Vascular endothelial growth factor is a survival factor for renal tubular epithelial cells

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Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen that promotes angiogenesis, increases vascular permeability, and is chemoattractant for monocytes (13, 14). VEGF has been shown to have a role in a wide variety of situations, including embryogenesis, placental growth, tumor growth, diabetes, wound healing, inflammatory responses, and tissue remodeling (13, 14). There are two known receptors for VEGF, previously described as flt-1 and KDR/flk-1, now designated VEGF receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2), respectively. Other VEGF-like receptors also exist, such as VEGF receptor 3 (VEGFR-3 or flt-4) (26, 27, 35, 39). All of these are type III tyrosine kinases, characterized by seven immunoglobulin-like loops within their extracellular domain and a split kinase domain within the cytoplasmic molety. VEGF and related factors such as placental growth factor and the recently described novel VEGF molecules VEGF-B, VEGF-C, and VEGF-D are ligands for VEGF receptors (6, 24, 26). VEGF receptors undergo dimerization and autophosphorylation after ligand binding, leading to activation of intracellular signaling molecules such as MAP kinase and phospholipase C (26, 41).

Until recently, most studies have described VEGF receptor expression as specific to endothelial cells. The discovery of flt-1 (VEGFR-1) on monocytes, and its ability to mediate monocyte chemotaxis in response to VEGF (3), was one of the first examples of nonendothelial cells possessing functional VEGF receptors. There are now several descriptions of nonendothelial cells expressing VEGF receptors, but most of these have not demonstrated function. For example, VEGF receptor protein or mRNA has been reported in rat mesangial cells (33), hepatocytes (VEGFR-1 and VEGFR-2) (30), Leydig and Sertoli cells (VEGFR-1 and VEGFR-2) (10), and in endometrial epithelium (VEGFR-2) (7) without demonstration of function. Cell lines that have been reported to express functional receptors include osteoblasts (22), human retinal pigment epithelial cells (19), pancreatic duct epithelium (VEGFR-2) (29), and uterine smooth muscle cells (5).

This study demonstrates the presence of VEGFR-1 and VEGFR-2 protein and mRNA on the renal tubular epithelial cell line NRK-52E, as well as histological evidence for VEGFR-1 and VEGFR-2 protein expression on rat renal tubular epithelium in vivo. In addition, this study demonstrates that VEGF can induce proliferation of these cells when serum deprived and protect against hydrogen peroxide-induced apoptosis and necrosis. These data suggest that VEGF and related ligands may function as survival factors for renal tubular epithelial cells in vivo.

METHODS

Cell culture. NRK-52E, a nontransformed tubular epithelial cell line from normal rat kidney (American Type Culture Collection no. CRL-1571), was maintained in Dulbecco’s modified Eagle’s medium supplemented with 15 mM HEPES buffer (GIBCO-BRL), 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Commonwealth Serum Laboratory) at 37°C in standard incubators (95% air-5% CO2). NRK-52E cells have been cloned from a mixed culture of normal rat kidney cells and possess characteristics of both proximal and distal tubular epithelial cells (8). Bovine aortic endothelial cells (BAEC) were grown under identical conditions with media further supplemented with cis-hydroxyproline (20 µg/ml) (Sigma Chemical, St Louis, MO).

Isolation of total RNA. Total RNA was extracted from cultured cells by using Trizol (Life Technologies, GIBCO-

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BRL, Melbourne, Australia) according to the manufacturer’s specifications. Sample RNA levels were quantitated by reading the absorbance at 260 nm. Final samples were stored at −70°C until required for RT-PCR and Northern blot analysis.

Northern blotting. VEGFR-1 and VEGFR-2 cDNA inserts were PCR amplified from reverse transcribed rat kidney RNA by using primer sequences obtained from the known rat receptor sequences (42, 43) (accession nos. D28488 and U93306, respectively). Primers for the VEGFR-1 insert (forward 5’-CAAGGGACTCTACACTTGTC-3’ and reverse 5’-CCGAATGCGGACAGATTTC-3’) resulted in a 240-bp product corresponding to a portion of the extracellular domain (amino acid residues 305–384). Primers for the VEGFR-2 insert were as described by Wen and co-workers (42) (forward 5’-GCCAATGAGGGGAACCTGAGAC-3’ and reverse 5’-TCTGACTCTGCTGATGCTGTC-3’). These produced a 537-bp product corresponding to the intracellular, NH2-terminal end of the tyrosine kinase domain of the receptor (amino acid residues 870–1049). The PCR products were cloned into pGEM-T Easy (Promega, Madison, WI), and the DNA sequences were confirmed by sequencing. The VEGFR-1 insert was excised from the vector by using the restriction enzyme EcoR I (Promega), whereas the VEGFR-2 insert was excised by using Nco I and Sal I (Promega). A murine GAPDH insert was obtained as a 1.2-kb Pst I fragment in clone pHcGAP (37). Total RNA samples (15 µg/well) were fractionated on a 1% agarose-formaldehyde gel and transferred to GeneScreen Plus membranes (NEN Life Sciences, Boston, MA). Membranes were cross-linked by using a Stratalinker (Stratagene, La Jolla, CA) and then prehybridized for 1 h at 65°C by using Rapid Hyb buffer (Amersham International).

Inserts were labeled by using the Megaprime DNA labeling system (Amersham, Bucks, UK) and added to fresh Rapid Hyb buffer at 2 × 106 counts/ml hybridization fluid. Membranes were hybridized for 2 h at 65°C and then washed three times for 20 min each [first wash in 2× standard sodium citrate (SSC)/0.1% SDS at 65°C, second wash in 1× SSC/0.1% SDS at 65°C, third wash in 0.1× SSC/0.1% SDS at room temperature] before exposure to X-ray film (Kodak).

RT-PCR. First-strand cDNA was synthesized from NRK52-E cell total RNA by using AMVRT and oligo(dT) (Promega). The subsequent PCR primer sequences were obtained from the VEGFR-1 and VEGFR-2 primers described earlier to produce cDNA inserts for Northern blotting experiments. PCR products were run on 1% agarose gels and analyzed under ultraviolet light. NRK52-E cell RNA samples without AMVRT were used as the negative control in PCR assays.

Laser scanning confocal fluorescence microscopy. All primary antibodies and blocking peptides described were purchased from Santa Cruz Biotechnology. Cells were grown on 22 × 22-mm glass coverslips until 70% confluent, washed with PBS, and then fixed in 3.2% paraformaldehyde. Paraformaldehyde was neutralized with 150 mM glycine in PBS. The cells were permeabilized with 0.3% Triton X-100 (BioRad), blocked with 5% BSA for 30 min, and then incubated overnight with anti-receptor antibodies in 0.3% Triton X-100 and 0.025% 3-(3-cholamidopropyl)-dimethylammonio) 1-propanesulfonate (CHAPS; Sigma Chemical). For VEGFR-1, C-20, a polyclonal rabbit antibody against amino acid residues 1326–1345; and N-931, a polyclonal anti-mouse antibody directed against amino acid residues 931–997. Negative controls were incubated with PBS, then the coverslips were mounted with a water-soluble mountant (Aquamount; BDH, Kilsyth, Victoria, Australia) and analyzed. Images were obtained and generated on a confocal laser scanning microscope (Bio-Rad MRC 1024, Bio-Rad Microscopy Division, Hemel, Hemstead, Herts, UK). BAEC were examined for expression of both receptors and compared with NRK52-E.

RT-PCR. First-strand cDNA was synthesized from NRK52-E cell total RNA by using AMVRT and oligo(dT) (Promega). The subsequent PCR primer sequences were obtained from the VEGFR-1, C-17, a 0.025% 3-(3-cholamidopropyl)-dimethylammonio) 1-propanesulfonate (CHAPS; Sigma Chemical). For VEGFR-1, C-17, a polyclonal antibody was used (directed against amino acid residues 1312–1328). For VEGFR-2, three different antibodies were used: 1) A-3, a monoclonal mouse anti-mouse antibody directed against amino acid residues 1158–1345; 2) C-20, a polyclonal rabbit anti-mouse antibody directed against amino acid residues 1326–1345; and 3) N-931, a polyclonal anti-mouse antibody directed against amino acid residues 931–997. Primary antibodies were incubated with their specific blocking peptide or with an irrelevant peptide for 2 h at room temperature (concentration of peptide 10 µg/ml). After incubation with primary antibodies, the cells were washed once with PBS containing 0.3% Triton X-100 and 0.025% CHAPS and then a further two times with PBS alone. Secondary immunofluorescent antibodies (all purchased from Molecular Probes, Eugene, OR) were goat anti-rabbit Texas red (to detect C-17), goat anti-rabbit Oregon green (to detect N-931 and C-20), and goat anti-mouse Oregon green (to detect A-3). Incubations were for 1 h at room temperature. Cells were washed a further three times with PBS, and then the coverslips were mounted with a water-soluble mountant (Aquamount; BDH, Kilsyth, Victoria, Australia) and analyzed. Images were obtained and generated on a confocal laser scanning microscope (Bio-Rad MRC 1024, Bio-Rad Microscopy Division, Hemel, Hemstead, Herts, UK). BAEC were examined for expression of both receptors and compared with NRK52-E.

PCR products were run on 1% agarose-formaldehyde gel and transferred to GeneScreen Plus membranes (NEN Life Sciences, Boston, MA). Membranes were cross-linked by using a Stratalinker (Stratagene, La Jolla, CA) and then prehybridized for 1 h at 65°C and then washed three times for 5 min each in TBS containing 0.05% wt/vol Tween (BioRad). Secondary antibody incubations were performed for 30 min at room temperature. VEGFR-1 membranes were incubated with horseradish peroxidase-linked protein A (Amersham,) at 1:5,000, and VEGFR-2 membranes were incubated with horseradish peroxidase-linked rabbit anti-mouse antibody (Dako) at 1:1,000, and VEGFR-2 membranes were incubated with horseradish peroxidase-linked rabbit anti-mouse antibody (Dako) at 1:1,000 in 5% rat serum. After a further three washes (5 min each in TBS containing 0.05% wt/vol Tween) immunoreactive proteins were detected according to the enhanced chemiluminescence protocol (Amersham). Blots were analyzed after exposure to autoradiography film (Hyperfilm ECL, Amersham).

Binding assay. NRK52-E were seeded in complete media in 24-well plates and grown to 90% confluence. Cells were washed with cold binding buffer (Dulbecco’s modified Eagle’s medium, 25 mM HEPES buffer, 1% BSA) and then incubated for 2 h at 4°C with binding buffer containing 10 pM [125I]-VEGF165 (specific activity 106 counts·min−1·ng−1; NEN). To compete with the binding of [125I]-VEGF165, unlabeled “competitor” growth factor was also added. Recombinant human VEGF165 (rhVEGF165; ligand for both receptors), recombinant human placental growth factor (rhPIGF; ligand for VEGFR-1 only), and epidermal growth factor (EGF; irrelevant control) were used at various concentrations (0, 0.1, 1, 10, and 100 ng/ml; R&D Systems, Minneapolis, MN). Supernatants were
subsequently removed, and the cells were washed twice in cold binding buffer and incubated for 30 min with 1% SDS in 0.4 M NaOH to lyse the cells. [125I]-VEGF165 binding was measured by using a gamma counter (Packard Cobra Autogamma 5005, Meriden, CT). Each competing rhVEGF165, rhPlGF, and EGF concentration was assessed in quadruplicate. Results were expressed as a percentage of maximum [125I]-VEGF165 binding (where no competitor was added).

Proliferation assay. Cells were seeded into 24-well plates (10^3/well), serum deprived, and then incubated with rhVEGF165 (0, 1, 10, 50, and 100 ng/ml; R&D Systems) in 1% BSA. The proliferation assay was performed in two different ways. In the first group of experiments, cells were seeded in 0.5% fetal calf serum, left overnight, and then incubated with rhVEGF165 and [3H]thymidine. To induce apoptosis, cells were washed and left overnight. To induce apoptosis, cells were washed and incubated with rhVEGF165 and [3H]thymidine (1 mCi/well; NEN). In the second group of experiments, cells were seeded in serum-free media, left overnight, and then incubated with rhVEGF165 and [3H]thymidine. To adequately control the experiments, an equivalent amount of BSA was added to each well (i.e., the same amount of protein as in the rhVEGF165 wells). As heparin has been shown to modulate VEGF receptor binding (18, 36), cells with and without heparin (0.1 ng/ml) were also assessed. At the end of the stimulation periods (24-, 48-, and 72-h incubation at 37°C), cells were washed with PBS, lysed with 200 µl of 1 M NaOH, and then filtered through glass-fiber filter paper by using a cell harvester (Inotech, Dottikin, Switzerland). Specific activity for each well was measured by using a beta counter (Packard Tri-Carb 1600 CA).

Annexin-V-FITC and propidium iodide binding. Apoptosis was induced by using a modification of previously used methods for NRK52-E (34). Briefly, cells were seeded into 25-cm² flasks in media containing 0.1% fetal calf serum and left overnight. To induce apoptosis, cells were washed and media containing 0.1% fetal calf serum was added along with hydrogen peroxide (0.5–1.0 mM) for 6 and 24 h. At the same time, rhVEGF165 (100 ng/ml) or BSA (control) was added. The amount of BSA added to each well (i.e., the same amount of protein as in the rhVEGF165 wells). As heparin has been shown to modulate VEGF receptor binding (18, 36), cells with and without heparin (0.1 ng/ml) were also assessed. At the end of the stimulation periods (24-, 48-, and 72-h incubation at 37°C), cells were washed with PBS, lysed with 200 µl of 1 M NaOH, and then filtered through glass-fiber filter paper by using a cell harvester (Inotech, Dottikin, Switzerland). Specific activity for each well was measured by using a beta counter (Packard Tri-Carb 1600 CA).

RESULTS

RT-PCR and Northern blots. Both RT-PCR and Northern blotting experiments demonstrated VEGFR-1 and VEGFR-2 mRNA transcripts in the NRK52-E. DNA bands from the RT-PCR were 240 (VEGFR-1) and 537 bp (VEGFR-2) as predicted (not shown). Bands obtained on Northern blots were of the correct size, ~7.2 kb for VEGFR-1 and 6.8 kb for VEGFR-2 (Fig. 1). A second mRNA species of ~4.2 kb was shown on VEGFR-1 blots. This may represent an alternatively spliced isoform and is in keeping with observations by other groups (6, 40).

Laser scanning confocal fluorescence microscopy. Fluorescence microscopy showed the presence of both VEGF receptors in NRK52-E (Fig. 2). VEGFR-1 staining was evident in the cytoplasm and on the membranous surface of the cells (Fig. 2A), with some showing prominent staining in vesicle-like structures within the cytoplasm. VEGFR-2 staining was evident with all three antibodies. The cytoplasmic staining was similar in all groups with a prominent perinuclear pattern.

![Fig. 1. Northern blot of total RNA samples from NRK52-E. Northern blot demonstrates mRNA for vascular endothelial growth factor receptor 1 (VEGFR-1; R1; left lane) and 2 (VEGFR-2; R2; right lane) in normal NRK52-E. A single mRNA species was identified for VEGFR-2 (6.8 kb). Two mRNA species were identified for VEGFR-1 (7.2 and 4.2 kb). Total RNA was ~15 µg/well.](http://ajprenal.physiology.org/)

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**Statistics.** Results from binding and proliferation assays were analyzed by using Instat 2.01 (GraphPad software). A one-way ANOVA was performed to determine whether there was a significant difference between experimental and control groups. Specific statistical tests used were multiple comparison Bonferroni (binding assay) and Dunnett (proliferation assay) tests. P values of <0.05 were deemed significant.
particularly with the A-3 and C-20 antibodies (Fig. 2, C and E). In addition, prominent nuclear staining was seen by using the N-931 antibody (Fig. 2F). Nuclear localization of VEGFR-2 has recently been observed in endothelial cells by others (11, 38), but the significance of our observation is unclear at this stage. The specific blocking peptides inhibited the staining observed (Fig. 2, B and D), whereas the use of irrelevant peptides did not (Fig. 2, A and C). No fluorescence was seen in negative control cells for A-3 and N-931 antibodies (not
BAEC showed similar staining to the NRK52-E with the use of the various antibody and peptide combinations. VEGF receptor staining in BAEC by using the C-17 and A-3 antibodies are shown (Fig. 2, G and H).

Western blots of NRK52-E cell protein. Blots of NRK52-E whole cell lysates showed bands corresponding to the known sizes of VEGFR-1 and VEGFR-2. Both receptors are ~200 kDa in size, but this may vary depending on glycosylation and whether the receptor is complexed with its ligand (18, 36, 41). Bands of ~200 and 170 kDa were demonstrated in the VEGFR-1 blots (Fig. 3A). Whole cell lysates prepared from BAEC were used as positive controls and demonstrated bands of ~200 and 180 kDa. VEGFR-2 blots also demonstrated two bands of the expected size in NRK52-E (~200 and 180 kDa), with a similar 180-kDa band found in BAEC (Fig. 3B).

Binding assay. Binding assay for [125I]-VEGF165 showed that both rhVEGF165 and rhPlGF bound strongly to the cells. Both growth factors were able to compete out the binding of [125I]-VEGF165 on the cells (Fig. 4). EGF had no effect on [125I]-VEGF165 binding. This further supports the presence of VEGFR-1 and VEGFR-2 on NRK52-E. Binding of [125I]-VEGF165 was decreased to ~25–40% of normal with the addition of $1 \text{ng/ml}$ of rhVEGF165 or rhPlGF ($P < 0.01$, Bonferroni multiple comparison test).

Proliferation assay. VEGF significantly stimulated NRK52-E proliferation in both sets of experiments (Fig. 5). Heparin (0.1 ng/ml) did not augment this response. The most significant effect occurred in cells seeded without serum and then serum deprived overnight before incubation with rhVEGF165 (Fig. 5A). After 24 h of incubation with rhVEGF165 at 1 and 10 ng/ml, cells had a 2.3-fold increase in [3H]thymidine incorporation ($P < 0.05$, Dunnnett multiple comparison test) and a 3.3-fold increase at 50 and 100 ng/ml of rhVEGF165 ($P < 0.01$, Dunnnett multiple comparison test). Where the cells were seeded in 0.5% fetal calf serum and then serum deprived for 72 h before incubation with rhVEGF165, the proliferative effect of VEGF was not evident until 72 h of rhVEGF165 incubation (Fig. 5B). At 24- and 48-h incubation, rhVEGF165-incubated cells and control cells (incubated in BSA) had similar levels of proliferation. A 1.5-fold increase in [3H]thymidine incorporation was seen at 72 h with rhVEGF165 concentrations of 50 and 100 ng/ml ($P < 0.05$ and $P < 0.01$, respectively, Dunnnett multiple comparison test). Results without heparin are shown (Fig. 5).

Annexin-V-FITC and propidium iodide binding. Cells incubated with rhVEGF165 were protected from hydro-
gen peroxide-induced apoptosis and necrosis. In cells
incubated with rhVEGF 165 for 6 h (Fig. 6), a lower
proportion showed annexin-V-FITC staining compared
with control cells (39 vs. 54%). The proportion of viable
cells (negative for both annexin-V-FITC and propidium
iodide) was also higher in the rhVEGF 165-incubated
group (50.5 vs. 39.7%). In cells incubated with rhVEGF 165
for 24 h (not shown), similar results were observed
compared with control (annexin-V-FITC positive cells:
28.4 vs. 38.9% viable cells: 67.5 vs. 57.3%). The major-
ity of annexin-V-FITC-positive cells showed double
staining with propidium iodide, indicating the cells
were necrotic or at a late stage of apoptosis. Few cells in
each group showed only single staining for annexin-V-
FITC (early apoptosis). Results shown are representa-
tive of three separate experiments using the same
conditions.

Immunohistochemical staining of normal rat kidney.
Normal rat kidney tubular epithelium showed staining
for both VEGFR-1 and VEGFR-2. Sequential staining
with the lectins AH (localizes DCT and collecting ducts)
and PHA-E (localizes PCT) was used to localize recep-
tor staining where this occurred. VEGFR-1 staining by
using the C-17 antibody was localized to both proximal
distal tubules of the cortex and to S3 segments of
the PCT in the outer medulla (Fig. 7, A-C). There was
prominent staining of the brush border in cells of the

Fig. 5. Proliferation of serum-deprived NRK52-E in response to
rhVEGF 165. Cells were seeded without serum (24 h; A) or seeded with
0.5% serum and then serum deprived (72 h; B). Addition of heparin
had no effect. Only results without heparin are shown. For detailed
description of conditions, see METHODS. When seeded without serum,
rhVEGF 165 (24-h incubation) significantly stimulated proliferation of
serum-deprived NRK52-E at all concentrations compared with cells
incubated with BSA alone. rhVEGF 165 (1 and 10 ng/ml) produced a
2.3-fold increase in [3H]thymidine incorporation (*P < 0.05); 50 and
100 ng/ml rhVEGF 165 produced a 3.3-fold increase in [3H]thymidine
incorporation (**P < 0.01). When seeded with serum and then serum
deprived (B), longer incubation with rhVEGF 165 was needed (72 h)
before an effect was seen. rhVEGF 165 (50 and 100 ng/ml) produced
a 1.5-fold increase in [3H]thymidine incorporation (*P < 0.05 and
**P < 0.01). DPMI, disintegrations/min.

Fig. 6. Survival effect of VEGF. Annexin-V-FITC and propidium
iodide staining of hydrogen peroxide-treated NRK52-E. NRK52-E
seeded in 0.1% fetal calf serum were incubated for 6 h with hydrogen
peroxide (0.75 mM) and either rhVEGF 165 (100 ng/ml; A) or BSA (B).
Proportion of annexin-V-FITC-stained cells was lower in cells treated
with VEGF compared with control (39 vs. 54%; right). Majority of
annexin-V-FITC-positive cells showed double staining with propidium
iodide (top right), indicating cells were late apoptotic or
necrotic. Proportion of cells that were viable (negative for both stains;
bottom left), stained with annexin-V-FITC alone (bottom right), and
propidium iodide alone (top left) are also shown. Proportion of viable
cells was higher in cells treated with VEGF compared with control
(50.5 vs. 39.7%).
In the medulla there was mild, generalized VEGFR-1 staining of tubular structures including collecting ducts and loop of Henle (not shown). There was little evidence of staining in glomeruli, peritubular capillaries, and larger vessels. Preincubation of C-17 antibody with C-17p blocking peptide inhibited staining (Fig. 7D). All three VEGFR-2 antibodies showed a similar staining pattern in the kidney, with prominent tubular epithelial staining. With the monoclonal antibody (A-3), VEGFR-2 staining was strongly localized to the macula densa and DCT of the cortex and to collecting ducts in the inner and outer medulla (Fig. 8, D-F). The localization of VEGFR-2 staining was very prominent, with a gradient of staining from the cortex down to the outer and inner medulla (Fig. 8D). Proximal tubules and the loop of Henle showed minimal staining with the A-3 antibody. The two polyclonal antibodies demonstrated tubular staining that was more generalized. The C-20 antibody demonstrated prominent staining of DCT and collecting ducts (Fig. 8A). Preincubation with C-20p blocking peptide inhibited this staining (Fig. 8B). Some nuclear staining was once again observed with the N-931 antibody, as were glomerular and peritubular capillary staining (Fig. 8C). Although endothelial cells in the kidney did not show prominent staining with all the antibodies, endothelial cell-specific staining was demonstrated in adult rat heart and lung specimens and a variety of neonatal rat specimens (not shown). The most prominent endothelial staining was observed by using the C-17 and N-931 antibodies, confirming the specificity of these antibodies to VEGF receptors on rat endothelium. All negative controls demonstrated no staining, in particular, controls using blocking peptides to the C-17 (VEGFR-1) and C-20 (VEGFR-2) antibodies. Preincubation of the C-17 antibody with the C-20 peptide, and the C-20 antibody with the C-17 peptide, did not inhibit staining.

**DISCUSSION**

VEGF is an important angiogenic growth factor that signals via VEGF receptors on endothelial cells (12, 14, 20). Recent evidence, however, supports a much wider role for VEGF with reports demonstrating receptors on a variety of nonendothelial cells (5, 19, 22, 29). This
study is the first to demonstrate functional VEGF receptors on nonendothelial cells of the kidney. The rat renal tubular epithelial cell line NRK52-E was found to express protein and mRNA for both VEGFR-1 and VEGFR-2. The sizes of the mRNA species are in keeping with published studies for both endothelial and nonendothelial cells (3, 5, 23, 35). Reports vary in terms of the accepted sizes of the
protein isoforms for the receptors with VEGFR-1, ranging from 170 to 210 kDa, and VEGFR-2 ranging from 180 to 235 kDa (17, 27, 36, 41). Part of this uncertainty may relate to the existence of alternatively spliced isoforms. Studies that have performed affinity cross-linking of VEGF receptors generally report the existence of several bands in the range of 170–235 kDa (35, 39). In addition, there are reports demonstrating a functional, truncated form of VEGFR-2 in rat retinal tissue (42) and a soluble variant of VEGFR-1 in human vascular endothelial cells (2).

VEGF induced a proliferative response in serum-deprived NRK52-E. In cells seeded without serum, the effect was observed 24 h after incubation with VEGF. In this group VEGF appeared to act as a survival factor, allowing the cells to survive and proliferate under conditions of extreme stress. Whether receptor activation mediated the survival and proliferative response directly, or through an effect on other growth factors is not clear. Studies have demonstrated upregulation of known proliferative growth factors in response to VEGF, such as heparin-binding epidermal growth factor-like growth factor (HB-EGF) and platelet-derived growth factor BB (PDGF-BB) (1).

To investigate the potential role of an antiapoptotic or survival response in the proliferative action of VEGF, the effect of VEGF on apoptosis was examined. Several reports describe the usefulness of annexin-V binding as a marker of apoptosis (4, 21). When combined with propidium iodide staining, cells can be subdivided into viable, early apoptotic and either late apoptotic or necrotic, on the basis of their staining characteristics on flow cytometry. VEGF had a small, protective effect on hydrogen peroxide-induced apoptosis and necrosis, with fewer cells showing staining for annexin-V and propidium iodide when incubated with VEGF, compared with control. Although these results may reflect a proliferative rather than a survival effect of VEGF, the short VEGF/peroxide incubation time should have minimized the degree of cell proliferation. In addition, the hydrogen peroxide concentrations were titrated to obtain a significant degree of apoptosis and necrosis, making cell proliferation under these conditions very difficult. Recent studies have shown a similar response in vascular endothelial cells, using concentrations of VEGF between 10 and 100 ng/ml (15, 16). This survival effect was shown to be regulated through VEGFR-2 with stimulation of the phosphatidylinositol 3'-kinase-Akt signal pathway (16). In the present study, it is difficult to ascertain which of the VEGF receptors may have mediated the survival and proliferative response observed in the serum-deprived cells. Both receptors have a high affinity for VEGF, although the affinity for VEGFR-1 is about 40-fold higher than that for VEGFR-2 (Kd values 16 vs. 760 pM) (23, 39, 41). The concentration of VEGF required to induce the proliferative response was low (≥10 or ≥260 pM) and in keeping with signaling through either of the two high-affinity receptors. Evidence that binding to VEGFR-2 and not VEGFR-1 relies more heavily on heparin modulation (9, 28) would support a role for VEGFR-1 in the proliferation assay, as there was no additional effect seen when cells were incubated with heparin. However, in endothelial cells and monocytes, VEGFR-1 appears to be responsible for target cell migration (3, 44), with evidence supporting more complex roles for VEGFR-2 in endothelial cells, such as mitogenesis, chemotaxis, actin reorganization, and changes in cell morphology (41).

Immunohistochemical staining demonstrated expression of both receptors in rat renal tubules in vivo. With two of the antibodies (C-17 and A-3), the distribution was unusual in that each receptor appeared to localize to specific areas of the nephron. VEGFR-2 staining was prominent in DCT and collecting ducts, whereas VEGFR-1 staining was more diffuse, involving both proximal and distal tubules, with more localized staining seen on the brush border of proximal tubules. These findings suggest VEGF may have a specific role in these parts of the kidney, although the exact nature of this remains unclear. Histological data presented in this study differs from the distribution of VEGF receptors in the kidney reported by another group (31, 32). In these reports, in situ hybridization localized VEGFR-1 and VEGFR-2 mRNA exclusively to renal endothelial cells. Immunofluorescence and in situ [125I]-VEGF binding was used to demonstrate receptor protein expression and this was also localized to endothelial cells. Apart from the fact that the techniques used differ, the reason for the discrepancy is not clear, although the reports refer to human kidney specimens only. A recent study, however, demonstrates evidence for VEGFR-1 on developing renal tubular epithelial cells (38). There are no other studies reporting results in the kidney with the VEGF receptor antibodies used here. The antibodies used in this study were directed against unique COOH-terminal, cytoplasmic portions of the receptors. Cross-reactivity with other tyrosine kinases has been excluded (Santa Cruz Biotechnology). Simon and co-workers (32) used antibodies raised against recombinant, soluble extracellular portions of the receptors. This discrepancy further raises the possibility of the existence of different receptor isoforms.

In conclusion, this study reports the presence of functional VEGF receptors on nonendothelial cells of the kidney, with VEGF exerting a survival effect on rat renal tubular epithelial cells in vitro. VEGF may promote renal tubular epithelial cell survival in vivo in situations associated with cellular stress, for example acute ischemia or toxic injury of the kidney. These data suggest an expanded role for VEGF in pathological conditions in the kidney.

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