss of 136 bp). The expected size for the full-length PECAM-1 cytoplasmic domain is 351 bp according to the human PECAM-1 cDNA sequence. However, the alternatively spliced isoforms have variable sizes, smaller than the full length. The absence of exon 15 in Δ15, Δ14&15, and Δ12,14&15 isoforms changes the reading frame, resulting in termination upstream of the commonly utilized termination codon.

Preparation of lectin-coated magnetic beads. To prepare lectin-coated magnetic beads, ~30 mg (1 ml) of Dynabeads M-450 Epoxy (Dynal ASA, Oslo, Norway) was rinsed three times in a solution of 0.019 M NaH2PO4–0.081 M Na2HPO4, pH 7.7. Next, 5 mg of lectin from Bandeiraea simplicifolia (B4-lectin, Sigma, St. Louis, MO) was dissolved in 1 ml of rinse solution and then mixed with the Dynabeads and incubated overnight at 4°C on a rocker. B4-lectin-coated Dynabeads were then washed three times with PBS (pH 7.4) and resuspended in 1 ml of Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and stored at 4°C.

Tissue preparation, isolation, and culture of mouse kidney EC. Kidneys from one litter (3–5 mice) of 4-wk-old PECAM-1 mice were minced into small pieces in a 60-mm tissue culture dish with sterilized razor blades and digested in 5 ml of collagenase type I (1 mg/ml in serum-free DMEM; Worthington, Lakewood, NJ) for 30–45 min at 37°C. After digestion, DMEM with 10% fetal bovine serum (FBS) was added and cells were pelleted. The cellular digests then were filtered through a double layer of sterile 400-μm nylon mesh (Sefar America, Fisher Scientific, Hanover Park, IL), centrifuged at 400 g for 10 min to pellet cells, and cells were washed twice with DMEM containing 10% FBS. The cells were resuspended in 1.5 ml of medium (DMEM with 10% FBS) and incubated with B4-lectin-coated magnetic beads for 3 h at 4°C with constant rocking. After affinity binding, magnetic beads were washed six times with DMEM with 10% FBS and bound cells in EC growth medium were plated into a single well of a 24-well plate precoated with 2 μg/ml of human fibronectin (BD Biosciences, Bedford, MA). Kidney EC were grown in DMEM containing 20% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% nonessential amino acids, 100 μg/ml streptomycin, 100 U/ml penicillin, freshly added heparin at 55 U/ml (Sigma), 100 μg/ml endothelial growth supplement (Sigma), and murine recombinant interferon-γ (R & D, Minneapolis, MN) at 44 units/ml. Cells were maintained at 33°C with 5% CO2. Cells were progressively passed to larger plates, maintained, and propagated on 1% gelatin-coated 60-mm dishes (38). The experiments described here were performed with three separate isolations of cells, with similar results.

Scratch wound assay. Cells (4 × 105) were plated on gelatin-coated 60-mm tissue culture dishes and allowed to reach confluence (2–3 days). After the medium was aspirated, cell layers were wounded with a 1-ml micropipette tip. Plates were then rinsed with PBS and fed with growth medium, and wound closure was monitored and photographed at 0, 24, 48, and 72 h. The distance migrated as percentage of total distance was determined for quantitative assessments as described previously (29). Similar assays were performed in the presence of 5-fluorouracil (10 μg/ml; Sigma) to rule out the potential contribution of differences in cell proliferation. These experiments were repeated at least twice, with similar results from two different isolations of cells.
Transwell migration assay. Transwell filters (Costar 3422) were coated with 2 μg/ml of fibronectin, rinsed with PBS, and then blocked with 2% BSA. Five hundred microliters of serum-free DMEM medium was added to the bottom of each well, and 1 × 10^5 cells in 100 μl of medium were added to the top of each well. Each condition was done in duplicate. After 3 h in a 37°C tissue culture incubator, the cells and medium were aspirated and the upper side of the membrane was wiped with a cotton swab. The cells that had migrated through the membrane were fixed with 4% paraformaldehyde and stained with hematoxylin and eosin. Ten fields of cells were counted for each condition, and averages and SD were determined.

Capillary morphogenesis in Matrigel. Matrigel (10 mg/ml; BD Biosciences) was applied at 0.5 ml per 35-mm tissue culture dish and incubated at 37°C for at least 30 min to harden. Cells were removed with trypsin-EDTA, washed with growth medium once, and resuspended at 1.0 × 10^5 cells/ml in serum-free growth medium. Cells (2 ml) were gently added to the Matrigel-coated plates, incubated at 37°C, monitored for 6–24 h, and photographed with a Nikon microscope equipped with a digital camera. For quantitative assessment of the data the mean numbers of branch points in 10 high-power fields (×100) were determined at 24 h. A longer incubation of the cells did not result in further branching morphogenesis.

Cell adhesion assays. Cell adhesion assays were performed as described previously (29). Briefly, varying concentrations of fibronectin, vitronectin, collagen type I, and laminin (all from BD Biosciences) prepared in TBS with 2 mM Ca^{2+} and 2 mM Mg^{2+} were coated on 96-well plates. As a control, wells were coated with 1% BSA overnight at 4°C. After being blocked with 1% BSA, 5 × 10^4 cells were removed by dissociation solution (Sigma), washed in serum-free medium, and added to each well, and the cells were allowed to adhere to the plate for 1.5 h at 37°C. The plate was washed with TBS containing 2 mM Ca^{2+} and 2 mM Mg^{2+} until no cells remained in the wells coated with BSA. The number of cells in each well was quantified by measuring the cellular acid phosphatase activity as previously described (29, 51). All samples were done in triplicate, and three different isolations of cells were used.

Western blot analysis. For fibronectin, tenasin-C, thrombospondin (TSP1), and TSP2 expression analysis, cells were plated at 4 × 10^5 on 1% gelatin-coated 60-mm dishes and allowed to reach nearly 90% confluence in 2 days. The cells were then rinsed once with serum-free medium and incubated with serum-free DMEM for 48 h. Conditioned medium was then collected and clarified by centrifugation. Equal volumes of samples were mixed with appropriate volumes of 6× SDS buffer and analyzed by SDS-PAGE (4–20% Tris-glycine gels; Invitrogen). The proteins were transferred to a nitrocellulose membrane, and the membrane was incubated with a rabbit anti-rat fibronectin polyclonal antibody (1:2,500; Invitrogen), a rabbit anti-chicken tenasin-C polyclonal antibody (1:3,000; Chemicon International, Temecula, CA); an anti-TSP1 monoclonal antibody (1:3,000; Clone A6.1, Neo Marker, Fremont, CA), or an anti-TSP2 monoclonal antibody (1:4,000; BD Biosciences). The membrane containing total cell lysate was also incubated with an antibody to β-catenin (1:3,000; BD Biosciences) as loading control. The blot was washed, incubated with appropriate secondary antibody, and developed with enhanced chemiluminescence (Amersham, Piscataway, NJ) (23).

FACScan analysis. FACScan analysis was performed essentially as previously described (20). The cells were washed once with PBS containing 0.04% EDTA and incubated with 3 ml of dissociation solution (Sigma) to remove the cells from the plate. The cells (1 × 10^6) were washed with TBS, blocked in TBS containing 1% goat serum on ice for 20 min, and incubated with 2 μg/ml of anti-PECAM-1 (MEC13.3; BD Transduction), anti-vascular endothelial...
(VE)-cadherin (Alexis Biochemicals, San Diego, CA), B2-lectin (Sigma), anti-α5 (555001; BD Pharmingen), anti-α6 (MAB1949; Chemicon), anti-αv (01521 D; BD Pharmingen), anti-β3 (SC-8978; Santa Cruz), anti-β1 (MAB1957; Chemicon), anti-αv/β3 (MAB1976Z; Chemicon), or control IgG (Chemicon). The cells were washed with TBS containing 1% BSA and then incubated with the appropriate FITC-conjugated secondary antibody (1:200; Pierce, Rockford, IL) on ice for 30 min. After the incubation, the cells were washed twice with TBS containing 1% BSA and resuspended in 0.5 ml of TBS containing 1% BSA. FACScan analysis was performed on a FACScan caliber flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Indirect immunofluorescence staining. For staining kidney sections, kidneys were surgically removed, fixed in 10% phosphate-buffered formalin, and embedded in paraffin. Kidney sections (5 μm) were deparaffinized in xylene, rehydrated in an ethanol gradient, washed in water, and boiled in antigen unmasking solution (Vector, Burlingame, CA). The sections were allowed to cool overnight at room temperature, followed by washing in water and PBS and incubation with blocking buffer (PBS with 1% BSA, 2% skim milk, and 0.3% Triton X-100). After blocking, the sections were incubated with anti-tenascin-C (1:400; Chemicon) followed by incubation with indocarbocyanin (CY3)-labeled secondary antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Sections were then photographed with a fluorescence microscope (Axiophot, Zeiss) in digital format.

For staining kidney EC, cells were plated on chamber slides (CC1; Falcon, Franklin Lakes, NJ) coated with 1% gelatin as described above. Cells were then rinsed with PBS, fixed with 3% paraformaldehyde for 10 min on ice, washed twice with PBS, and incubated with anti-vinculin antibody (1:100; Sigma) for 30 min at 37°C. After incubation, slides were washed three times with TBS, and cells were incubated with appropriate CY2-conjugated secondary antibody (Jackson ImmunoResearch) and tetramethylrhodamine isothiocyanate-labeled phalloidin (Sigma) at 37°C for 30 min. Cells were washed three times with TBS, mounted, and photographed with a Zeiss fluorescence microscope (Axiophot) in digital format.

Construction of adenoviruses and expression of PECAM-1 isoforms in EC. PECAM-1 isoform cDNAs coding for full-length and Δ14&15 were prepared as previously described (33). Full-length or Δ14&15 PECAM-1 cDNA was obtained as an Hin dIII/ Nor I fragment, blunted, and ligated with the intermediate plasmid (pShuttle; Stratagene, La Jolla, CA) digested with Eco RV. The ligation was transformed into E. coli DH5α-competent cells (Invitrogen), and positive clones were confirmed by restriction digestion and DNA sequencing. The functionality of the construct was further confirmed by expression and Western blotting in HEK-293 cells. For construction of a recombinant adenovirus, pShuttle/mPECAM-1 isoforms were linearized by Pac I and introduced into E. coli BJ5183-AD-1 (Strategene), which contains the viral plasmid, and the recombinant clones were screened by Pme I digestion. The isolated recombinant viral plasmid DNA was then digested with Pac I to release the viral DNA. This viral DNA was then introduced into HEK-293 cells for viral packaging and amplification. The amplified virus was then titered and used for expression studies. For adenoviral infection, 3 × 10⁵ EC were plated in a 35-mm culture dish. The next day, cells were rinsed twice with serum-free medium and infected with adenoviral vectors containing the cDNA encoding full-length or Δ14&15 PECAM-1 isoform or empty vector in the presence of Lipofectin (Invitrogen) for 5 h. After incubation, plates were rinsed with growth medium to remove Lipofectin solution and fed with growth medium. To confirm the effectiveness of adenoviral infection, the levels of PECAM-1 expression were analyzed by FACScan 3 days after infection. PECAM-1 isoform-transfected PECAM-1−/− kidney EC were used in wound migration, Transwell migration, and capillary morphogenesis assays as described above.

Statistical analysis. Statistical differences between groups were evaluated with Student’s unpaired t-test (2-tailed). Means ± SD are shown. P values <0.05 were considered significant.

RESULTS

Expression pattern of PECAM-1 isoforms during kidney vascular development. We previously showed (35) that multiple isoforms of PECAM-1 are expressed in kidneys from adult mice and that the expression of these isoforms may be developmentally regulated. Here we determined the identity of PECAM-1 isoforms in kidney vasculature of wild-type mice

Fig. 5. Altered expression of extracellular matrix (ECM) proteins in PECAM-1−/− kidney EC and kidney sections. A: fibronectin, tenascin-C, thrombospondin (TSP)1, and TSP2 levels were determined in conditioned medium prepared from PECAM-1+/+ and PECAM-1−/− kidney EC by Western blot analysis. Note increased expression of fibronectin and decreased expression of tenascin-C and TSP1 in PECAM-1−/− kidney EC. TSP2 levels were similar in these cells. These experiments were repeated twice with 2 different isolations of EC, with similar results. Molecular masses of fibronectin dimer (220 kDa), TSP1 and TSP2 trimers (~450 kDa), and tenascin-C pentamer (~650 kDa) are indicated. B: kidney sections prepared from postnatal day 21 PECAM-1+/+ and PECAM-1−/− mice were stained with an antibody to tenascin-C and photographed under identical conditions as described in MATERIALS AND METHODS. Note the significant decrease in expression of tenascin-C in kidney sections prepared from PECAM-1−/− mice (arrows indicate kidney glomeruli). These experiments were repeated twice with kidneys from 3 different mice. In the absence of primary antibody, no staining was observed (not shown).
from E13 to P20 and the frequency at which they occurred (Table 1). We found that multiple isoforms of PECAM-1 were expressed at different stages of kidney development with similar identity to the isoforms observed in kidneys of adult mice (35). However, the frequency at which these isoforms occurred during kidney development varied significantly. PECAM-1 lacking exons 14 and 15 was the most frequently detected isoform during embryonic and postnatal kidney development, except at E13, when the Δ12,14&15 isoform was predominant. These data, along with our previous findings, suggest that PECAM-1 isoforms may have different functions during renal vascular development and angiogenesis.

Isolation and characterization of PECAM-1+/+ and PECAM-1−/− kidney EC. PECAM-1 plays important roles during angiogenesis. Unfortunately, little is known about the molecular and cellular mechanisms that mediate PECAM-1 function during angiogenesis. To determine PECAM-1 isoform-specific functions in kidney endothelium, we isolated kidney EC from PECAM-1+/+ and PECAM-1−/− mice. Figure 1 demonstrates that kidney EC prepared from PECAM-1+/+ and PECAM-1−/− mice and plated on gelatin-coated plates exhibit a similar morphology. To confirm that these cells maintain EC characteristics, we examined the expression of EC markers in PECAM-1+/+ and PECAM-1−/− kidney EC. Both PECAM-1+/+ and PECAM-1−/− kidney EC expressed VE-cadherin and were positive for B2-lectin (Fig. 2). PECAM-1 was expressed by PECAM-1+/+ kidney EC but not PECAM-1−/− kidney EC, as expected (Fig. 2).

PECAM-1−/− kidney EC are impaired in their ability to undergo capillary morphogenesis. PECAM-1 is involved in the assembly of EC into capillary-like structures in Matrigel. Antibodies to PECAM-1 block EC capillary morphogenesis in vitro and angiogenesis in vivo (7, 33). To determine whether capillary morphogenesis is affected in the absence of PECAM-1, PECAM-1+/+ and PECAM-1−/− kidney EC were plated in Matrigel. PECAM-1+/+ kidney EC began forming a capillary network at 6 h and demonstrated a well-formed branched network by 24 h (Fig. 3A). In contrast, PECAM-1−/− kidney EC failed to organize into any significant capillary-like network in Matrigel. A quantitative assessment of the data after 24 h of incubation is shown in Fig. 3B. A longer incubation of the cells did not result in further branching morphogenesis. These results are consistent with our previous PECAM-1 antibody blocking experiments (33).

PECAM-1−/− kidney EC are less migratory. EC migration is a key step during angiogenesis. The inability of PECAM-1−/− EC to form capillary-like networks in Matrigel may suggest potential defects in cell migration. We examined migration of PECAM-1+/+ and PECAM-1−/− kidney EC with both wound migration and Transwell assays. For wound migration, the confluent monolayers of EC were wounded and wound closure was monitored in the presence of 5-fluorouracil (10 μg/ml) to prevent proliferation, as described in MATERIALS AND METHODS. PECAM-1+/+ kidney EC migrated effectively and closed the wound after 48 h (Fig. 4A). However, in PECAM-1−/− kidney EC a significant area of wound re-
mained uncovered by 48 h (Fig. 4A). The quantitative assessment of the data is shown in Fig. 4B (P < 0.01).

We also examined migration of PECAM-1+/+ and PECAM-1−/− kidney EC with a Transwell assay. The membranes were coated with fibronectin (2 µg/mL), and the number of cells that migrated through the membrane to the bottom side was determined 3 h later. We observed a significant decrease in the number of PECAM-1−/− kidney EC that migrated through the membrane compared with their PECAM-1+/+ counterparts (Fig. 4C; P < 0.01). Thus PECAM-1−/− kidney EC migrate slower than PECAM-1+/+ kidney EC.

PECAM-1−/− kidney EC secrete increased amount of fibronectin and decreased amount of tenasin-C and TSP1. ECM proteins are implicated in regulation of angiogenesis, including EC adhesion and migration. In particular, fibronectin, tenasin-C, TSP1, and TSP2 play important roles in EC migratory and proliferative events and regulation of angiogenesis (4, 21, 23, 32, 50). We investigated secretion of ECM proteins by PECAM-1+/+ and PECAM-1−/− kidney EC. PECAM-1−/− kidney EC secreted increased amounts of fibronectin compared with PECAM-1+/+ kidney EC. In contrast, we observed decreased secretion of tenasin-C and TSP1 in PECAM-1−/− cells compared with PECAM-1+/+ cells (Fig. 5A). The level of TSP2 was not significantly different in these cells. The decreased production of tenasin-C is consistent with reduced migratory properties of PECAM-1−/− kidney EC and their inability to form capillary networks in Matrigel. Decreased expression of tenasin-C was also observed in kidney sections prepared from PECAM-1−/− mice compared with PECAM-1+/+ mice (Fig. 5B). The levels of β-catenin in cell lysates were used to show similar loading of samples (Fig. 5A).

Expression of integrins in kidney EC. Interaction of ECM proteins and EC plays a major role in EC behavior such as adhesion, migration, and capillary morphogenesis. We next investigated EC-related integrin expression on PECAM-1+/+ or PECAM-1−/− kidney EC by FACScan analysis. Expression of αvβ3-integrin and, α5β1, and β3-integrin subunits on PECAM-1+/+ and PECAM-1−/− kidney EC were similar. Neither PECAM-1+/+ nor PECAM-1−/− kidney EC expressed α1-integrin subunit (Fig. 6).

PECAM-1−/− kidney EC are less adherent on vitronectin. Altered integrin expression and/or activity may affect cell adhesion and migration. We observed similar expression levels of integrins in PECAM-1+/+ and PECAM-1−/− kidney EC. However, the observed differences in migratory properties of these cells suggested that alteration in cell adhesive properties may exist. We next assessed cell adhesion to fibronectin, vitronectin, collagen type I, and laminin of PECAM-1−/− kidney EC. In contrast, both PECAM-1+/+ and PECAM-1−/− kidney EC adhered well to fibronectin and vitronectin, with PECAM-1+/+ cells adhering better to vitronectin while PECAM-1−/− cells adhered better to fibronectin.

We also examined actin cytoskeleton organization and the distribution of focal adhesions by phalloidin and vinculin staining, respectively. PECAM-1+/+ kidney EC exhibited numerous actin stress fibers and small and well-defined focal adhesions (Fig. 8, A and B), similar to PECAM-1−/− kidney EC expressing Δ14&15 PECAM-1 (Fig. 8, G and H). However, PECAM-1−/− kidney EC exhibited a peripheral cortical
actin organization with less numerous and larger focal adhesions (Fig. 8, C and D), similar to PECAM-1/H11002/kidney EC expressing full-length PECAM-1 (Fig. 8, E and F). These data are consistent with the slower migration of PECAM-1−/− kidney EC. Thus PECAM-1 expression may affect activity of integrins impacting cell adhesive and migratory properties in an isoform-specific manner.

Expression of Δ14&15, but not full-length, PECAM-1 restores migratory and capillary morphogenesis defects observed in PECAM-1−/− kidney EC. The impact of specific PECAM-1 isoforms in migration and capillary morphogenesis of kidney EC has not been previously addressed. PECAM-1−/− kidney EC were infected with adenoviruses that express full-length PECAM-1, Δ14&15 PECAM-1, or empty vector. PECAM-1+/+ kidney EC were also infected with empty vector as a control. PECAM-1 expression in PECAM-1−/− kidney expressing Δ14&15 PECAM-1 is shown in Fig. 9, A and B. The expression of full-length and Δ14&15 PECAM-1 isoforms in PECAM-1−/− kidney EC is shown in Fig. 9, C and D, respectively. These results confirmed similar levels of PECAM-1 expression in PECAM-1−/− kidney EC compared with PECAM-1+/+ kidney EC.
We next evaluated the effects of specific PECAM-1 isoform expression on kidney EC migration with the wound migration assay. Monolayers of kidney EC expressing empty vector (Fig. 10, A and B), full-length PECAM-1 (Fig. 10C), or Δ14&15 PECAM-1 (Fig. 10D), were wounded, and wound closure was evaluated after 48 h. A significant portion of the wound remained uncovered in PECAM-1+/− kidney EC expressing the empty vector (Fig. 10B) or full-length PECAM-1 (Fig. 10C). In contrast, PECAM-1+/− kidney EC expressing Δ14&15 PECAM-1, like PECAM-1+/+ kidney EC, completely covered the wound after 48 h (Fig. 10, A and D). Quantification of the data is shown in Fig. 10E (P < 0.01). Similar results were observed in the Transwell migration assay (Fig. 10F; P < 0.01). Thus expression of Δ14&15, but not full-length, PECAM-1 restores the migratory defect observed in PECAM-1+/− kidney EC.

We then evaluated the ability of kidney EC expressing empty vector or PECAM-1 isoforms to undergo capillary morphogenesis in Matrigel (Fig. 11). As expected, PECAM-1+/+ kidney EC expressing the empty vector formed a capillary network (Fig. 11A), while PECAM-1+/− kidney EC expressing the empty vector failed to do so (Fig. 11B). These data are consistent with those shown in Fig. 3. PECAM-1+/− kidney EC expressing full-length PECAM-1 attempted to organize but failed to form an extensive network (Fig. 11C). In contrast, PECAM-1+/− kidney EC that expressed Δ14&15 PECAM-1 organized into an extensive capillary-like network (Fig. 11D). The quantitative assessment of the data is shown in Fig. 11E. Thus expression of Δ14&15, but not full-length, PECAM-1 restored the capillary morphogenesis defect observed in the absence of PECAM-1.

**DISCUSSION**

Appropriate and sufficient organ vascularization plays an integral role during development and repair of tissue damage (12). PECAM-1 is an EC adhesion molecule with important roles in angiogenesis. Multiple isoforms of PECAM-1 are present in the adult kidney. However, the identity and distribution of these isoforms during embryonic and postnatal kidney vascular development have not been previously examined.

To gain further insight into the role PECAM-1 and its specific isoforms play during kidney vascular development, we determined the identity and frequency at which isoforms of PECAM-1 are expressed in murine kidney at different stages of development. Here we show that multiple isoforms of PECAM-1 are expressed in murine kidney at different stages of development. We show that multiple isoforms of PECAM-1 are present during kidney vascular development, similar to isoforms detected in adult mice (35). However, the frequencies at which these isoforms were detected varied significantly during embryonic and postnatal kidney development. Δ14&15 PECAM-1 was the most frequently detected isoform during embryonic and postnatal kidney vascular development. In addition, PECAM-1+/− kidney EC showed reduced migration, failed to undergo capillary morphogenesis in Matrigel, and had altered ECM expression and integrin activity. Expression of Δ14&15, but not full-length, PECAM-1
was sufficient to restore migration and capillary morphogenesis defects observed in PECAM-1−/− kidney EC. Thus the complex interaction of cell adhesive molecules and ECM proteins may play an important role in organization of microvascular endothelium during kidney development and angiogenesis, and it is influenced by alternative splicing of PECAM-1.

PECAM-1 plays an important role during EC monolayer formation and capillary morphogenesis. Antibodies to PECAM-1 block EC capillary morphogenesis in vitro (33) and angiogenesis in mouse corneal pocket assay (7). Mice that lack PECAM-1 are born but exhibit significant defects in angiogenesis and inflammation (9, 15, 37). Here we show that kidney EC prepared from PECAM-1−/− mice fail to undergo capillary morphogenesis in Matrigel. These cells also were less migratory and exhibited altered expression of various ECM proteins and integrin activity impacting their adhesive and migratory properties (8, 16, 17). Thus these data suggest that

Fig. 10. Migration of PECAM-1−/− kidney EC expressing a specific PECAM-1 isoform. The migration of PECAM-1+/+ kidney EC (A), PECAM-1−/− kidney EC infected with empty virus (B), and PECAM-1−/− kidney EC infected with adenovirus expressing full-length (C) or Δ14&15 (D) PECAM-1 was evaluated in wound migration assays as described in MATERIALS AND METHODS. Note that PECAM-1−/− kidney EC expressing Δ14&15, but not full-length, PECAM-1 migrated and closed the wound similar to PECAM-1+/+ EC. E: quantitative assessment of the data. Data are the mean number of branch points from 10 high-power fields (×100). Note that PECAM-1−/− kidney EC expressing Δ14&15, but not full-length, PECAM-1 organize an extensive capillary-like network, very similar to PECAM-1+/+ kidney EC (P < 0.05). These experiments were repeated twice with 2 different isolations of kidney EC, with similar results.

Fig. 11. Capillary morphogenesis of PECAM-1−/− kidney EC expressing a specific PECAM-1 isoform. Capillary morphogenesis of PECAM-1+/+ kidney EC (A), PECAM-1−/− kidney EC infected with empty adenovirus (B), and PECAM-1−/− kidney EC infected with adenoviruses expressing full-length (C) or Δ14&15 (D) PECAM-1 was evaluated on Matrigel as described in MATERIALS AND METHODS. E: quantitative assessment of the data. Data are the mean number of branch points from 10 high-power fields (×100). Note that PECAM-1−/− kidney EC expressing Δ14&15, but not full-length, PECAM-1 organize an extensive capillary-like network, very similar to PECAM-1+/+ kidney EC (P < 0.05). These experiments were repeated twice with 2 different isolations of kidney EC, with similar results.
expression of PECAM-1 impacts not only cell-cell interactions but also cell-matrix interactions that are important during angiogenesis.

We previously showed (35, 44, 45) that multiple isoforms of PECAM-1 are present in vascular beds of various tissues of adult mice, including the kidney. Using an exon 14-specific antibody, we showed that not only are the products of these isoforms present but also their expression is developmentally regulated (35, 47, 48). We also showed that these patterns are maintained in adult animals and EC prepared from these mice (35, 45, 48). Here we show that multiple isoforms of PECAM-1 are present in embryonic and postnatal developing kidney vasculature and that Δ14&15 PECAM-1 is the predominant isoform detected. This is consistent with our previous observations in adult kidney and mouse endothelium in general, as well as mouse hematopoietic cells and platelets (35, 44, 45), where Δ14&15 PECAM-1 is the most frequently detected isoform. However, the specific function of PECAM-1 isoforms during vascular development and angiogenesis remains unknown.

Using PECAM-1+/− kidney EC, we determined the impact of expression of full-length and Δ14&15 PECAM-1 on EC migration and capillary morphogenesis. The expression of Δ14&15, but not full-length, PECAM-1 restored the migratory and capillary morphogenesis defects observed in PECAM-1−/− kidney EC. This is consistent with our observation that full-length PECAM-1 occurs at a very low frequency in mouse kidney endothelium. Furthermore, expression of full-length human PECAM-1 in NIH3T3 (lack PECAM-1), ECV304 (lack PECAM-1), and bovine aortic EC (have PECAM-1) slowed their migration (20, 31). However, expression of the Y-to-F mutation at residue Y686 (in exon 14) of full-length human PECAM-1 in these cells had a promoting effect on their migration. These data suggest that the lack of exon 14 and/or its tyrosine phosphorylation has enhanced migratory effects. Thus the presence of multiple isoforms of PECAM-1 in the endothelium may suggest potential cross talk among PECAM-1 isoforms and regulation of EC phenotype and angiogenesis in a finely balanced process.

PECAM-1 is now recognized as a signaling molecule that functions as a scaffold and/or adaptor protein for organizing signaling complexes that impact EC phenotype. The murine PECAM-1 contains five tyrosine residues in its cytoplasmic domain whose phosphorylation provides docking sites for a number of signaling proteins, including SHP2, Src, Shc, Crk-L, and Grb2 (46). A major emphasis has been put on tyrosine residues in exons 13 and 14, which form the immunoreceptor tyrosine-based inhibitor motifs (ITIMs) (24). ITIMs are involved in recruitment of signaling molecules that are involved in downregulation of signals from tyrosine kinase receptors. However, the role these motifs play in modulation of intracellular signaling in EC during angiogenesis remains unexplored.

Previous studies indicated that the presence or absence of PECAM-1 exon 14 and/or its tyrosine phosphorylation impacts its homophilic interactions in mouse lung fibroblasts (10, 49). We showed that an isoform of PECAM-1 with exon 14 (Δ15 PECAM-1), but not Δ14&15 PECAM-1 (lacks exon 14), can activate the MAPK/ERKs when expressed in an epithelial cell line (34). This was associated with alterations in cell adhesive and migratory properties. We later showed that PECAM-1 associated with SHP2, Shc, Crk-L/C3G, and Grb2/SOS in an exon 14-dependent manner, while its association with Src was exon 14 independent (46). PECAM-1 has been recently shown to activate MAPK/ERKs in EC in response to shear stress in a SHP2-dependent manner (13, 40).

Expression of PECAM-1 in kidney EC affected their adhesion to ECM proteins, particularly vitronectin, a ligand of αvβ3-integrin. Our integrin expression studies indicated minimal changes in integrin expression suggesting changes in the integrin activity. Ligation of PECAM-1 on the surface of leukocytes and platelets modulates the activities of β1-, β2-, and β3-integrins (2, 6, 26, 41, 42). In addition, αvβ3-integrin interacts with PECAM-1 with potential impacts on EC function during angiogenesis and inflammation (3, 27). We recently showed (46) that interaction of αvβ3-integrin with PECAM-1 is isoform specific. The association of αvβ3-integrin with Δ15 PECAM-1 resulted in its lack of localization to focal adhesions and reduced adhesion to vitronectin. Thus isoform-specific association of PECAM-1 with αvβ3-integrin may modulate integrin localization and/or activity. PECAM-1 may also modulate integrin activity through activation of small GTPase Rap1 (11, 28). The role of Rap1 in modulation of integrin activity by PECAM-1 has been demonstrated in platelets and leukocytes. However, the components of the bidirectional signaling pathways that mediate the interactions between integrins and PECAM-1, especially in EC, remain to be determined.

In summary, PECAM-1 is a critical modulator of kidney EC adhesion, migration, and capillary morphogenesis. PECAM-1+/− kidney EC are less migratory and fail to undergo capillary morphogenesis in Matrigel. However, expression of PECAM-1 is sufficient to restore these defects in an isoform-specific manner. Therefore, PECAM-1 isoforms may exhibit different adhesive and signaling properties during vascular development and angiogenesis. Further studies are needed to determine how these different adhesive properties are modulated by PECAM-1 isoforms and what intracellular signaling pathways are engaged to regulate angiogenesis.

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