Angiopoietin 1 and Vascular Endothelial Growth Factor Modulate Human Glomerular Endothelial Cell Barrier Properties

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Abstract. Normal glomerular filtration depends on the combined properties of the three layers of glomerular capillary wall: glomerular endothelial cells (GEnC), basement membrane, and podocytes. Podocytes produce endothelial factors, including angiopoietin 1 (ang1), and vascular endothelial growth factor (VEGF), whereas GEnC express their respective receptors Tie2 and VEGFR2 in vivo. As ang1 acts to maintain the endothelium in other vascular beds, regulating some actions of VEGF, these observations suggest a mechanism whereby podocytes may direct the unique properties of the glomerular endothelium. This interaction was investigated by studies on the barrier properties of human GEnC in vitro. GEnC were examined for expression of endothelium-specific markers by immunofluorescence and Western blotting and for characteristic of the glomerular endothelium. TEER and passage of labeled albumin. Responses to a cAMP analogue and thrombin were examined before those to ang1 and VEGF. Results confirmed the endothelial origin of GEnC and their expression of Tie2 and VEGFR2. GEnC formed monolayers with a mean TEER of 30 to 40 Ω/cm². The cAMP analogue and thrombin increased and decreased TEER by 34.4 and 14.8 Ω/cm², respectively, with corresponding effects on protein passage. Ang1 increased TEER by 11.4 Ω/cm² and reduced protein passage by 45.2%, whereas VEGF reduced TEER by 12.5 Ω/cm² but had no effect on protein passage. Both ang1 and VEGF modulate GEnC barrier properties, consistent with potential in vivo roles; ang1 stabilizing the endothelium and resisting angiogenesis while VEGF induces the high permeability to water, characteristic of the glomerular endothelium.

Angiopoietins (ang) and vascular endothelial growth factors (VEGF) form two groups of growth factors that are of particular importance in directing endothelial cell (EnC) behavior. These molecules are widely expressed and have essential and coordinated actions in physiologic regulation of vascular development, maturation, and permeability (1). They are also key players in pathologic angiogenesis and alterations in permeability (2) and as such have been implicated in major diseases, including cancer, diabetic retinopathy, and arteriosclerosis. Ang1 acts via the endothelial Tie2 receptor and in the adult continued expression of ang1 in periendothelial cells (3), and the phosphorylation of Tie2, even in quiescent vessels, suggests a role in vascular maintenance (4). Ang1 is unique in its ability to reduce EnC permeability (5) and seems to regulate some of the actions of VEGF, stabilizing the endothelium and resisting permeability increases, inflammation, and angiogenesis in the mature endothelium (5, 6). Ang2 is a competitive antagonist of the actions of ang1 on the Tie2 receptor, and in the adult its expression is limited to tissues in which there is active remodeling (7). VEGF-A (often referred to simply as VEGF) is the most widely expressed member of the VEGF family and is present in a variety of biologically active isoforms arising by differential splicing, VEGF₁₆₅ being most abundant (8). Like ang1, VEGF is expressed by mural cells (including vascular smooth muscle cells [VSMC] and pericytes), which are well positioned to act as paracrine regulators of endothelial behavior. VEGF has powerful angiogenic and permeability-inducing effects acting through endothelial VEGF receptors (VEGFR1 to 3) (8).

We and others have shown that ang1 and VEGF are produced by podocytes in the adult renal glomerulus, whereas the adjacent glomerular EnC (GEnC) express the cognate receptors Tie2 and VEGFR1 and 2 (9–12). This suggests a relationship, analogous to that seen between mural cells and EnC in other vessels, whereby podocytes may influence endothelial phenotype and hence their contribution to the glomerular filtration barrier. Evidence in support of such an interaction is accumulating. Both experimental administration of neutralizing antibodies to VEGF and podocyte-specific reduction in VEGF production cause GEnC abnormalities, including swelling, vacuolation, and detachment along with proteinuria (13–15). The similar glomerular lesion of pre-eclampsia may be caused by reductions in circulating free VEGF (16), and use of a
VEGF-neutralizing antibody in clinical trials in treatment of metastatic carcinoma has been associated with proteinuria (17). We investigated the nature of this interaction further by examining effects of these mediators on barrier properties of human GEnC in culture.

Much debate has focused on which part of the glomerular filtration barrier is primarily responsible for restricting protein flux, but models indicate that the barrier functions as a whole, with each part making an essential contribution (18, 19). In vivo, GEnC form a continuous inner layer of the glomerular capillaries. Away from the nucleus, the cytoplasm is attenuated to 200 nm and is punctuated by numerous fenestrae: transcellular pores, 60 to 80 nm in diameter (20). Novel imaging techniques have demonstrated a GEnC glycocalyx of 200 to 400 nm in thickness, which covers both fenestrae and interfenestral domains (20). Whereas fenestrations are essential for the high hydraulic conductivity of the glomerular filtration barrier, the glycocalyx is likely to offer significant resistance to filtration of macromolecules (19, 21).

GEnC have been little studied in comparison with other glomerular and EnC from other origins. This is largely because GEnC have been difficult to culture, and few have published evidence of their successful isolation and propagation; fewer still have done so using human cells (22). However EnC from different organs, types of blood vessels, and species have different morphologies, behavior, and patterns of protein expression (23–25), indicating the importance of selecting the relevant EnC type for in vitro studies. GEnC have recently become available from a commercial source. We characterized these cells to confirm their endothelial origin and investigated their expression of Tie2 and VEGF receptors. We developed a system for studying the effects of ang1 and VEGF on GEnC permeability properties in vitro and hence to confirm the ability of these mediators to modulate GEnC characteristics that may be relevant to their barrier function in vivo. GEnC monolayers were grown on porous supports, and barrier formation was assessed by measurement of transendothelial electrical resistance (TEER) and passage of labeled albumin. Responses to mediators that are known to alter permeability of other EnC monolayers (a cAMP analogue and thrombin) (26, 27) were examined to validate the experimental system before the effects of ang1 and VEGF were studied.

**Materials and Methods**

**Culture of GEnC**

GEnC derived from decapsulated glomeruli isolated from normal human kidney (according to the supplier’s data sheet) were obtained at passage 2 from the Applied Cell Biology Research Institute (Kirkland, WA). Cells were cultured in endothelial growth medium 2–microvascular (EGM2-MV; Cambrex, Wokingham, UK), made up from endothelial basal medium 2 (EBM2; Cambrex) and FCS (5%), antimicrobial agents, and growth factors as supplied. Cells that were being prepared for or being used in experiments were cultured in EGM2-MV without VEGF. Cells were used in experiments up to passage 8.

**Expression of Tie2 and VEGF Receptors**

Polyclonal antibodies were used to examine expression of Tie2 and VEGFR1 and 2 (all Santa Cruz) in cultured GEnC by immunofluorescence (IF) and Western blotting as above.

**Culture of GEnC in Tissue-Culture inserts**

Polycarbonate supports (0.4-μm pore size, 0.5-cm² surface area) in tissue-culture inserts (1-cm diameter, Nalge Nunc International,
Rochester, NY) were seeded with GEnC at passages 5 to 8 at 100,000 cells/cm². Inserts were placed in 24-well plates and were used for experiments between Days 5 and 12 after seeding.

**Measurement of TEER**

TEER was measured using an EVOMx voltohmeter (World Precision Instruments, Sarasota, FL) connected to an Endohm 12 electrode chamber and cap (World Precision Instruments). The Endohm is designed for accurate quantitative measurements of lower resistance monolayers (e.g., endothelial rather than epithelial tissue cultures). The chamber and cap each contain a pair of concentric electrodes: a voltage-sensing silver/silver chloride pellet in the center and an an- nular stainless steel current electrode. The symmetrically apposing circular disc electrodes, situated above and beneath the membrane, allow uniform current density to flow across the membrane. The height of the upper Endohm 12 electrode was adjusted to 1 mm above the cell layer with an insert in place. The Endohm chamber was filled with 3 ml of medium, and the top electrode was replaced. Tissue-culture inserts were removed sequentially from culture plates and placed in the chamber, and the resistance was recorded after 10 s. The resistance of the endothelial monolayer was calculated as the total resistance measured minus the mean resistance of control inserts, with correction for surface area. Formation of a satisfactory monolayer was indicated by a TEER ≥20 Ω/cm².

**Measurement of Transendothelial Protein Passage**

Transendothelial permeability to macromolecules was assessed by measuring passage of FITC-labeled BSA (Sigma) across the monolayer. The method was based on a published protocol (28). Medium in both well and insert containing GEnC monolayers was replaced with serum-free medium (SFM; EBM2). After 1 h, the medium in the insert was replaced with 500 µl of SFM containing 0.5 mg/ml FITC-labeled BSA; that in the well was replaced with 500 µl of SFM containing 0.5 mg/ml unlabeled BSA (Sigma). At 1, 2, and 3 h, 100-µl aliquots were removed and replaced with 100 µl of SFM containing unlabeled BSA (0.5 mg/ml). The fluorescence of the aliquots was measured as above, and the concentration of FITC-BSA was calculated by reference to a standard dilution.

**Effect of cAMP and Thrombin on GEnC Monolayer Barrier Properties**

The effect of a cAMP analogue was examined as a control for the system as increasing intracellular cAMP decreases permeability in other EnC (26). Cell membranes are impermeable to cAMP, so a cell membrane-permeable cAMP analogue, 8-(4-chlorophenylthio) (pCPT)-cAMP (Sigma), was used in combination with a cAMP-specific phosphodiesterase inhibitor, RO-20-1724 (CN Biosciences, Nottingham, UK) to elicit a maximal response. Conversely, thrombin increases permeability in other EnC monolayers (27). Culture medium in both wells and inserts was replaced with SFM. Baseline TEER was measured after 1 h, and the culture medium was again replaced, this time with SFM containing 20 µM RO-20-1724 and 300 µM pCPT-cAMP ("cAMP medium"), 1 U/ml thrombin (Sigma), or control medium. TEER was measured at 1 h. The effects of cAMP and thrombin on FITC-BSA passage were measured as above, adding the agonists in the above concentrations with the BSA in SFM solutions.

**Effect of Ang1 and VEGF on Monolayer Barrier Properties**

Effects of recombinant Ang1 (100 ng/ml; R&D Systems) and recombinant VEGF₁₆₅ (50 ng/ml; R&D Systems) on TEER of GEnC monolayers were measured as for cAMP and thrombin above. TEER was measured at various time points. In some experiments, a soluble Tie2/Fc receptor chimera (1 µg/ml; R&D Systems) was added to the Ang1 solution 30 min before application to monolayers to bind Ang1 and block its interaction with cell-surface Tie2 receptors. In other experiments, Ang1 and VEGF were added sequentially. Experiments to examine the effect of Ang1 and VEGF were repeated using serum-containing medium (EGM2-MV). The effects of Ang1 and VEGF on FITC-BSA passage were measured as above, adding the agonists in the above concentrations with the BSA in SFM solutions.

**Statistical Analyses**

Microsoft Excel software (Microsoft Corporation, Redmond, WA) was used for simple statistics, including means and t tests. SPSS 11.0 (SPSS, Chicago, IL) was used for other tests, including SEM and ANOVA. P < 0.05 was taken to indicate statistical significance.

**Results**

**GEnC Culture and Characterization**

Commercially available GEnC had typical endothelial appearances on light microscopy, having bright perinuclear halos and growing to form confluent monolayers of polygonal cells at passage 8 or less. At later passages, cultures began to show signs of senescence. IF confirmed expression of endothelial-specific proteins in the expected distribution (Figure 1). vWF staining was cytoplasmic, whereas the adhesion molecules PECAM-1 and VE-cadherin were expressed at cell junctions. Western blotting for the same proteins confirmed their expression in GEnC (Figure 2). vWF and VE-cadherin were expressed equally in GEnC and HMVEC, but PECAM-1 expression was lower in GEnC.

Treatment of GEnC with TNF-α increased expression of e-selectin by IF (Figure 3) but had no effect on binding of an irrelevant primary antibody (not shown). This typical EnC

![Image](https://via.placeholder.com/150)

**Figure 1.** Immunofluorescence staining showing expression of endothelial cell (EnC)-specific markers by human glomerular EnC (GEnC) cultured on coverslips. Magnification, ×200.
response was shown to be dose dependent by cell-based fluorescence immunoassay (Figure 4). 10 ng/ml TNF-α caused a 3.8-fold increase in e-selectin expression over 6 h.

Expression of Tie2 and VEGF Receptors by Cultured GEnC
GEnC were shown to express VEGFR2 and Tie2 by IF (not shown) and Western blotting (Figure 5). Although VEGFR1 seemed to be expressed on IF, this was not confirmed by Western blotting, whereas there was a strong band for VEGFR2. Tie2 consistently appeared in Western blotting as a triplet in cultured cell lysates and a single band in tissue lysates, suggesting differences in posttranslational processing.

Culture of GEnC in Tissue-Culture Inserts
GEnC monolayers reached a mean TEER of 30 to 35 Ω/cm² within 7 d with an additional, more gradual increase to 35 to 40

Ω/cm² at 14 d (data not shown). FITC-BSA passage across monolayers decreased correspondingly.

Effects of cAMP and Thrombin on Monolayer Barrier Properties
Increasing the effective intracellular concentration of cAMP by use of the cAMP medium increased the mean TEER of GEnC monolayers relative to controls by 34.4 Ω/cm² over 1 h, whereas thrombin decreased TEER by 14.8 Ω/cm² (Figure 6). Treatment with cAMP medium caused a corresponding decrease in passage of FITC-BSA (by 51% over 3 h), whereas thrombin produced the opposite effect (increasing FITC-BSA passage over 3 h by 301%; Figure 7).

Effect of Ang1 and VEGF on Monolayer Barrier Properties
Ang1 increased TEER of GEnC monolayers by a maximum of 11.4 Ω/cm², whereas VEGF had the opposite effect, decreasing TEER by 12.5 Ω/cm² (Figure 8). Addition of the Tie2/Fc chimera reduced the effect of ang1 on TEER by 64.3%
at 1 h ($P < 0.005$ by repeated measures ANOVA, not shown), confirming that the effect of ang1 on TEER was predominantly due to Tie2 binding. When the experiment was repeated using serum-containing medium, similar, although less pronounced, effects were observed (Figure 9). When ang1 and VEGF were added sequentially, both agonists exerted effects comparable in magnitude and direction with those observed in previous experiments (Figure 10).

Ang1 decreased FITC-BSA passage across GEnC monolayers over 3 h by 45.2% (Figure 11), consistent with the observed increase in TEER. VEGF did not increase the passage of FITC-BSA despite its effects on TEER, which were of similar magnitude to those of both thrombin and ang1 (in the same and opposite directions, respectively). However, concurrent addition of VEGF partially abrogated the effect of ang1 in decreasing protein passage.

**Discussion**

Commercially available human GEnC were derived from sieved glomeruli, and contamination with nonglomerular endothelium therefore is negligible. Our results show that these cells maintain endothelial phenotype and function *in vitro*. The expression of Tie2 and VEGFR2 by cultured GEnC is consistent with observations of their expression *in vivo*. In the mature animal, the majority of VEGF actions on EnC, including survival, proliferation, chemotaxis (all important in angiogenesis), and induction of permeability, are mediated through VEGFR2 (8).

GEnC formed monolayers in culture with mean TEER of 30 to 40 $\Omega/cm^2$, comparable both with other microvascular EnC studied in our laboratory (HMVEC as above; data not shown) and with published data using other EnC types. TEER are generally reported in the range of 6.1 to 69 $\Omega/cm^2$ (29). Endothelia that form a highly restrictive blood-organ barrier *in vivo* such as brain and retinal microvascular endothelia show the highest TEER in culture with values of 186.6 $\Omega/cm^2$ recorded in bovine retinal EnC (29, 30). Because a variety of different tracers and methods have been used for measuring macromolecular permeability, it is difficult to compare directly results of protein passage measurements. TEER is a measure of ion flux and is often used as an indicator of the pathways across a cell layer open to water and small molecules. These pathways include those that are accessible to macromolecules, known to be mainly interendothelial gaps in other cultured EnC monolayers (29). Variations in fractional area of these gaps between different monolayers are likely to explain differences in TEER and protein passage between individual monolayers. GEnC have a unique phenotype *in vivo*, yet no previously published studies have described their *in vitro* permeability characteristics.

The cAMP medium and thrombin provided good controls, demonstrating the ability of this system to detect changes in ion flux and macromolecular passage across GEnC monolayers. GEnC behavior in response to these mediators was similar to that reported for other EnC (26, 27). Both cAMP and thrombin effect monolayer integrity through modulation of the size of intercellular gaps, determined by the balance between cytoskeletal tension, which tends to pull cell junctions apart, and...
the actions of junctional adhesion molecules, including VE-cadherin (31). Cytoskeletal tension is dependent on the activity of nonmuscle myosin light chain (MLC) kinase, an enzyme required to initiate actin-myosin interaction by phosphorylating MLC. Increased cAMP is accompanied by a reduction in MLC phosphorylation and therefore also in contractility (32). Thrombin induces calmodulin-dependent phosphorylation of MLC and inhibition of MLC phosphatase (27).

Ang1 caused an increase in TEER of GEnC monolayers and a corresponding decrease in protein passage, indicating an increase in the integrity of the monolayer. The effect on TEER was of a similar magnitude to that of VEGF, although in the opposite direction. These are the first data confirming that ang1 has effects on permeability measures in cultured microvascular EnC and on TEER of any cells. The one previous investigation of ang1 effects on barrier function in vitro used macrovascular cells and studied protein passage only, showing a similar effect (33). The current study adds the additional information that ang1 does not prevent VEGF from having an effect, as suggested in Gamble’s paper (33); rather the barrier function of monolayers treated with both agonists was the result of their combined actions. It is intriguing that despite decreasing TEER, VEGF had no effect on FITC-BSA passage in these experiments, suggesting that in GEnC it has a greater effect on small-diameter pathways across the monolayer than on those open to macromolecules. This is consistent with an in vitro role for VEGF in the induction of the high hydraulic conductivity of GEnC. Taken together, these observations confirm the potential of ang1 and VEGF to have effects on GEnC phenotype and maintenance of barrier properties as a result of their combined actions in vivo.

The reduction in magnitude of the effects of ang1 and VEGF when examined in serum-containing medium may be due to blockade by serum proteins or specific inhibitors (e.g., soluble VEGFR1 and Tie2 (16, 34)). Such inhibitors may moderate systemic effects of these mediators while allowing paracrine...
actions. Both ang1 and VEGF have matrix-binding properties and so may become incorporated in the glomerular basement membrane, whereas EnC have the ability to utilize these matrix-bound mediators (35, 36).

It is likely from this and previous studies that the observed effect of ang1 was mediated through effects on junctional proteins, strengthening cell–cell adhesion (33). This effect is consistent with a stabilizing action on GEnC in vivo. Similarly, the decrease in TEER caused by VEGF suggests a parallel effect in vivo. In other studies, VEGF has been shown to have various potentially relevant effects, including phosphorylation of VE-cadherin (weakening cell–cell adhesion) and induction of transcellular holes, vesiculovacuolar organelles, and fensetrations (37). Any of these may have contributed to the effects observed here, although, as above, the results suggest a greater effect on small-diameter pathways. Furthermore, that thrombin, acting on cell junctions, both decreased TEER and increased protein passage suggests an alternative mode of action for VEGF in these experiments. The action of ang1 on intercellular junctions while VEGF acts primarily via effects on another transmonolayer pathway suggests a way in which the seemingly opposing actions of these mediators may be complementary in vivo. Future ultrastructural studies will examine in detail the mechanisms of the observed effects. This model will be useful for further studies of GEnC barrier properties and their contribution to glomerular permselectivity.

That GEnC are responsive to these important mediators in vitro suggests that some of their actions (on EnC survival, proliferation, and barrier properties) may have therapeutic applications in glomerular disease. Endothelial damage is a central component of glomerular diseases, including hemolytic-uremic syndrome (22) and pre-eclampsia (16), and may also play a role in lupus nephritis (38). The generalized endothelial dysfunction that accompanies the microalbuminuria seen in early diabetic renal disease also suggests a contribution from a GEnC lesion (39). Endothelial damage is important in experimental immune glomerulonephritis (40) and in remnant kidney models, where it may initiate glomerulosclerosis (41). In some cases, endothelial regeneration accompanies glomerular repair (42) and may be essential for it (43). These observations suggest that endothelial growth factors may mitigate endothelial damage and promote recovery. Repair of glomerular capillaries in thy-1 nephritis is associated with increased VEGF expression (42), and endothelial damage caused by VEGF blockade is associated with upregulation of podocyte VEGF expression (44). Blocking VEGF slows recovery from experimental mesangioproliferative nephritis (44), whereas VEGF administration hastens recovery in a thy-1 habu snake venom model (43) and in experimental thrombotic microangiopathy (45). However, in models of diabetic nephropathy (where increased VEGF expression may be pathogenic), blockade of VEGF attenuates development of hyperfiltration, albuminuria, and the upregulation of endothelial nitric oxide synthase in GEnC (46, 47). These observations suggest that where there is significant GEnC loss, VEGF may be used to promote recovery, but where there is excess VEGF production, blockade may be appropriate. Ang1 may have particular application where GEnC integrity is compromised, including where there is excess VEGF. In an animal model of diabetic retinopathy, ang1 had a profound effect in restoring integrity of the retinal EnC permeability barrier, normalizing VEGF levels, and countering many of the actions of VEGF (48). Thus, different combinations of mediators could be tailored for different glomerular diseases according to the particular pathology. Although further in vitro and in vivo work is required to define precisely the potential of such approaches in glomerular disease, VEGF is already in use in clinical trials in peripheral and cardiovascular disease (49).

In summary, these data confirm the potential of GEnC to respond to both ang1 and VEGF. The increase in GEnC monolayer integrity induced by ang1 is consistent with a role for ang1 in vivo, stabilizing the endothelium and resisting angiogenesis while allowing VEGF to induce the high permeability to water and small molecules that is characteristic of the glomerular endothelium. This study provides further evidence of the biologic plausibility of podocyte-produced factors crossing the glomerular basement membrane to have effects on the glomerular endothelium.

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References


