Functional evidence that vascular endothelial growth factor may act as an autocrine factor on human podocytes

Rebecca R. Foster,1 Rachel Hole,2 Karen Anderson,3 Simon C. Satchell,3 Richard J. Coward,4 Peter W. Mathieson,3 David A. Gillatt,5 Moin A. Saleem,4 David O. Bates,1 and Steven J. Harper1,3

1Microvascular Research Laboratories, Department of Physiology, University of Bristol, Preclinical Veterinary School, Bristol BS2 8EJ; and 2Department of Pathology, 3Academic and Children's Renal Unit, University of Bristol, and 4Bristol Urological Institute, Southmead Hospital, Westbury on Trym, Bristol BS10 5NB, United Kingdom

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Foster, Rebecca R., Rachel Hole, Karen Anderson, Simon C. Satchell, Richard J. Coward, Peter W. Mathieson, David A. Gillatt, Moin A. Saleem, David O. Bates, and Steven J. Harper. Functional evidence that vascular endothelial growth factor may act as an autocrine factor on human podocytes. Am J Physiol Renal Physiol 284:F1263–F1273, 2003. First published March 4, 2003; 10.1152/ajprenal.00276.2002.—Vascular endothelial growth factor (VEGF) is expressed by renal glomerular epithelial cells (podocytes) and is thought to be protective against nephrotoxic agents. VEGF has been shown to be an autocrine survival factor in neuropilin-1-positive, VEGF receptor-negative breast carcinoma cells. Normal human podocytes are also known to express neuropilin-1, VEGF, and are VEGF-R2 negative. Here, we investigated whether a similar VEGF autocrine loop may exist in podocytes. Podocyte cytosolic calcium concentration ([Ca2+]i) was analyzed in primary cultured and conditionally immortalized podocytes using ratiometric fluorescence measurement. Cytotoxicity was determined by lactate dehydrogenase assay, proliferation by [3H]-thymidine incorporation, and cell counts by hemocytometric assay. VEGF decreased [Ca2+]i in primary podocytes (from 179 ± 36 to 121 ± 25 nM, P < 0.05) and conditionally immortalized podocytes (from 95 ± 10 to 66 ± 8 nM, P < 0.02) in the absence of extracellular calcium. The type III receptor tyrosine-kinase inhibitor PTK787/ZK222584 abolished this reduction. VEGF increased podocyte [3H]-thymidine incorporation (3,349 ± 283 cpm, control 2,364 ± 301 cpm, P < 0.05) and cell number (4.5 ± 0.7 × 10^4/ml, control 2.6 ± 0.5 × 10^4/ml, P < 0.05) and decreased cytotoxicity (5.9 ± 0.7%, control 12 ± 3%, P < 0.05), whereas a monoclonal antibody to VEGF increased cytotoxicity. Electron microscopy of normal human glomeruli demonstrated that the glomerular VEGF is mostly podocyte cell membrane associated. These results indicate that one of the functions of VEGF secreted from podocytes may be to act as an autocrine factor on calcium homeostasis and cell survival.

intracellular calcium; apoptosis; glomerulus; cell survival; immunogold

The glomerulus is a unique functional unit characterized by differential permeability, high to water and electrolytes and low to protein. The podocytes (visceral glomerular epithelial cells; GECs) are believed to play a crucial role in the maintenance of this selective barrier. Podocyte dysfunction, either genetic (20) or acquired in glomerular disease (13), results in loss of the macromolecular selectivity of the glomerular filtration barrier and proteinuria. How podocytes exert their influence is poorly understood, but the available data suggest that podocytes contribute structurally by the provision of slit diaphragm and glomerular basement membrane (20, 22) and functionally by production of molecules known to affect endothelial permeability in other vascular beds. Examples of these molecules include vascular endothelial growth factor (VEGF) (2), known to increase microvascular permeability (5, 6), and angiopoietin-1 (33), the only podocyte-secreted molecule that has been shown to decrease macromolecular extravasation (38).

Despite the production of VEGF by podocytes at high levels, the detailed role of VEGF in normal glomerular physiology and its potential contribution to glomerular macromolecular permeability remain controversial. Normal VEGF biology is complex. Differential exon splicing of the VEGF gene results in a number of mRNA species, which code for a series of isoforms containing different numbers of amino acids termed VEGF188 and VEGF165 (the most widespread isoform and also that found predominantly in the renal glomerulus) and VEGF121 (10). VEGF isoform expression in glomeruli is heterogeneous. Individual human glomeruli express one, two, or all three of these main isoforms at the mRNA level (40). Minor VEGF mRNA splice variants (VEGF206, VEGF183, VEGF148, and VEGF145) have also been reported, but they are less well characterized (18, 19, 29, 40). In addition, evidence for a new set of almost identical sister molecules of inhibitory

Address for reprints and other correspondence: D. O. Bates, Microvascular Research Laboratories, Dept. of Physiology, Univ. of Bristol, Preclinical Veterinary School, Southwell St., Bristol BS2 8EJ, UK (E-mail: Dave.Bates@bristol.ac.uk).
VEGF isoforms in the renal cortex has recently been described by this laboratory (4).

VEGF signals through two receptors. The primary targets of VEGF on vascular endothelial cells are the class III receptor tyrosine-kinases, VEGFR-1 (flt-1) and VEGFR-2 (KDR), both of which are expressed by the glomerular endothelium (9). The latter initiates angiogenesis, cell migration, and permeability changes. VEGFR-1 also exists in a soluble form, sVEGFR-1 (sFlt), which is inhibitory when bound to free VEGF. In addition, the neuropilins have been shown to bind specific isoforms of VEGF, although their signaling properties remain unknown (14, 16, 35, 36). Neuropilin-1 (Np-1), for example, facilitates the binding of VEGF165 to VEGFR-2 (12) enhancing VEGFR-2-mediated effects.

VEGF, one of the most potent mediators of angiogenesis and endothelial permeability known, is produced at a high level by the podocytes 200–300 nm from its receptors on the glomerular endothelial cells. A paracrine action for VEGF would therefore appear clear (7). For this to occur, however, VEGF needs to act against a significant filtration of fluid across the glomerular barrier. It has therefore been suggested that VEGF might act on cells other than the glomerular endothelium. This led us previously to investigate VEGF-R expression by human podocytes themselves. Although we were unable to detect tyrosine-kinase VEGFR-2 expression, we demonstrated the expression of Np-1 by normal human podocytes in vitro and in vivo (17). These results suggest that podocytes may have the potential to bind the VEGF they secrete. We therefore hypothesized that the potential VEGF-Np-1 interaction may be important in terms of an autocrine loop or in VEGF sequestration in podocytes (17).

Although Np-1 has been considered as a nonsignaling VEGF coreceptor, VEGF has more recently been identified as an autocrine survival factor for Np-1-positive, VEGF tyrosine-kinase receptor-negative breast carcinoma cells (1). Because VEGF has been shown to stimulate increases in cytosolic calcium concentration [Ca\(^{2+}\)]\(_i\) in endothelial cells, we were prompted to investigate intracellular cytosolic calcium responses of cultured human podocytes to exogenous VEGF to address the hypothesis that VEGF may play a role as a podocyte autocrine factor. We studied these potential functional responses in proliferating dedifferentiated primary culture podocytes and nonproliferating differentiated podocytes in vitro. In addition, we investigated the potential of exogenous VEGF to act as a survival factor for dedifferentiated proliferating podocytes. Finally, we determined the distribution of VEGF within the region of the glomerular filtration barrier using transmission electron microscopic (TEM) analysis of colloidal gold immunohistochemistry on normal human glomeruli.

**MATERIALS AND METHODS**

VEGF used in these experiments was recombinant VEGF165, a kind gift of N. Ferrara (Genentech). All chemicals/solutions were from Sigma unless otherwise stated.

**Primary culture podocytes.** Nephrectomy tissue was supplied by the Department of Urology, Southmead Hospital, from patients undergoing nephrectomy for unipolar renal tumor (age range 43–68 yr). All patients were nondiabetic, normotensive with normal excretory renal function and no urinary sediment. Cells and mRNA from human tissue were derived from material removed at surgery and the excess to diagnostic requirements or postmortem. Informed consent was obtained from patients or relatives as appropriate.

Podocytes were isolated from the nonmalignant histologically normal pole of renal cell carcinoma nephrectomy specimens by sieving and cultured under standard conditions as previously described (26). Cells grown by this method demonstrate a typical polyhedral shape with a cobbledstone appearance on confluence and have been characterized as positive for cytokeratin and Wilms tumor protein-1 (WT-1) by immunofluorescence; positive for VEGF, WT-1, and synaptopodin by RT-PCR; and negative by RT-PCR for von Willebrand factor, CD45, and smooth muscle myosin, excluding contamination by endothelial cells, leukocytes, or mesangial cells, respectively, as previously described by ourselves and co-workers (17, 26). This phenotype was confirmed by regular sampling of cells studied.

**Conditionally immortalized podocytes.** This cell line has been conditionally immortalized from normal human podocytes with a temperature-sensitive mutant of immortalized SV40 T antigen. These cells have been previously characterized in detail elsewhere (32). At the “permissive” temperature of 33°C, the SV40 T antigen is active and allows the cells to proliferate rapidly. Thermoswitching the cells to the “nonpermissive” temperature of 37°C silences the transgene and the cells become growth arrested and differentiated. Under these conditions, they express antigens appropriate to in vivo arborized podocytes. Cells were grown on coverslips for a period of 14 days to ensure growth arrest and differentiation.

**Intracellular calcium studies.** Podocytes were grown on coverslips to confluence. Cells were incubated with fura 2-AM (10 μM) for 90 min in DMEM at room temperature, and the coverslip was then placed in a holder. The holder was then mounted on a rig consisting of an inverted fluorescence microscope (DM IRB, Leica) equipped with a UV source (Cairn Instruments, World Precision Instruments) with filters for excitation at 340 and 380 nm. Fast switching was achieved using a rotary filter wheel at 50 Hz and a spectrophotometer for photometric measurement (Cairn Instruments). The spectrophotometer received emitted light via a 400-nm dichroic filter and a 510- to 530-nm barrier filter in front of the photometer. Powerlab software was used for analysis and graphic display.

Experiments were conducted in HBSS media containing 1.3 mM calcium (i.e., normal extracellular calcium concentration, [Ca\(^{2+}\)]\(_o\)) and in nominally calcium-free HBSS (Gibco BRL). Test samples of 1 nM VEGF, 30 μM ATP, used as a positive control, and HBSS, used as a negative control, were left to wash and record for 5 min. To ensure that changes in [Ca\(^{2+}\)]\(_i\) were effectively detected, 5 μM ionomycin were added to stimulate Ca\(^{2+}\) entry into the cells. One millimolar manganese chloride (MnCl\(_2\)) in the continued presence of 5 μM ionomycin was then used to quench the calcium-sensitive fura to determine the background (Ca\(^{2+}\) independent) fluorescence signal. Three washes with appropriate HBSS were used between stimuli, and cells were allowed to rest for 20 min. VEGF was used at 1 nM, because this concentration has been shown to produce physiological responses in our previous in vivo experiments (3, 6, 27).

Emission fluorescent measurements (I\(_f\)) were taken 50 times a second. The ratio of the I\(_f\) measured during 340-nm
excitation to that during 380-nm excitation (R), proportional to the calcium concentration, was calculated from

$$R = \frac{I_{\text{f}380}}{I_{\text{f}340}} = \frac{(I_{\text{f}340} - B_{340})/(I_{\text{f}380} - B_{380})}{I_{\text{f}340} - I_{\text{f}380}}$$

where $R_{\text{exp}}$ = (I$_{\text{f}340}$ - B$_{340}$)/(I$_{\text{f}380}$ - B$_{380}$), I$_{\text{f}340}$ is the I$_{\text{f}}$ measured during excitation at 340 nm, I$_{\text{f}380}$ is the I$_{\text{f}}$ measured during excitation at 380 nm, and B$_{340}$ and B$_{380}$ are the background I$_{\text{f}}$ values measured during excitations at 340 and 380 nm, respectively (measured as the I$_{\text{f}}$ after Mn$^{2+}$ quenching). $R_{\text{min}}$ is the in vitro ratio for zero [Ca$^{2+}$]i/[[Ca$^{2+}$]i], was calculated from the following formula

$$[\text{Ca}^{2+}] = K_d p_3 \times (R - 0.85)/(0.85 \cdot [\text{Ca}^{2+}]_\text{max} - R)$$

Where $K_d$ (the product of the fura dissociation constant from bound-to-free calcium and the ratio of minimal-to-minimal I$_{\text{f}380}$) was calculated from an in vitro calibration curve.

The order in which the test samples were added was varied between experiments. Inhibition studies were conducted in which conditionally immortalized cells were challenged with 1 nM VEGF after preincubation for 10 min with the class III tyrosine-kinase receptor inhibitor PTK787/ZK222584 (100 nM) (a kind gift from J. Wood, Novartis, Basle, Switzerland), a response recorded, the cells washed three times with HBSS (minimal calcium) and allowed to rest for 20 min.

Table 1. Primers and primer sequences

<table>
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<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Annealing Temperature</th>
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<tbody>
<tr>
<td>VEGF R1</td>
<td>5’-ATG ATG CCA GCA AGT GGG AGT TTG C-3’</td>
<td>55°C</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-GCA CCA ACC CAG CCA AAG GCC GTG-3’</td>
<td>55°C</td>
</tr>
<tr>
<td>VEGF R2</td>
<td>5’-ATG ATG CCA GCA AGT GGG AGT TTG C-3’</td>
<td>55°C</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-GCA CCA ACC CAG CCA AAG GCC GTG-3’</td>
<td>55°C</td>
</tr>
<tr>
<td>VEGF R3</td>
<td>5’-ATG ATG CCA GCA AGT GGG AGT TTG C-3’</td>
<td>55°C</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-GCA CCA ACC CAG CCA AAG GCC GTG-3’</td>
<td>55°C</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-ACC ACC ACC ATG GAG CAG-3’</td>
<td>55°C</td>
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Cytoxotoxicity assays. Ninety-four wells of a 96-well plate (Costar) were seeded with primary cultured podocytes and 100 μl 20% FBS-RPMI. Podocytes were left for 48 h, and then media were removed and replaced with FBS-free media. After 24 h, 100 μl media were removed from each well and cytotoxicity was assayed using a lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche) and quantified using a Bichrometric Multi-scan plate reader (Labelys). These samples were used for background LDH measurement ($T_{\text{min}}$). The media of half of the wells were replaced with 100 μl FBS-free media, the other half with FBS-free media containing 1 nM VEGF$_{165}$. Twenty-four hours later, 100 μl media were again removed from each well and cytotoxicity was assayed and quantified. These samples were used to determine the cytotoxicity ($T_{\text{exp}}$). Finally, 100 μl of 2% Triton X-100/1 × PBS (final concentration 1%) were added to each well and left for 10 min to completely lyse the cells. One hundred microliters were removed and cytotoxicity was again assayed and quantified. This enabled determination of the maximum LDH from the well ($T_{\text{max}}$). Percent cytotoxicity ($T_x$) was calculated for each well as

$$\left(\frac{T_{\text{exp}} - T_{\text{min}}}{T_{\text{exp}} - T_{\text{max}}}\right) \times 100$$

The fold-increase in cytotoxicity was calculated as $T_x$ (treatment)/$T_x$ (control). The percent reduction in cytotoxicity was calculated as $T_x$ (VEGF) × 100/$T_x$ (control).

RT-PCR. Total RNA was extracted as previously described (8) using the TRizol method from the conditionally immortalized human podocyte cell line, human thyroid, brain, and kidney. Reverse transcription was carried out using 1 μg RNA and 5 μM oligo dT (Promega) in 10 μl RNase-free water (Sigma). This mixture was incubated at 65°C for 5 min and immediately placed on ice. The reaction mixture was then altered to 1× first-strand synthesis buffer (Roche), 10 mM DTT (Roche), 2.5 mM dNTPs (Promega), 1 U RNA guard (Amersham), and 2.5 U expand RT (Roche) in a total of 20 μl RNase-free water (Sigma). This was incubated at 42°C for 2 h. PCR was performed using the primers as detailed in Table 1. The PCR mixture consisted of 1× PCR buffer (Abgene), 1.25 mM MgCl$_2$ (Abgene), 375 μM dNTPs, 10 μM forward primer, 10 μM reverse primer (except for GAPDH where 5 μM of each primer were used), 1 μl CDNA, and 1 U Taq (Abgene) in 20 μl RNase-free water. A standard PCR cycle was used, i.e., 55°C, 35 cycles (Hybaid). RT-PCR products were run on 2% agarose (Roche) gels in the presence of 0.5 μg/ml ethidium bromide (Invitrogen). Gels were photographed under UV transillumination (Gibco). A 100-bp ladder (Sigma) was used to visualize bands from 100 to 1,000 bp.
Western blot analysis. Confluent primary cultured podocytes from a T75 flask were left untreated or treated with VEGF (1 nM) for 30 min. The cells were then trypsinized, rinsed in PBS, pelleted, and the protein was extracted in 0.2% (vol/vol) SDS, 300 mM NaCl, 20 mM Tris, 10 mM ethyldiamine tetraacetic acid, 2 mM Na3VO4, 1 mM phenylmethlysulfonyl fluoride, 10 μg/ml leupeptin, 20 μM E64, 2 μg/ml aprotinin, 62.5 mM β-glycerophosphate, and 1 μM pepstatin A. Protein quantification was performed spectrophotometrically using Bio-Rad dye. Equal amounts of protein were electroblotted to polyvinylidene difluoride membrane. Membranes were blocked in 10% (wt/vol) nonfat dry milk (Marvel) in 1× PBS-Tween 0.1% (vol/vol) (PBST) for 1 h and incubated with primary antibody (1:300 goat anti-VEGF-R1, SC-316, Santa Cruz) for 1 h in 1× PBST plus 5% (wt/vol) Marvel. Unbound primary antibody was removed by five washes in 1× PBST (5 min/wash). The membrane was incubated for 1 h with 1× PBST plus 5% (wt/vol) Marvel and secondary antibody (1:3,000 donkey anti-goat IgG). Washes were performed as previously described, and the protein was detected by enhanced chemiluminescence.

TEM. One-millimeter cubed pieces of renal cortex were taken from the normal pole of nephrectomy samples taken for unipolar cancer and fixed in 0.2% glutaraldehyde (Agar Scientific) and 0.2 M phosphate buffer at pH 7.4 at room temperature for 30 min. The tissue was then stored in 0.2 M phosphate buffer until processed. Specimens were partially dehydrated using a 10-min wash in 50% IMS followed by three 10-min washes in 70% methylated spirits (IMS). Specimens were infiltrated with LR white hard grade resin (London Resin) in a 2:1 ratio with 70% IMS for 30 min. Specimens were infiltrated with LR white resin for four 30-min periods. The specimens were then embedded in LR white resin plus an accelerator (London Resin) in size 00 gelatin capsules (Agar Scientific) and left to polymerize via a cold catalytic process at 4°C for at least 2 h. The blocks were then transferred to a 50°C oven for 2 h. The capsules were then exposed to the air and left to set. Sections were cut at 0.5–0.9 μm on a Leica Reichert Ultratrac S ultramicrotome and placed on a glass slide and stained with 1% toluidine blue in 1% borax to determine whether the tissue was suitable for further investigation. Appropriate tissue was cut into 90-nm sections and mounted onto 300 mesh hexagonal nickel grids (Agar Scientific) and left to air dry. Grids were washed in 0.01 M PBS (pH 7.4) for 10 min and then incubated in polyclonal rabbit anti-VEGF antibody (A. Menarini) in PBS (pH 7.4) and 0.6% BSA at 1:10 in Antibody diluent (A. Menarini) for 60 min at room temperature. Sections were then washed for 1 min in PBS (pH 7.4) and PBS (pH 8.2). The secondary antibody was 15-nm gold-conjugated goat anti-rabbit IgG (BioCell at Agar Scientific) 1:10 dilution, applied for 60 min. Grids were washed in PBS (pH 8.2) and deionized H2O for 1 min and then stained with a saturated solution of uranyl acetate for 20 min; sections were then washed in deionized water and stained with lead citrate for 1 min. The grids were then rinsed with deionized water and left to air dry. Grids were viewed under an electron microscope (Philips CM10). Podocyte (intracellular or membrane associated), glomerular basement membrane, and glomerular endothelial cell-associated gold particles were enumerated in 16 random fields from four different kidneys. Colloidal gold particles were considered membrane associated if they were within two particle widths (i.e., 30 nm) of the membrane on either side.

Statistics. Data are presented as means ± SE. Two-tailed, paired t-tests were used to compare paired data on the same cells, and unpaired t-tests were used to compare separate cell populations treated differently. ANOVA was used to compare distribution of gold particles on podocyte foot processes.

Fig. 1. Effect of HBSS and ATP on podocyte and HK2 cell calcium. A: example of no significant change of cytosolic Ca2+ concentration ([Ca2+]i) in primary culture human podocytes in response to 1 μl HBSS in the presence of 1.3 mM extracellular Ca2+ concentration ([Ca2+]o). B: means ± SE of [Ca2+]i change in podocytes and human tubular epithelial cells in response to 1 μl HBSS in the presence of 1.3 mM [Ca2+]o. C: transient increase in [Ca2+]i in primary culture human podocytes in response to 30 μM ATP. D: means ± SE of ATP-stimulated response compared with baseline values (n = 6, **P < 0.005, paired t-test).
RESULTS

Primary cultured podocytes. Figure 1 shows the effect of HBSS on $[\text{Ca}^{2+}]_i$ in primary cultured podocytes (Fig. 1A) and human tubular epithelial cells (HK2). HBSS did not change calcium in either cell line or in human tubular epithelial cells (Fig. 1B) in either the presence or absence (not shown) of extracellular $\text{Ca}^{2+}$. ATP, on the other hand, caused a transient rapid increase in $[\text{Ca}^{2+}]_i$ on all occasions (Fig. 1C). $[\text{Ca}^{2+}]_i$ increased from 113.2 ± 22.1 to 209.5 ± 41.0 nM ($P < 0.01$; Fig. 1D) peaking at 30 ± 10 s and returning to baseline after 3.2 ± 0.5 min. Surprisingly, although VEGF did not alter $[\text{Ca}^{2+}]_i$ in the presence of extracellular $\text{Ca}^{2+}$ (Fig. 2A), VEGF produced a slow and sustained reduction in $[\text{Ca}^{2+}]_i$, which was significantly different from baseline in minimal extracellular calcium (Fig. 2B). There was a significant reduction in the ratio (R) in minimal, but not normal extracellular calcium (Fig. 2C), which corresponds to a reduction in $[\text{Ca}^{2+}]_i$ from 178.9 ± 35.6 to 121.1 ± 25.4 nM with VEGF ($P < 0.05$). A minimum was reached after 5 ± 1 min.

Conditionally immortalized podocytes. ATP administration stimulated a transient increase in $[\text{Ca}^{2+}]_i$ in conditionally immortalized cells from 102.2 ± 34.5 to 142.1 ± 35.8 nM postexposure, $P < 0.05$. In a similar pattern to that seen in primary culture podocytes, VEGF did not alter $[\text{Ca}^{2+}]_i$ in differentiated podocytes in the presence of $[\text{Ca}^{2+}]_o$ (Fig. 2D) but again produced a slow but sustained significant reduction in $[\text{Ca}^{2+}]_i$ when these differentiated podocytes were incubated in minimal extracellular calcium (Fig. 2E). There was a significant reduction in the ratio (Fig. 2F), which corresponds to a reduction in $[\text{Ca}^{2+}]_i$ from 94.7 ± 9.8 to 66.1 ± 8.4 nM ($P < 0.02$).

To determine whether VEGF was acting on type III tyrosine kinases such as VEGF-R2 or VEGF-R1, we performed the experiments after preincubating the cells for 10 min with the type III tyrosine-kinase receptor inhibitor PTK787/ZK222584. To our surprise, the addition of VEGF to cells preincubated in PTK787/ZK222584 resulted in a small but significant increase in $[\text{Ca}^{2+}]_i$ (Fig. 3A), whereas addition of PTK787/ZK222584 to cells did not result in any change in intracellular calcium by itself (Fig. 3B). This VEGF-induced increase in the presence of PTK787/ZK222584 was consistent in all six sets of experiments, with a mean ± SE increase from 78.8 ± 35 to 115.2 ± 50.6 nM.
Therefore, the reduction in $\text{Ca}^{2+}$ stimulated by VEGF (0.69 ± 0.06-fold) was reversed by this inhibitor (1.47 ± 0.15-fold, $P < 0.001$; Fig. 3D), suggesting that the VEGF-dependent reduction in $[\text{Ca}^{2+}]_i$ may be a constitutive event in podocytes, mediated by one or more type III receptor tyrosine kinases.

**Proliferation and cytotoxicity.** In primary cultured podocytes, addition of 1 nM VEGF to culture medium resulted in a significant increase in $[^{3}\text{H}]$thymidine incorporation (from 2,364 ± 301 to 3,349 ± 283 cpm, $P < 0.05$; Fig. 4A). Assuming that the $[^{3}\text{H}]$thymidine incorporation is the same for each cell for each division, then $[^{3}\text{H}]$thymidine incorporation gives the number of cells dividing within a defined time. If more cells are surviving, then there will be more cells present to undergo the normal rate of division. Therefore, to determine whether this increase in $[^{3}\text{H}]$thymidine incorporation was due to increased proliferation rate or due to an increase in the survival of VEGF-treated cells (and hence increased cell number), we measured the number of cells in each well. The cell number also increased from 2.6 ± 0.5 to 4.5 ± 0.7 $\times$ 10^4/ml ($P < 0.05$; Fig. 4B) with VEGF treatment. $[^{3}\text{H}]$thymidine incorporation calculated per cell was therefore not affected by VEGF (untreated 0.1 ± 0.015 cpm/cell, treated 0.125 ± 0.029 cpm/cell, not significant; Fig. 4C), suggesting that
VEGF was acting not by increasing proliferation rate but by reducing cell death. To assess independently whether VEGF could reduce cytotoxicity, the effect of VEGF on LDH release into the media (which occurs when cells lyse) was carried out. VEGF stimulated a reduction in cytotoxicity from 12.5 ± 3.0 to 5.9 ± 0.67% (P < 0.05; Fig. 4D).

To determine whether this decrease in cytotoxicity was also brought about by endogenous VEGF, proliferating primary cultured podocytes were incubated with a neutralizing antibody to VEGF. This resulted in a significant increase in cell death, which was abolished by addition of VEGF. The effect of exogenous VEGF, furthermore, was abolished by the addition of PTK787, although this concentration of PTK787 alone did not significantly increase endogenous cytotoxicity (Fig. 5). The reduction in cytotoxicity appeared to occur through phosphatidylinositol (PI3)-kinase activation, because the reduction in cytotoxicity was blocked by treatment with the PI3 kinase inhibitor Wortmannin (Fig. 6).

Transmission electron microscopy. Immunogold transmission electron microscopy was carried out to detect the subcellular localization of VEGF in isolated human glomeruli derived from the normal pole of nephrectomy specimens. The protocol described to detect VEGF by colloidal gold resulted from a compromise between fixation, morphology, and antigen detection, optimized finally for antigen detection. A short fixation with 0.2% glutaraldehyde was the only fixation protocol to result in antigen detection.

Colloidal gold particles were seen throughout the glomerular filtration barrier, within the podocyte foot processes (77.9 ± 1.81%), glomerular basement membrane (11.9 ± 1.2%), and what were taken to be glomerular endothelial cells (10.2 ± 1.6%) (Figs. 8 and 9A). Unfortunately, the endothelial morphology was poor with this technique, despite good morphological preservation of podocytes and basement membrane, so we were unable to determine whether the staining was predominantly luminal or abluminal. Of the podocyte foot process-bound VEGF, 63.15 ± 3.29% was membrane associated in contrast to 36.85 ± 3.29% (P < 0.03), which was intracellular (Fig. 9B). Particles were seen throughout the glomerular basement membrane and on both luminal and abluminal surfaces of the glomerular endothelial cells. The differential expression of VEGF at progressively further distances from the membrane was highly significant (P < 0.0001, ANOVA; Fig. 9C).

Colloidal gold particles were only identified within Bowman’s space when associated with podocyte cell debris. Negative controls (no primary antibody in-
cluded) revealed no gold particles at all within the glomeruli, although occasional scattered particles were seen in tubular cells (not shown).

DISCUSSION

Characteristics of primary cultured and conditionally immortalized podocytes. Despite the extensive use of primary cultured podocytes for renal research, two longstanding criticisms remain. First, there is the observation that podocytes alter their phenotype in culture, becoming dedifferentiated and proliferative. This contrasts with the growth-arrested, differentiated podocytes in vivo. We therefore studied both phenotypes. Second, there is the issue of purity. We did our utmost to ensure podocyte purity of primary cultures. There has been some debate over the origin of epithelial cells grown using the sieving method particularly with regard to the effective removal of parietal epithelial cells. We therefore studied both phenotypes. Second, there is the issue of purity. We did our utmost to ensure podocyte purity of primary cultures. These issues are fully covered by ourselves and co-workers elsewhere (17, 26). We showed that the vast majority of cells isolated using this technique have identical expression characteristics of visceral GECs but cannot exclude a minority of cells being parietal in origin. For both of these reasons, we also chose to study a conditionally immortalized podocyte cell line in addition to primary cultured podocytes. The conditionally immortalized cell line has a differentiated phenotype and is a pure population (32). Both primary cultured podocytes and conditionally immortalized, differentiated podocytes demonstrated a similar fall in [Ca\textsuperscript{2+}]\textsubscript{i} in response to VEGF\textsubscript{165} when intra- and extracellular calcium concentrations are similar. Furthermore, in the event of significant mesangial or glomerular endothelial cell contamination of primary cell cultures, an increase in [Ca\textsuperscript{2+}]\textsubscript{i} in response to VEGF\textsubscript{165} would be expected rather than a decrease. Both mesangial and glomerular endothelial cells have been shown to respond to a variety of agents with increased [Ca\textsuperscript{2+}]\textsubscript{i} (reviewed in Ref. 24). Moreover, it is well characterized that the VEGF-mediated increase in permeability of systemic capillaries in vivo is mediated via an increase in [Ca\textsuperscript{2+}]\textsubscript{i} (6, 28).

What are the physiological roles for VEGF in the glomerulus? The physiological role of podocyte-derived VEGF is still poorly understood. It has, however, been hypothesized that glomerular VEGF may have an important function in the maintenance of the glomerular endothelium (including maintaining fenestration) and/or selective permeability to macromolecules (7, 30). In fact, we previously showed in vivo that VEGF can effectively increase hydraulic conductivity (permeability to water) without reducing macromolecular selectivity in the mesenteric microcirculation, exactly that scenario present in the glomerular endothelial barrier (3). Continued controversy over the role of VEGF in normal glomerular physiology stems from a number of anomalies, however, not least of which is the apparent minimal disruption that results from the in vivo administration or inhibition of VEGF\textsubscript{165} in normal animals (25, 39). Although both of these reports only administered or inhibited VEGF\textsubscript{165} (one species among many potential isoforms), neither study demonstrated any abnormality save for VEGF-associated hypotension (39).

In addition, despite a high level of podocyte VEGF production and associated high permeability of the glomerular filtration barrier to water, the glomerulus is not a site of new vessel formation in healthy subjects. It is clear then that under normal circumstances, the proangiogenic properties of VEGF must be modified by other factors. Angiopoietin-1, VEGF\textsubscript{165b}, and other members of the inhibitory family of isoforms are good candidate molecules (5, 33). It has therefore been sug-

![Fig. 7. A: expression of VEGFR-R1, VEGF-R3, and NP-1 mRNA in human conditionally immortalized podocyte cell line. VEGF-R2 was not detected. B: VEGF R2 and GAPDH mRNA expression in human kidney tissue. C: Western blot for VEGF-R1 with and without treatment with VEGF.](image-url)

![Fig. 8. Identification of VEGF expression by immunogold TEM in a normal human glomerulus. Gold particles can be clearly seen on the edge of the podocyte foot processes. BS, Bowman’s space; GBM, glomerular basement membrane; PFP, podocyte foot process.](image-url)
gested that VEGF may have no physiological role in health but, in contrast, may only be important in glomerular disease (stimulating endothelial cell proliferation). Evidence for this, however, is only apparent from animal models rather than in human pathology (37), and it does not explain why VEGF is so strongly expressed in normal human glomeruli. Although VEGF decreases cytotoxicity of podocytes grown in culture, the relevance of this finding to healthy kidneys in vivo is still unclear. Future studies addressing the amounts of specific isoforms of VEGF including the inhibitory isoforms of VEGF (VEGF<sub>165b</sub>) produced by podocytes in vivo will help clarify this issue.

Furthermore, the microanatomic positioning of glomerular VEGF production and receptor expression suggests that VEGF has to diffuse against a significant filtration gradient to bind to its target molecules (receptor binding studies would suggest there are no VEGFRs in the distal nephron (34)). Therefore, the synthesis of some isoforms of VEGF, for example VEGF<sub>121</sub>, which has little or no heparin-binding properties (and therefore no ability to sequestrate into the glomerular basement membrane), would appear redundant since the glomerular filtration would tend to wash such molecules into Bowman's space. Our colloidal gold TEM finding of significant localization to endothelial cells would suggest that at least some VEGF isoforms are able to travel against the gradient of glomerular filtration down a concentration gradient.

The above paradox, in conjunction with the identification of Np-1 podocyte expression, led us to study potential VEGF-podocyte autocrine responses. In this report, we provide the first functional data to support the notion that podocyte-derived VEGF may have autocrine potential in addition to its other putative roles. Not only have we shown that exogenous VEGF acts directly on cultured human podocytes, but inhibition of endogenous VEGF, by a neutralizing monoclonal antibody, increases cytotoxicity of podocytes, an effect that is overcome by exogenous VEGF. Interestingly, however, this response is not mimicked by VEGF receptor inhibitors, suggesting that the endogenous effect may circumvent receptor inhibition (possibly by activation of an internal autocrine loop (15)). Furthermore, we provide the first TEM studies of VEGF expression in human renal glomerulus. These indicate that most VEGF within the glomerular filtration barrier is podocyte cell membrane associated. This phenomenon could be explained either by an accumulation of VEGF protein before secretion or by the sequestration of VEGF onto the podocyte cell surface, via chemical or receptor binding. Therefore, we have evidence that supports the hypothesis that one of the roles of VEGF in the glomerulus is to act as an autocrine survival factor for podocytes.

How does VEGF act on podocytes? [Ca<sup>2+</sup>]<sub>i</sub> is an important second messenger in most cells and, certainly in endothelial cells, plays an important role in VEGF-mediated permeability, mitogenesis, and vasodilatation. The nature of the [Ca<sup>2+</sup>]<sub>i</sub> response we demonstrated in podocytes, however, is atypical. This is the first evidence that VEGF can stimulate a reduction in [Ca<sup>2+</sup>]<sub>i</sub> under any circumstances in any cells. The conditions under which reductions in [Ca<sup>2+</sup>]<sub>i</sub> are seen in podocytes in response to VEGF are nonphysiological (i.e., minimal calcium). The fact that VEGF can stimulate a reduction in calcium under low external calcium conditions, however, suggests that VEGF is activating calcium extrusion or sequestering mechanisms.

![Fig. 9. Distribution of VEGF expression by immunogold in a normal human glomerulus. A: means ± SE% of gold particle distribution within the 3 components of the glomerular filtration barrier. B: gold particle distribution within podocyte foot processes, either membrane associated or intracellular. C: means ± SE% of gold particle distribution at 25-nm intervals from the membrane, with results significantly different using ANOVA.](http://ajprenal.physiology.org/doi/10.220.32.247)
It is possible that VEGF stimulates sarcoplasmic reticulum calcium or plasmalemmal calcium ATPases (SERCA or PMCA). Activation of either of these pumps would reduce [Ca\(^{2+}\)], but this effect would normally be masked by normal calcium homeostasis. The functional significance of this VEGF-mediated reduction in [Ca\(^{2+}\)] in podocytes is not clear, but there are a number of possibilities. VEGF is known to act as an autocrine survival factor in breast carcinoma cells that express the same VEGF receptor profile as do podocytes. Bacherler et al. (1) showed that VEGF inhibits the apoptosis of tyrosine-kinase VEGF receptor-negative, neuropilin-positive breast cancer cells via stimulation of PI3-kinase. In addition, other studies showed that PI3-kinase activity mediates the in vitro inhibition of cyclosporin A-induced podocyte apoptosis via Bcl-X (11). The evidence described above is consistent with a role of PI3-kinase in this VEGF-mediated reduction in cytotoxicity. It is well recognized that intracellular subcellular Ca\(^{2+}\) localization plays an important role in regulating apoptosis (21). Our findings, that VEGF acts as a survival factor for podocytes when dedifferentiated and proliferative, support this hypothesis. Further details of the signaling mechanisms that underlie this mechanism await further investigation.

Alternatively, the effect of VEGF on the calcium-handling properties of podocytes may be to modify the response of podocytes to other agents. Although many molecules (including bradykinin, thrombin, arginine, vasopressin, and serotonin) have been shown to have no effect on podocytes [Ca\(^{2+}\)] (24), other studies have highlighted a number of agents that result in a dose-dependent increase in podocyte [Ca\(^{2+}\)]. These include polycations (in primary culture and conditionally modified mouse podocytes) (31) and angiotensin (24). This latter effect is thought to be mediated via angiotensin receptors because angiotensin receptors signal by increasing [Ca\(^{2+}\)], and the response is inhibited by the ANG II type 1 receptor blocker losartan. Continued injury of podocytes participates in the progression of chronic renal lesions. It is generally accepted that ANG II accelerates this process, because inhibition of the renin-angiotensin system produces benefits in renal survival in both animal models and humans. If the VEGF-induced reduction in [Ca\(^{2+}\)] is functionally important in vivo, then it may explain the mechanism by which VEGF acts as a cytoprotective agent in such lesions by counteracting the ANG II-driven increases in podocyte [Ca\(^{2+}\)].

Our results prompt questions concerning which receptor(s) or intracellular pathway(s) mediate the response we identified. We demonstrated that human podocytes in vitro (primary culture) and in vivo express Np-1. Np-1 has no signaling domain, however, nor does it have a commercial inhibitor. Podocytes are not believed to express tyrosine-kinase VEGF receptors (VEGF-R1 or R2) but we demonstrated that although VEGF-R2 could not be identified, VEGF-R1 and VEGF-R3 mRNA and VEGF-R1 protein are in fact expressed in the conditionally immortalized human podocyte cell line. We therefore addressed the possibility that VEGF may act through a type III tyrosine-kinase receptor (for example, VEGF-R1). The podocyte response to exogenous VEGF was inhibited by PTK787/ZK222584. In fact, the addition of this inhibitor produced a significant increase in podocyte [Ca\(^{2+}\)]. Because PTK787/ZK222584 is a class III receptor tyrosine-kinase inhibitor that has been shown to inhibit all such tyrosine kinases in the submicromolar range, including PDGF-R, c-kit, VEGF-R2 (41), we cannot use our data to show that VEGF acts on podocytes via VEGF-1 with or without neuropilin (the literature suggests that NP-1 acts as a coreceptor for VEGF-R2 but not VEGF-R1 (23)). The likelihood is, however, that in podocytes VEGF either acts on VEGF-R1 and/or VEGF-R3 or via another unidentified podocyte expressed class III tyrosine-kinase receptor. These data suggest that these receptors may play a role in normal podocyte function, and this possibility requires further investigation.

In conclusion, we showed that VEGF can act as an autocrine factor for podocytes, acting via an alteration in calcium handling of the cells and reducing cell death. This appears to be true for proliferating primary cultured cells and growth-arrested, differentiated cells. The details of the receptor and intracellular regulatory pathways involved in this phenomenon and any potential effect on cell function or survival in vivo remain to be determined. In vivo studies including conditional knockouts of specific VEGF receptors expressed on the podocytes will need to be done to clarify further the role of VEGF in an adult glomerulus.

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