Human Podocytes Express Angiopoietin 1, a Potential Regulator of Glomerular Vascular Endothelial Growth Factor

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Abstract. Vascular endothelial growth factor (VEGF) is abundantly expressed by podocytes, but its role in glomeruli is unknown. Angiopoietins are endothelial cell growth factors that function in concert with VEGF but have not previously been observed in human glomeruli. Angiopoietin 1 (Ang1) acts via the endothelial receptor Tie2 to promote maturation and stabilization of blood vessels, resisting angiogenesis and opposing some actions of VEGF. Ang1, Ang2, Tie2, and VEGF expression in normal human renal cortex was examined with immunofluorescence and immunohistochemical analyses. High-power, multiple-color, immunofluorescence analyses and additional antibodies (specific for particular components of the glomerular filtration barrier) were used to determine the exact locations of Ang1 and Tie2 in the glomerular capillary wall. Immuno-electron-microscopic analysis of rat glomeruli was used to further localize endothelial Tie2 expression. RNA and protein extracted from human glomeruli, cultured human podocytes, and cultured human endothelial cells were analyzed for Ang1, Ang2, and Tie2 by using reverse transcription-PCR and Western blotting. Ang1 was detected in podocytes in normal glomeruli and, with VEGF, was concentrated in podocyte foot processes. Tie2 was demonstrated on glomerular capillary endothelial cells, particularly on the abluminal surface. Reverse transcription-PCR and Western blotting analyses confirmed the expression of Ang1 and Tie2 in glomeruli and of Ang1 in cultured podocytes. These findings suggest a role for Ang1 in the maintenance of the glomerular endothelium and make it a good candidate to be a regulator of the actions of VEGF on glomerular permeability, resisting angiogenesis while allowing the induction of high permeability to water and small solutes.

Vascular endothelial growth factor (VEGF) is abundantly expressed in glomeruli by podocytes (visceral glomerular epithelial cells) (1), but its functional role and the factors responsible for its regulation are poorly understood. Angiopoietins are endothelial cell-specific growth factors that function in concert with VEGF in the development and maintenance of the vascular endothelium and in the regulation of vascular permeability (2). We hypothesized that angiopoietins may have important roles in adult renal glomeruli in these respects. In systemic vessels, angiopoietin 1 (Ang1) is produced by periendothelial cells, including vascular smooth muscle cells, and acts via the endothelial cell Tie2 tyrosine kinase receptor (3). Ang1 promotes vascular development and maturation (4), and its continued expression, along with Tie2 phosphorylation, in adults suggests a role in vascular maintenance (5). Ang1 reduces endothelial permeability both in adult mice (6) and in cultured human cells (7). Ang2 is produced principally by endothelial cells and antagonizes Ang1 by competitive inhibition at the Tie2 receptor (8). In adults, Ang2 is expressed in tissues undergoing vascular remodeling, where it causes loosening of the mature vascular structure, which may be a prerequisite for new vessel growth induced by VEGF (9).

Angiopoietins have not previously been observed in mature kidneys of any species, but mRNA for Ang1 and Tie2 (10) and Ang2 transgene activation (11) have been detected in the glomerular tufts of embryonic mouse kidneys, where they are proposed to be involved in glomerular development. In adult glomeruli, Ang1 has the potential to play an ongoing role in the maintenance of the glomerular permeability barrier, by stabilizing endothelial cells and allowing them to resist the potent angiogenic stimulus provided by VEGF.

We examined the expression and distribution of angiopoietins and Tie2 in adult human glomeruli by using immunofluorescence (IF) and immunohistochemical techniques and in cultured endothelial cells and podocytes by using reverse transcription (RT)-PCR and Western blotting. We further defined endothelial expression of Tie2 by using immuno-electron-microscopic (IEM) analysis of rat glomeruli. We have demonstrated that Ang1 is expressed by podocytes and is localized to foot processes and that Tie2 is
expressed by glomerular endothelial cells, particularly on the abluminal membrane.

**Materials and Methods**

**Nephrectomy Tissue**

Human renal tissue was obtained from patients undergoing nephrectomies because of polar renal tumors. Within 30 min after nephrectomy, samples of cortex from the normal pole were obtained for preparation of frozen sections and paraffin sections and for extraction of glomeruli by sieving.

**Immunostaining**

Frozen sections were air-dried before immunostaining with polyclonal antibodies to Ang1, Ang2, Tie2, or VEGF (Santa Cruz Biochemicals, Santa Cruz, CA). Control sections were incubated with same-species Ig or with specific antibodies that had been preincubated with blocking peptide (Santa Cruz). Secondary antibodies were FITC-labeled anti-goat IgG (for Ang1 and Ang2) or FITC-labeled anti-rabbit IgG (for Tie2 and VEGF). Formalin-fixed paraffin sections were stained with the nitro-blue tetrazolium method, as previously described (12), using the same antibodies. The exact localization of angiopoietins and Tie2 within glomeruli was determined by high-power, multiple-color, IF analysis (at a magnification of 1000, with oil immersion), using monoclonal antibodies specific for particular components of the glomerular filtration barrier, *i.e.*, platelet-endothelial cell adhesion molecule-1 (R&D Systems, Minneapolis, MN) for endothelial cells, type IV collagen α3 chain (Wieslab AB, Lund, Sweden) for the glomerular basement membrane, and nephrin (a gift from Karl Tryggvason, Karolinska Institute, Stockholm, Sweden) for podocyte foot processes. Anti-mouse IgG secondary antibodies were labeled with FITC, tetrarhodamine isothiocyanate, or aminomethylcoumarin acetic acid.

**IEM**

Blocks of renal cortex from perfusion-fixed (4% paraformaldehyde in phosphate buffer, pH 7.2) rat kidneys were dehydrated in ethanol and embedded in K4M Lowicryl (Polysciences Inc., Warrington, PA) (13). Ultrathin sections were incubated with antibodies to Tie2 (as described above), followed by goat anti-rabbit IgG-gold (10 nm) conjugate. In control sections, primary antibodies were omitted or

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Table 1. Primers and conditions for PCR analyses

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Corresponding Nucleotides</th>
<th>Annealing Temperature (°C)</th>
<th>Product Length (bp)</th>
</tr>
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<tr>
<td>Ang1</td>
<td>forward</td>
<td>AGAACCACACGGCTACCATGCT</td>
<td>671 to 692</td>
<td>410</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>TGTGCCATACGCTCCAGTTGC</td>
<td>1059 to 1080</td>
<td></td>
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<tr>
<td>Ang2</td>
<td>forward</td>
<td>AGCTGTGATCTTGTCTTGGC</td>
<td>377 to 396</td>
<td>445</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>GTTCAGTCTGTCGTGCTG</td>
<td>802 to 821</td>
<td></td>
</tr>
<tr>
<td>Tie2</td>
<td>forward</td>
<td>CCTTAATGAACCAGCACCAGG</td>
<td>335 to 356</td>
<td>460</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>ACTTCTGGGTCTCACATCCTC</td>
<td>773 to 794</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>forward</td>
<td>GCCAAAGGGTCATCATCTTC</td>
<td>422 to 440</td>
<td>286</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>GTAGAGGCAGGGATGATGTT</td>
<td>688 to 707</td>
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</tr>
</tbody>
</table>

**Figure 1.** Immunofluorescence (IF) staining of consecutive fresh-frozen sections of normal cortex. Magnification, ×400. (A) Glomeruli stain positively for angiopoietin 1 (Ang1). (B) Glomeruli are negative for Ang2.

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*Ang1, angiopoietin 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.*
replaced by irrelevant antibodies. Sections were stained with lead citrate and examined with a JEOL 1010 electron microscope (JEOL, Tokyo, Japan). Rat kidney sections were also immunostained for Tie2 as described above for human kidneys, to confirm similar Tie2 distributions.

**Cell Culture**

Human microvascular endothelial cells (HMVEC) (derived from adult lung tissue; Clonetics Corp., San Diego, CA) were obtained at passage 3 and used for experiments up to passage 7. A conditionally immortalized human podocyte cell line was cultured as described previously (14).

**RT-PCR**

RNA was extracted from sieved glomeruli and from cultured cells by using a standard phenol-chloroform method and was reverse-

*Figure 2.* Immunohistochemical analysis of paraffin sections of normal cortex. Magnification, ×400. (A) Glomeruli and vascular smooth muscle cells are positive for Ang1. (B) Staining for Ang1 is lost after preincubation of antibody with blocking peptide.

*Figure 3.* IF staining of normal cortex for Tie2. (A and B) Consecutive sections are presented. Magnification, ×200. (A) Tie2 is detected in glomeruli. (B) The control antibody-treated section does not exhibit glomerular staining. (C) Tie2 is distributed in a capillary endothelial pattern (arrows). Magnification, ×1000.
transcribed with a kit (Promega, Madison, WI) before PCR amplification of sequences specific for Ang1, Ang2, and Tie2 (Table 1). The identity of the PCR products was confirmed by forward and reverse sequence analysis (ABI Prism 377; ABI, Madison, WI).

**Western Blotting**

Cultured cells (HMVEC and podocytes) and sieved glomeruli were lysed in Laemmli sample buffer, and solubilized protein concentrations were determined (bicinchoninic acid assay; Pierce Chemical

**Figure 4.** Multiple-color, high-power, IF analysis of sections through individual glomerular capillary loops, stained with various antibodies. b, Bowman’s space; c, capillary lumen. Magnification, ×1000. (A) Ang1 (green) is external to endothelial platelet-endothelial cell adhesion molecule-1 (red). (B) Ang1 (green) is also external to type IV collagen (red) in the glomerular basement membrane. (C) Ang1 (green) colocalizes with nephrin (red) in the same layer of the glomerular capillary wall (arrow), confirming expression of Ang1 in podocyte foot processes. (D) Vascular endothelial growth factor (VEGF) (green) is similarly distributed in podocyte foot processes, external to endothelial platelet-endothelial cell adhesion molecule-1 (red). (E and F) Tie2 (green) is expressed on the endothelium, internal to podocyte foot processes, which are labeled for nephrin (red) (E), and internal to the glomerular basement membrane (red) (F). (G and H) Ang1 (green) in podocyte foot processes is external to the glomerular basement membrane (blue), whereas endothelial Tie2 (red) is internal.
Co., Rockford, IL). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and were blotted onto nitrocellulose membranes. The membranes were air-dried and blocked in 5% fat-free milk before incubation with antibodies to Ang1, Ang2, Tie2, and actin (Sigma Chemical Co., St. Louis, MO), as described above. After incubation with horseradish peroxidase-conjugated secondary antibodies, bands were detected by using the ECL chemiluminescence system (Amersham, Biotech Ltd., Bucks, UK). As with PCR, HMVEC were included principally as a positive control. Recombinant Ang1 (15) (a gift from Regeneron Pharmaceuticals Inc., Tarrytown, NY) and Ang2 (R&D Systems) were used as additional positive controls.

Results

Ang1 was detected in a linear pattern in adult glomeruli in frozen and paraffin-embedded sections (Figures 1 and 2), but Ang2 was not detected. Ang1 was also detected in vascular smooth muscle cells, as in other tissues (3). Tie2 was detected in a predominantly endothelial distribution in glomeruli (Figure 3). Using high-power, multiple-color, IF analyses, we demonstrated that Ang1 was localized externally to the glomerular basement membrane in the capillary wall and that its distribution was identical to that of nephrin, confirming its expression in podocyte foot processes (Figure 4, A to C, G, and H). VEGF was distributed in a similar pattern (Figure 4D), whereas Tie2 was expressed on the glomerular endothelium (Figure 4, E to H). As assessed in IEM analyses of normal rat glomeruli, Tie2 was expressed on both surfaces of the endothelium but predominantly on the abluminal surface (Figure 5). Some Tie2 was also detected on podocyte foot processes.

RT-PCR (Figure 6) and Western blotting (Figure 7) confirmed the expression of Ang1 and Tie2 in glomeruli and the expression of Ang1 in cultured podocytes, at both the mRNA and protein levels. These findings are in accordance with the aforementioned histologic data indicating that podocytes are the source of glomerular Ang1. Ang2 mRNA was detected at low levels in glomeruli, but Ang2 protein was not detected in either glomeruli or cultured podocytes, again consistent with IF observations. Ang1, Ang2, and Tie2 mRNA and protein were detected in cultured endothelial cells, serving as a useful positive control, although we observed no evidence of Ang1 or Ang2 expression by glomerular endothelium in vivo.
Discussion

We have demonstrated that Ang1 is expressed by podocytes both in culture and in mature human glomeruli. This is an important development based on previous reports of the detection of Ang1 mRNA in embryonic mouse glomerular tufts (10) and is consistent with the production of Ang1 by periendothelial cells in other tissues (3). Furthermore, Ang1 seems to be concentrated in podocyte foot processes in vivo, as is VEGF. Taken together with the presence of Tie2 (described here) and VEGF receptor 2 (16) on glomerular endothelium, these findings have implications for the functional roles of these molecules in the regulation and maintenance of the glomerular endothelium. The transport of Ang1 and VEGF within podocytes to the foot processes suggests that these molecules are released to have some local action, most obviously on the adjacent endothelium.

The known actions of Ang1 that may be important in glomeruli include stabilization of capillary structure, promotion of endothelial cell survival, and reduction of endothelial permeability. Ang1 reduces permeability via the paracellular route, by increasing the integrity of interendothelial cell junctions (4, 7). This action of Ang1 may at first appear inconsistent with the high permeability to water and small molecules exhibited by the glomerular endothelium. However, this high permeability is largely attributable to extensive transcellular endothelial fenestrations, which may be induced by VEGF produced by podocytes. This juxtaposition is analogous to that in other tissues, such as the choroid plexus, where endothelial fenestrations are induced by VEGF produced by the underlying epithelium (17). Ang1 may stabilize the capillary structure while allowing VEGF to promote high permeability to water and small molecules. The presence of Ang1 helps explain why high concentrations of glomerular VEGF in adults do not cause angiogenesis in the glomerular capillaries.

The IEM data indicating Tie2 expression predominantly on the abluminal surface of the glomerular endothelium are the first to differentiate between luminal and abluminal growth factor receptor expression in these cells. These findings are consistent with our hypothesis that Ang1 from podocytes acts on glomerular endothelial cells, and they are analogous to findings of VEGF receptor 2 expression on the abluminal surface of other endothelia (16). The detection of Tie2 on rat podocytes suggests that there may also be an autocrine loop.

The failure to detect Ang2 protein in normal adult glomeruli is consistent with previous observations in embryonic mice (11) and in human subjects, where Ang2 is readily detected only in tissues undergoing active vascular remodeling or repair (8). However, Ang2 is likely to be important in the endothelial response to glomerular injury. Both Ang1 and Ang2 were detected in cultured endothelial cells (in addition to Tie2), although there was no evidence of their expression in glomerular endothelial cells in vivo. This is consistent with previous reports indicating that, whereas endothelial cells may produce angiopoietins when stressed by injury or culture, they do not usually do so in stable tissue (3, 8).

Dysregulation of angiopoietins may be important in glomerular disease (as has been demonstrated for VEGF) (18) and may provide a therapeutic target in diseases associated with increased glomerular permeability or glomerular capillary damage. In animal models, exogenous VEGF has been demonstrated to augment glomerular repair (19) but optimal results may be produced with the combination of Ang1 and VEGF (20).

Acknowledgments

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References


