Vascular endothelial growth factor and nephrin interact and reduce apoptosis in human podocytes

Rebecca R. Foster, Moin A. Saleem, Peter W. Mathieson, David O. Bates, and Steven J. Harper. Vascular endothelial growth factor and nephrin interact and reduce apoptosis in human podocytes. Am J Physiol Renal Physiol 288: F48–F57, 2005. First published August 31, 2004; doi:10.1152/ajprenal.00146.2004.—Vascular endothelial growth factor (VEGF) is anti-cytotoxic in podocytes. Moreover, it has been suggested that nephrin, a cell adhesion molecule of the podocyte slit diaphragm, can contribute to antiapoptotic mechanisms in these cells. We therefore investigated whether VEGF signals to reduce apoptosis and the role of nephrin in this survival mechanism. Flow cytometry showed that podocytes with nephrin mutations had a significantly greater proportion of apoptosis. Although VEGF reduced apoptosis in human conditionally immortalized podocytes [wild-type (WT)] by 18.1% of control ($P < 0.001$), it was unable to do so in nephrin-deficient human conditionally immortalized podocytes. Moreover, Western blotting and immunodetection with an anti-nephrin antibody showed that the phosphorylation of nephrin, compared with serum-starved WTs, was significantly increased (ratio of 3.36 $\pm$ 1.2 to control, $P < 0.05$) by VEGF treatment and significantly reduced by treatment with a neutralizing VEGF monoclonal antibody (mAb) (ratio of 0.2 $\pm$ 0.09 to control, $P < 0.05$). The AKT signaling pathway has been implicated in nephrin-mediated inhibition of apoptosis in transfected cells, but the role of this pathway has not previously been shown in podocytes. Surprisingly, exogenous VEGF decreased AKT/PKB phosphorylation in normal podocytes but increased it in nephrin-deficient podocytes. We suggest therefore that both exogenous and endogenous (podocyte derived) VEGF can stimulate the phosphorylation of nephrin and through this action may prevent podocyte apoptosis. However, the involvement of AKT in this survival response in normal human podocytes is not clear.

Glomerular filtration rate; nephritis; VEGF; filtration; foot process

The glomerular filtration barrier is formed by the trilayer of endothelial cells, glomerular basement membrane, and the podocyte foot processes. The slit diaphragms between adjacent foot processes contain what is believed to be the final restriction to fluid and macromolecular flux as they filter blood to form urine. This slit diaphragm is composed of repetitive multiple protein units, the core of which is nephrin, a member of the immunoglobulin family (42). Nephrin is thought to form interdigitating polymers with itself and with NEPH1 (30) within the slit diaphragm (24). Mutations in NPHS1, the gene that encodes nephrin, results in congenital nephrotic syndrome of the Finnish type (23). Many functionally significant nephrin mutations have been identified but the most common are mutations in Exon 2 (Finmajor), which results in a complete lack of nephrin, and in exon 26 (Finminor), a nonsense mutation resulting in a stop at Arg-1109 (5) (Fig. 1). This also results in a lack of functional nephrin protein expression probably due to protein misfolding of the protein, which becomes trapped in the endoplasmic reticulum and consequently is not expressed in the plasma membrane (31). Nephrin has nine intracellular tyrosine residues (23). This has led to the hypothesis that nephrin acts as a signaling molecule between the slit diaphragm and the podocyte cytoskeleton. Recent data have suggested that nephrin phosphorylation is mediated via the Src family kinases such as Fyn (26, 43) and that nephrin activation may influence podocyte survival via a nephrin-CD2AP-podocin-phosphoinositol (PI) 3-kinase/AKT axis (20).

Although the podocyte is the site of vascular endothelial growth factor (VEGF) production within the glomerulus in vivo (3), the role and/or interaction of podocyte-derived and circulating VEGF in glomerular health and disease remains contentious. VEGF receptors (VEGF-R1 and VEGF-R2) are expressed on glomerular endothelial cells (11, 35), so a paracrine function for podocyte-derived VEGF is therefore likely. However, VEGF is a potent endothelial mitogen and angiogenic factor, and yet angiogenesis does not occur in the normal healthy glomerulus. It is therefore possible that VEGF may act on targets other than the endothelial cells of the glomerulus. Receptors for VEGF family members have now also been identified on podocytes themselves (neuropilin-1, VEGF-R1 and VEGF-R3, but not VEGF-R2) (14, 19). VEGF stimulates an autocrine signaling pathway in podocytes that results in increased calcium sequestration and reduced cell death in a VEGF receptor-dependent manner (14). A similar mechanism of VEGF-dependent autocrine survival has been described in other nonendothelial cells such as hematopoietic cells (16), prostate cancer cells (22), and breast cancer cells (1), which express similar VEGF receptor profiles. In systemic endothelial cells, VEGF is known to stimulate a survival pathway through the phosphorylation of VEGF-R2, which results in phosphorylation of AKT at serine 473 and the upregulation of the antiapoptotic protein Bcl-2 (15). Survival signaling through VEGF-R1 or neuropilin in cells that do not express VEGF-R2, e.g., microglial cells (13), monocytes (8), and breast cancer cells (1), appears less well defined, in that in some cells [e.g., microglial cells (13)] phosphorylated AKT is not increased, but in others it appears to be required (1). Some recent reports also show that VEGF-R1 agonists can stimulate AKT phosphorylation and reduce apoptosis in endothelial cells (7) expressing both receptors. VEGF is also known to stimulate Fyn activa-
tion in endothelial cells (29). Furthermore, VEGF receptors are known to colocalize with caveolin-1 in the plasma membrane of endothelial and mesangial cells (41), and caveolin-1 is co-immunoprecipitated with nephrin and CD2AP in podocytes (39). However, an association of VEGF receptors and nephrin has not been demonstrated.

Our previous report showed a reduction in podocyte cytotoxicity in response to VEGF165, which was blocked by the PI3 kinase inhibitor wortmannin and a VEGF neutralizing antibody (14). These results, together with studies suggesting that nephrin may be able to signal through PI3 kinase to reduce apoptosis, led us to test the hypothesis that VEGF165 can reduce apoptosis in podocytes and stimulate the phosphorylation of nephrin and AKT.

METHODS

VEGF165 was a kind gift of N. Ferrara (Genentech). The anti-nephrin antibody was a kind gift of K. Tryggvasson (Karolinska Institute). All chemicals/solutions were from Sigma unless otherwise stated.

Human conditionally transformed podocytes. Two cell lines from normal human podocytes isolated from two different patients, conditionally transformed with a temperature-sensitive mutant of immortalized SV-40 T antigen, were used. Cells have additionally been transfected with a telomerase construct (34). These cells have previously been characterized in detail elsewhere (37). Initial studies showed no difference between these two cell lines, and so data were pooled. At the “permissive” temperature of 33°C, the SV-40 T antigen is active and allows the cells to proliferate rapidly. Thermoswitching the cells to the “nonpermissive” temperature of 37°C inactivates the T antigen and the cells become growth arrested and terminally differentiated to express antigens appropriate to in vivo arborized podocytes.

To assess the significance of nephrin in the functional responses investigated, two other cell lines were studied. A human podocyte cell line was conditionally immortalized as above from an infant with steroid-resistant congenital Finnish nephrotic syndrome (Finmajor) [nephrin-deficient podocytes (NDs)]. The tissue from which these cells were derived was kindly donated from Dr. J. Merenmies, Helsinki, Finland with appropriate consent and ethical approval. A second human podocyte cell line was conditionally immortalized as above, from an infant with a mutation in exon 11 of the nephrin gene, resulting in a single base pair R460Q mutation at nt1379 [nephrin-mutant podocytes (NMs)]. Both of these mutations are illustrated in Fig. 1. All cells were grown in 10% RPMI media containing 1% penicillin/streptomycin (GIBCO) and 1% ITS liquid media, and passages were used between 10 and 20.

Apoptosis assay. Wild-type (WT), NM, and ND podocytes were serum starved for 16 h, treated for 4 h with 1 nM VEGF, or left untreated. Cells were then scraped into a cell suspension and left on a roller for a further 4 h. NDs showed significantly reduced cell number and very high cell death after a total of 24-h serum starvation (total cell count <25% of control), so to investigate whether NDs underwent apoptosis more quickly than WTs, NDs were serum starved for 4 h, scraped into a cell suspension, and then rolled for a
further 4 h (8-h total serum starvation). Each treatment was split four ways and incubated with either serum-free media, FITC-conjugated annexin V (AV) alone, propidium iodide (PI) alone, or a combination of both AV and PI according to the manufacturer’s instructions (Bender Medsystems). Cells were analyzed using a flow cytometer (Beckman Coulter), with excitation at 488 nm and emission collected at 525 nm (FITC-conjugated AV-labeled cells) and 620 nm (PI-labeled cells). Single labeling was used to gate and control for bleed through. The cell population was characterized according to whether it was labeled with neither AV nor PI (viable), PI alone (necrotic), AV alone (apoptotic), or both PI and AV (late apoptotic).

Due to the robust nature of the normal differentiated podocytes in culture, the baseline level of apoptosis in normal WTs was too low in the acute WT assay to see a VEGF-induced reduction. Therefore, a chronic serum starvation protocol was used. This involved serum starvation for a total of 48 h, including treatment with VEGF as above.

**Immunoprecipitation.** To determine the effect of exogenous VEGF on nephrin phosphorylation, WT cells were serum starved for 16 h and then treated with 1 nM VEGF for 20 min or left untreated. To determine the effect of endogenous VEGF on nephrin phosphorylation, WT cells were serum starved for 16 h and then treated for 24 h with a neutralizing mouse monoclonal anti-VEGF IgG2b antibody or left untreated. Previous experiments showed that mouse IgG by itself has no effect on either cytotoxicity or calcium handling of these cells. Protein was then extracted from cells after they had been trypsinized (1 X trypsin for 5 min) and spun down. The cell pellet was treated with lysis buffer (containing 1 X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM Na2VO4, and 20 μg/ml aprotinin) and left to rock for 20 min at 4°C. The samples were then incubated on ice for 1 h, spun at 13,000 rpm for 15 min at 4°C, and the pellet was discarded. Protein was quantified using a Bio-Rad protein assay according to the manufacturer’s instructions. One-hundred microliters of protein lysate from each sample were pre-cleared, first with 2 μl of washed A/G agarose beads (Santa Cruz Biotechnology) alone for 30 min, and then, after spinning for 10 min at 13,000 rpm and removing the lysate, with 2 μl of washed A/G agarose beads plus 0.25 μg/ml normal mouse IgG. The sample was respun for 10 min at 13,000 rpm, the lysate was kept, and the pellet was discarded. The lysate from each sample was then incubated with 2 μg/ml of mouse monoclonal anti-phosphotyrosine antibody (250 μg/ml, R&D PY20) overnight, rocking at 4°C. Two microliters of washed A/G agarose beads were then added to each of the samples and left to incubate for 2 h, rocking at 4°C. Samples were then spun

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**Fig. 2.** Mutations in nephrin signaling result in increased apoptosis in podocytes in response to serum starvation. Comparison of annexin V (AV) and propidium iodide (PI) labeling of serum-starved wild-type (WTs; A), NDs (B), and NMs (C). Pie charts show percentage of cell populations in serum-starved WTs compared with NMs and NDs for this experiment. D: % apoptosis in serum-starved nephrin-mutated and -deficient cells was significantly increased compared with WTs at the same time point. ++P < 0.01 unpaired t-test. *P < 0.01 unpaired ANOVA, n ≥ 3.
for 10 min at 13,000 rpm at 4°C, the supernatant was removed and retained, and the precipitate was washed twice with lysis buffer. Equal quantities of supernatant or the entire precipitate of each protein sample lysate was mixed with 1× SDS-loading buffer (100 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.2% bromophenol blue) and boiled for 5 min. Samples were run on a 7.5% SDS-PAGE gel at 90 V and were then transferred to a polyvinylidene-fluoride (PVDF) membrane over a period of 4 h at 250 mA. Membranes were blocked in 3% BSA, and immunodetection was carried out using 1:5,000 mouse monoclonal anti-phosphotyrosine IgG (R&D, PY20) and 1:30,000 horseradish peroxidase (HRP)-conjugated goat polyclonal anti-mouse IgG antibody (10 μg/ml, Pierce) diluted in 1.5% BSA. Bands were visualized using a Supersignal West Femto Maximum Substrate kit (Pierce). The membrane was stripped as above and reprobed with either 1:500 rabbit polyclonal anti-nephrin antibody and 1:2,000 HRP-conjugated goat polyclonal anti-rabbit antibody (Pierce). Bands were analyzed using densitometry.

**Western blotting.** WT cells were serum starved for 16 h, then treated with 1 nM VEGF for 20 min, or left untreated. Protein was then extracted and quantified as above. Equal quantities of cell lysate were then mixed with SDS-loading buffer, run on a 12% SDS-PAGE gel, and transferred to a PVDF membrane as above. The PVDF membrane was blocked with 10% Marvel and then probed with 1:200 mouse monoclonal anti-phospho-AKT antibody (p-ser 472/473) (200 μg/ml, BD Bioscience) in 5% dried milk (Marvel) overnight, rocking at 4°C. The membrane was then washed a minimum of 5 × 5 min with 0.05% PBS-Tween (PBS-T) and then probed with 1:2,000 HRP-conjugated goat polyclonal anti-mouse IgG antibody (Pierce) in 5% Marvel. The washes were repeated and the bands were visualized using the SuperSignal West Femto Maximum Substrate kit (Pierce). The membrane was then stripped with stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 1 M Tris), washed in PBS-T, and the immunodetection was repeated this time using 1:200 mouse monoclonal anti-AKT antibody (200 μg/ml) and 1:2,000 of the HRP-conjugated secondary antibody (Pierce), both diluted in 5% Marvel and visualized as before. Bands were analyzed using densitometry, and phosphorylated AKT was expressed as a percentage of total AKT protein.

**Statistics.** Data are presented as means ± SE. A Bonferroni test was used to compare subpopulations of cells in the apoptosis assays. Two-tailed paired Student’s t-tests were used to compare paired data on the same cells, and unpaired t-tests were used to compare cells treated differently on blots. A P value of <0.05 was taken as indicative of significance.

**RESULTS**

**Human podocyte apoptosis is nephrin dependent.** Serum starvation did not increase apoptosis in WT cells when serum starved for 8 h (apoptosis 1.5 ± 0.4% of cells) or 24 h (2.2 ± 0.4%; Fig. 2A). In cells deficient in nephrin, 24-h serum starvation resulted in widespread cell death (>50% of cells). To determine whether this was due to rapid apoptosis, the effect of 8-h serum starvation was investigated. After 8-h serum starvation, significantly more cells were undergoing apoptosis than in WT cells subjected to 8-h serum starvation (22.1 ± 4.7%; Fig. 2B), indicating that in the absence of nephrin, serum starvation induces rapid apoptosis. Moreover, in cells with a mutation in the extracellular domain of nephrin (NM cells), apoptosis was significantly greater than in WT cells after 24-h serum starvation (25.3 ± 2.0%; Fig. 2C).

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**Fig. 3.** Vascular endothelial growth factor (VEGF) reduces apoptosis in chronically serum-starved normal human podocytes. Comparison of AV and PI labeling of serum-starved WTs (A) compared with 1 nM VEGF-treated (B), serum-starved WTs. C: compared with serum starvation (SS) alone, VEGF treatment significantly reduced apoptotic cells (paired t-test, *P < 0.05, n = 3).
Figure 2D shows the mean results of three experiments showing that ND cells undergo significantly more apoptosis than WT cells, even after only 8-h serum starvation, and mutations in the extracellular domain also result in more cells undergoing apoptosis than WT cells. It has previously been shown that WT conditionally immortalized, but dedifferentiated (i.e., before thermoswitching), podocytes do not form foot processes, and nephrin expression is restricted to the perinuclear region. This alters during differentiation to result in normal nephrin localization (27). To determine whether apoptosis in WT cells with abnormal nephrin localization is also exaggerated, dedifferentiated cells were serum starved for a total of 24 h and apoptosis was measured as above. Apoptosis was significantly greater than in differentiated cells (15.7 ± 4.6%, P < 0.05, unpaired t-test).

VEGF reduces apoptosis only in podocytes with intact nephrin intracellular domains. Although the level of apoptosis was low in WT cells serum starved for 24 h, apoptosis could be induced to a significant degree in normal podocytes by chronic serum starvation for a total of 48 h (Fig. 3A). This apoptosis was attenuated by treatment with VEGF (Fig. 3B). In chronically serum-starved cells, 1 nM VEGF significantly decreased the percentage of cells undergoing apoptosis from 37.6 ± 5.6 to 19.5 ± 5.6%, P < 0.05 (Fig. 3C), suggesting that VEGF reduces cytotoxicity by reducing apoptosis. In nephrin-deficient cells serum starved for 24 h and with a similar percentage of apoptosis, however, VEGF was unable to inhibit apoptosis induced by serum starvation (Fig. 4, A and B). Treatment with VEGF had no effect on the distribution of cells undergoing apoptosis or necrosis in this cell type (Fig. 4C). Interestingly, VEGF was still able to reduce apoptosis induced by a total of 24 h in cells with a mutation in the Ig5 motif of the extracellular domain of nephrin (NMs), an example of which is shown in Fig. 5, A and B. Treatment with VEGF significantly decreased the percentage of cells undergoing apoptosis from 25.32 ± 2.01 to 15 ± 6.35% (Fig. 5C). To investigate whether the VEGF dependence of the reduction in apoptosis was associated with nephrin localization, dedifferentiated WT cells (i.e., before thermoswitching) were treated with VEGF as above and compared with serum-starved cells. VEGF did not reduce apoptosis compared with untreated cells (12.5 ± 2.8, compared with 15.7 ± 4.6%, P > 0.1), suggesting that correct nephrin localization may be necessary for the inhibition of apoptosis.

VEGF stimulates phosphorylation of nephrin. Because VEGF-mediated inhibition of apoptosis was dependent on the presence of nephrin, we determined whether VEGF could stimulate phosphorylation of nephrin. Figure 6A shows proteins from serum-starved podocytes that were immunoprecipitated with a phosphotyrosine antibody. In the absence of VEGF, nephrin was detected in equal proportions in the supernatant and the precipitate (lanes 4 and 5). In the presence of VEGF, most of the detection occurred in the precipitate (lanes

![Fig. 4. VEGF has no significant effect on serum-starved, nephrin-deficient podocytes (NDs). Comparison of AV and PI labeling of serum-starved NDs (A) compared with 1 nM VEGF-treated NDs (B). C: compared with serum starvation alone, VEGF treatment had no significant effect on any of the cell populations (not significant, Bonferroni, n = 5).](http://ajprenal.physiology.org/)}
2 and 3), indicating that nephrin is either phosphorylated in the presence of VEGF or is coprecipitated with other tyrosine-phosphorylated proteins. Unfortunately, the antibody to nephrin could not successfully be used to precipitate nephrin directly. Because VEGF is produced by these podocytes, it is possible that the tyrosine phosphorylation was being induced by an autocrine mechanism. To determine whether this was the case, the experiments were repeated using a neutralizing monoclonal antibody to VEGF in the absence of exogenous VEGF (mAb). In the presence of the neutralizing antibody, the density of the nephrin band in the immunoprecipitate was attenuated (Fig. 6, lanes 6 and 7, n = 4, VEGF/AKT/PKB 0.05). When the densities of the bands were compared, it was clear that VEGF treatment resulted in a 3.35 ± 1.2-fold increase in nephrin phosphorylation compared with control (n = 6, e.g., Fig. 6A, lane 2 vs. lane 4), whereas mAb treatment resulted in a decrease in nephrin phosphorylation by 0.21 ± 0.09-fold compared with control (n = 4, Fig. 6A, lane 6 vs. lane 4). There was therefore a significant difference in nephrin phosphorylation between VEGF and mAb-treated cells (P < 0.05, unpaired t-test; Fig. 6B).

**VEGF stimulates AKT phosphorylation only in the absence of nephrin.** Because 1) VEGF is known to stimulate AKT/PKB in some cell types, 2) the results described show that VEGF reduces apoptosis and induces the phosphorylation of nephrin, and 3) previous data showed that the VEGF-mediated reduction in podocyte cytotoxicity was wortmannin dependent, we postulated that VEGF might reduce apoptosis through nephrin-dependent activation of AKT. Normal and nephrin-deficient podocytes were therefore treated with VEGF and AKT phosphorylation was measured using a phospho-AKT-specific antibody. First, we observed that basal phospho-AKT levels were not comparable between normal podocytes and nephrin-deficient podocytes. AKT phosphorylation in serum-starved cells (normalized to the positive control band, i.e., phosphorylated AKT in ethanol-treated human umbilical vein endothelial cells) was significantly reduced in nephrin-deficient podocytes (0.7 ± 0.29-fold of control, n = 5) compared with normal podocytes (2.9 ± 0.93-fold of control, n = 3, P < 0.05, unpaired t-test; Fig. 7). In addition to our surprise, serum-starved normal podocytes treated with VEGF showed a highly significant and reproducible reduction in AKT phosphorylation (0.43 ± 0.06-fold, n = 5) compared with cells serum starved alone (Fig. 8A). Moreover, when we treated nephrin-deficient cells with VEGF, we were also surprised to see that VEGF significantly increased AKT phosphorylation 1.7 ± 0.29-fold compared with untreated cells (n = 5, P < 0.001, unpaired t-test; Fig. 8B). These changes in AKT phosphorylation were reproducible, significant (P < 0.001; Fig. 8C), consistent, and were detected using two different phospho-AKT antibodies. To ensure that another possible pathway was not involved, we investigated MAPK phosphorylation. There was no difference in MAPK phosphorylation between VEGF-treated and control-treated cells (data not shown).
DISCUSSION

Because most mutations in nephrin result in nephrotic syndrome and proteinuria, it has been regarded as a key structural component of the slit diaphragm, regulating the macromolecular permeability of the glomerulus. More recently, the potential of nephrin to act as a signaling molecule has opened new lines of inquiry. There are a number of potential mechanisms for its phosphorylation and for its downstream targets. For example, in a similar manner to VEGF in endothelial cells, nephrin can facilitate the phosphorylation of AKT. The COOH-terminal domain of nephrin associates with the p85 subunit of PI 3-kinase and recruits it to the plasma membrane (20). This allows the phosphorylation of AKT on serine 473 [the site of VEGF-mediated AKT phosphorylation in endothelial cells (15)]. The intracellular signaling pathways through which nephrin acts, for example to influence podocyte survival, may therefore play a key role in maintaining barrier permeability in addition to, or perhaps in some circumstances instead of, a direct junctional permeability restriction.

The role of VEGF in glomerular biology and pathology remains unclear. Recent in vivo studies suggest that a balance of VEGF expression may be required for normal glomerular function (12). The potential paracrine role highlighted above would appear to be a not unreasonable notion. There are observations, however, that still require explanation; for example, the paradox of the evident production of VEGF by podocytes but the absence of overt angiogenesis in normal glomeruli despite the fact that glomerular endothelial cells express VEGF-R2. We demonstrated that this may be because mature podocytes express significant levels of distally spliced isoforms of VEGF (10) that have been shown to be inhibitory (3) and antiangiogenic (45). In addition, the presence of neuropilin-1 and VEGF-R1 by podocytes themselves suggest a potential autocrine role for podocyte-derived VEGF as with many other “nonendothelial” cells. We initially addressed this possibility by a study of VEGF-induced calcium responses and cell survival in human podocytes (14). Here, we show that this increased cell survival is due to a reduction in apoptosis mediated by VEGF. VEGF has been shown to reduce apoptosis in a large number of cells and cell lines, many of which express VEGF-R1. These include cancer cells, e.g., breast, prostate, and multiple myeloma cells (1, 22, 27) as well as normal neurons (33), monocytes (36), skeletal myoblasts (18) in mammals, and hematopoietic cells in both mammals and Drosophila (6, 16). The mechanisms through which VEGF reduces apoptosis, as shown here, and increases podocyte cell survival (14) are unknown. Although VEGF is known to induce phosphorylation of MAP kinases in transformed endothelial cells overexpressing VEGF-R1 (21) and MAP kinases have been linked with both induction and inhibition of apoptosis (as reviewed in Ref. 44), we did not see any change in MAPK phosphorylation. This is an area that needs further investigation in podocytes.

Nephrin has previously been suggested to aid podocyte survival, because animals deficient in the nephrin-anchoring protein CD2AP have increased podocyte apoptosis and reduced cell survival (38). However, this is the first direct evidence that nephrin is a survival factor for human podocytes.
This leads to the possibility that deficiencies in nephrin due to some nephrin mutations might result in increased proteinuria, not only through nephrin-dependent slit diaphragm structure but also through a failure of nephrin-dependent podocyte survival signaling.

These results suggest that nephrin can stimulate a signaling pathway that affects the apoptotic state of the podocyte. Moreover, VEGF can also stimulate a signaling pathway that affects the apoptotic state of the podocyte but does not do so when intracellular nephrin signaling is disrupted, either by mutation or mislocalization. This occurred at doses of VEGF that have been shown to be active on endothelial cells to increase permeability (4), angiogenesis (28), and vasodilatation (25).

There is therefore a distinct possibility that the two pathways are closely interlinked and that VEGF could signal via nephrin to prevent apoptosis and hence may ultimately affect the dynamics of the glomerular filtration barrier. Interestingly, in mice treated with neutralizing antibodies to VEGF, nephrin expression in the glomerulus appeared to be reduced (40), and proteinuria occurred, again suggesting a link between nephrin, VEGF, and renal function.

The interaction of VEGF with nephrin is not clear. However, the cytoplasmic tail of nephrin can be tyrosine phosphorylated by Src family members (20), particularly Fyn (43). Another Src family member, Yes, is also associated with nephrin, although it does not appear to increase its phosphorylation. In endothelial cells, Fyn and Yes have shown preferential binding to VEGF-R1 [expressed on podocytes (14)], resulting in their phosphorylation on VEGF binding (9). It has also been suggested that VEGF-R1-Fyn/Yes complexes localize to caveolae fractions of endothelial cells (21), which might also be the case in podocytes. Of interest, caveolin-1, the principal protein that identifies caveolae in endothelial cells, is located in the foot processes of podocytes (39) and is therefore colocalized with nephrin. VEGF phosphorylation of its receptor in podocytes could induce the phosphorylation of the Src family of protein kinases, e.g., Fyn, and subsequently induce the phosphorylation of nephrin.

Surprisingly, although VEGF signaled through AKT, it did so in the opposite manner to that expected, i.e., reduced AKT phosphorylation in the presence of nephrin, but increased AKT phosphorylation in its absence. This puzzling finding is at odds with data (20) from transfected human embryonic kidney (HEK) cells or mouse podocytes. Interestingly, nephrin expression in those two cell types was unusual, in that it was overexpressed in HEK cells, and its expression in mouse podocyte cell lines was reduced compared with normal. Although we are not able to readily explain this finding, it was highly reproducible. One possibility is that HEK293 cells in the above study transfected only with nephrin lack other slit diaphragm machinery, i.e., slit diaphragm-associated proteins that may modify nephrin-dependent signaling. In addition, these authors highlight the tendency of receptor or adaptor protein overexpression to initiate signaling in the absence of ligand binding. An alternative explanation is that promoter driven overexpression of nephrin in HEK293 cells or podocytes activates different pathways compared with nephrin expressed at a constitutive level and/or it responds to activation by a specific ligand.

Of note, previous studies found that VEGF also induced a small reduction in AKT phosphorylation in microglial cells, which, like podocytes, express VEGF-R1 but not VEGF-R2 (13). It is possible that in the presence of nephrin, AKT phosphorylation is constitutively active in serum-starved, normal podocytes, supported by the observation that serum-
starved levels of phospho-AKT are much higher in normal podocytes than nephrin-deficient cells. VEGF has been shown to activate VEGF-R-associated tyrosine phosphatases such as SHP2. It is therefore possible that in the presence of nephrin, VEGF cannot further activate PI 3-kinase, but it can activate tyrosine phosphatases that act on AKT (possibly via VEGF-R1 as VEGF-R1 has been shown to activate tyrosine phosphatases such as SHP2 (32)), hence reducing AKT phosphorylation. In the absence of nephrin, VEGF can potentially stimulate PI 3-kinase to a greater extent than it activates its phosphatase, hence increasing AKT phosphorylation (Fig. 8). Presumably, this is not sufficient to rescue apoptosis, because VEGF does not reduce apoptosis in nephrin-deficient cells. Clearly, VEGF-nephrin signaling may act through one or more other survival related signaling pathways, but the full signaling pathways are not yet known, nor the effect of VEGF treatment on VEGF-induced gene expression in cell types that express VEGF to act in an autocrine fashion. The effect of VEGF on VEGF expression and splicing in these cells is unknown but may be an important regulatory pathway.

Here, we showed for the first time that normal, constitutive, nephren expression prevents apoptosis in human podocytes. Moreover, VEGF can inhibit apoptosis and also stimulates phosphorylation in the intracellular domain of nephrin. In conclusion, nephren appears to be involved in signaling in human podocytes to prevent apoptosis. VEGF reduces apoptosis, possibly by a nephren-mediated pathway, stimulates phosphorylation of nephren, but does not stimulate AKT phosphorylation in the presence of nephren. VEGF may therefore act, in normal adult human podocytes, as an autocrine survival factor by nephrin-dependent inhibition of apoptosis.

**GRANTS**

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