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VEGF Expression in Hypoxia and Hyperglycemia: Reciprocal Effect on Branching Angiogenesis in Epithelial-Endothelial Co-Cultures

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Abstract. Vascular endothelial growth factor (VEGF), an angiogenic factor for endothelial cells, is produced by glomerular and tubular epithelia. Using immunoelectron microscopy, VEGF expression by podocytes (GEC) and the proximal tubular epithelium of rat kidney was confirmed. To elucidate the mechanisms of VEGF production and its physiologic consequences, studies were performed in cultured GEC and proximal tubular epithelial cells (RPTEC). Both GEC and RPTEC expressed VEGF-120 and 164 mRNA, as detected by quantitative RT-PCR. Hypoxia resulted in an increase in mRNA abundance, more robust in RPTEC than in GEC, and an increase in VEGF expression by 1.9- and 1.6-fold, respectively. 30 mM D-glucose, but not 30 mM L-glucose, resulted in the elevation of VEGF mRNA in RPTEC, but not in GEC, although both cell types showed a comparable modest increase in VEGF expression. Combined treatment (hypoxia and 30 mM D-glucose) resulted in an increase of VEGF mRNA only in RPTEC; however, an enhanced protein expression was detectable in both cell types. To investigate the role of VEGF in branching angiogenesis, "sandwich" co-cultures were applied with endothelial cells and capillary tube formation was compared under the above conditions. Both RPTEC and GEC induced VEGF-dependent capillary tube formation by co-cultured endothelial cells and in both cell types hypoxia further augmented angiogenesis. In contrast, 30 mM D-glucose suppressed angiogenesis in co-cultures with both cell types despite increased mRNA for VEGF receptors 1 and 2. This study shows (*1*) that VEGF produced by GEC and RPTEC is necessary for branching angiogenesis and (*2*) that hypoxic environment stimulates VEGF production by both epithelial cell types and augments branching angiogenesis, whereas (*3*) hyperglycemic microenvironment, although also stimulatory for VEGF production, fails to augment angiogenesis.

Vascular endothelial growth factor (VEGF) gene family is comprised of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF). VEGF-A, initially described as a vascular permeability factor (1), is expressed by various cell types, including tumor cells (2), cardiomyocytes (3), monocytes (4), and smooth muscle cells (5), as one of five major isoforms: $VEGF_{120}$, $VEGF_{144}$, $VEGF_{164}$, $VEGF_{188}$, and $VEGF_{205}$ (6). $VEGF_{164}$ is a major isoform of VEGF-A, forming homodimeric heparin-binding glycoprotein with molecular weight of 45 kD. VEGF can be expressed as a secretory isoform $VEGF_{120}$ or cell surface- or extracellular matrix-bound isoforms, $VEGF_{188}$ and VEGF $_{205}$ (7). Two distinct receptor tyrosine kinases, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1), are receptors for VEGF expressed on endothelial cells. A third receptor, VEGFR3 (Flt-4) binds VEGF-C and VEGF-D but not VEGF-A. PlGF and VEGF-B bind with high affinity only to VEGFR1. VEGF-A, -C, -D, and -E are established ligands for VEGFR2. Neuropilin-1 (NP-1) is a recently recognized receptor for $VEGF_{164}$, but not $VEGF_{120}$, VEGF-B, VEGF-E, or PlGF (6).

Endothelial cells are the major target for all members of the VEGF family. VEGF-A is a potent endothelial-specific mitogen and a stimulator of angiogenesis. Furthermore, VEGF is a vascular permeability factor, possibly acting by promoting endothelial fenestration (8). The role of VEGF in the maintenance of highly permeable and extensive vasculature in different tumors and in endocrine organs has been established (9).

In the kidney, the peculiar anatomy of microvasculature is characterized by the first division of arterioles into glomerular capillaries, which is followed by the "rete mirabile" of the second subdivision of the efferent arterioles into peritubular capillaries. Both the glomerular and peritubular capillary endothelium is characterized by extensive fenestration and high permeability to solutes. This analogy with the microvasculature of endocrine organs and tumors provided an impetus for investigations into the production of VEGF in the different nephron segments of the kidney (9). VEGF has been found to be produced by the glomerular epithelial cells, podocytes, where it has been tentatively linked to the formation of

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fenestrae in glomerular endothelial cells. Little is known, however, about peritubular capillaries and the mechanisms of their extensive branching, maintenance, and fenestration. In the remnant kidney model, Kang *et al.* (10) observed a strong correlation between the number of peritubular capillaries and tubular VEGF expression. Changes in VEGF production have been also studied under hypoxic and diabetic conditions, yielding some controversial findings (11,12).

It is difficult, however, to examine the role of VEGF, especially in the presence of other growth factors, in the maintenance of glomerular and peritubular capillaries *in vivo*; furthermore, both heterozygous and homozygous VEGF knockout mice are not viable (13), which effectively limits investigative manipulation of VEGF production *in vivo*. For this reason, we elected to study epithelial-endothelial cell interactions *in vitro* in a co-culture system. Here, we demonstrate (*1*) that VEGF produced by glomerular epithelial or proximal tubular cells in culture is necessary and sufficient for branching angiogenesis under control conditions and (*2*) that hypoxic environment stimulates VEGF production by both epithelial cell types and augments branching angiogenesis, whereas (*3*) hyperglycemic microenvironment, although also stimulatory for VEGF production, fails to augment angiogenesis.

Materials and Methods

Cell Cultures

Human renal proximal tubular epithelial cells (RPTEC) were characterized by and obtained from Dr. Lorraine Racusen (Johns Hopkins University School of Medicine, Baltimore, MD) (14). Cells were grown in Renal Epithelial Cell Basal Medium (REBM, Clonetics, San Diego, CA) supplemented with REGM complex (0.5 μl/ml hydrocortisone, 10 pg/ml hEGF, 0.5 μg/ml epinephrine, 6.5 pg/ml triiodothyronine, 10 μg/ml transferrin, 5 μg/ml insulin, 1 μg/ml gentamicin sulfate, and 2% FBS). At confluency, media was replaced with serum-free and growth factor-free media. Cells were exposed to (*1*) hypoxia for 24 h (hypoxic environment was achieved by incubating the cells in the BBL GasPak Pouch, and the completeness of oxygen consumption was confirmed in each experiment [Becton Dickinson, Sparks, MD]; (*2*) to high D-glucose (30 mM) or (*3*) to the equivalent concentration of L-glucose (30 mM) for 24 h; or (*4*) to the combination of high D-glucose and hypoxia. Control cells were cultured under normoxic and euglycemic (5 mM) conditions. After 24 h, cells incubated under different experimental conditions were harvested for isolation of mRNA, immunoprecipitation, and Western blot analyses or used for *in vitro* angiogenesis experiments.

Rat glomerular epithelial cells (GEC) were established by Dr. Esai Noiri (University of Tokyo, Japan), as described previously (15). Briefly, primary GEC were plated on collagen IV-coated 3-cm dishes and maintained in K-1 medium (Nipro, Osaka, Japan) supplemented with 2% NuSerum I (Collaborative Biomedical Products, Bedford, MA), Insulin/Transferrin/Selenium (Collaborative Biomedical Products), 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, BRL, Gaithersburg, MD). After colonies were formed, the medium was aspirated and high-titer (10^8 virus/ml) wild-type simian virus-40 (SV-40) was added for 60 min. GEC were isolated by limiting cloning. To validate the authenticity of the immortalized GEC clone, the expression of several markers of these cells and the lack of markers characteristic of endothelial and mesangial cells were examined. The selected clone of SV-40–transformed GEC expressed large T-antigen (results not shown), confirming the adequacy of transfection. Several markers of GEC were expressed by these cells. The Wilms tumor protein has been detected *in situ* in podocytes, and immunocytochemical staining also showed the presence of this marker in GEC. Moreover, nephrin mRNA was detected in GEC. A monoclonal antibody GSA3 recognizing a cell-specific surface antigen on podocytes (19) showed positive immunostaining of GEC. Furthermore, puromycin exerted cytotoxic effect in GEC, and cells lacked markers characteristic of endothelial and mesangial cells—von Willebrand factor and Thy-1 antigen (not shown). These findings collectively identify the clone of SV-40–transformed GEC as podocytes and rule out any possible contamination of the cells with other resident glomerular cells—endothelial and mesangial cells. GEC were grown in K-1 medium (Nipro). At confluency, medium was replaced with the serum-free and growth factor-free medium (EBM-2, Clonetics). Cells were divided to five experimental groups similar to those described for the RPTEC.

Human umbilical vein endothelial cell (HUVEC) were obtained from Clonetics Corp and grown in endothelial basal medium-2 (EBM-2, Clonetics) as preiviously detailed (15).

RPTEC-HUVEC or GEC-HUVEC Sandwich Co-Cultures

To examine the effect of RPTEC or GEC on the phenotype of endothelial cells, we applied the co-culture model using these two cell lines. RPTEC or GEC were plated in 24-well clusters and cultured for 24 h in REBM or K-1, respectively. Cells were subjected to hypoxia, or 30 mM D-glucose or L-glucose or normoxia for 24 h, as described above. After 24 h under the above conditions, culture medium was aspirated, 100 ul of growth factor–depleted basement membrane matrix (Matrigel; Fisher Scientific, Pittsburgh, PA) diluted with growth factor-free EMB-2 was used as an overlay and was allowed to gel for 30 min in the incubator at 37°C. This step was followed by the addition of 1 ml of EBM-2 serum-free and growth factor–free medium. Thereafter, 30,000 HUVEC cells were added to each well to complete a sandwich co-culture, as described previously (15). Co-cultures were incubated for additional 24 to 96 h without addition of any growth factors or serum.

To study the role of VEGF in capillary tube formation, parallel cultures were treated with 1 μg/ml neutralizing anti-VEGF antibody (PeproTech, Rocky Hill, NJ), which was added at the time of HUVEC seeding.

Angiogenesis (capillary cord formation) was evaluated after 24, 48, and 96 h in co-culture. To quantify the rate of angiogenesis, digital images of 5 fields/well in each group were obtained under the low-power objective using an inverted Nikon microscope and a CoolPix camera (Nikon, Japan). Images were superimposed on a digital grid using Photoshop Pro 5 program (Adobe, Mountain View, CA). The number of capillaries crossing the grid was calculated for each image, and the average number per each field in the experimental group was calculated according to the previously described technique (16). We referred to this number as the angiogenesis score.

Transmission Immunoelectron Microscopy

To visualize the VEGF distribution in the kidney, immunoelectron microscopy of rat kidney sections was performed as previously detailed (15). Rat kidneys were fixed in 4% paraformaldehyde/0.5% glutaraldehyde (GA) in phosphate buffered saline (PBS), pH 7.4. For immunohistochemistry using electron microscopy (EM), 1-mm^3 tissue blocks of GA-fixed kidneys were washed with PBS, dehydrated in ethanol, and embedded in LR-white resin (Polysciences, Washington,

PA). For EM morphology, similar tissue blocks were post-fixed with 1% OsO4 in Veronal-acetate buffer, pH 7.4, for 1 h at 4°C, dehydrated in ethanol and propylene oxide, and embedded in araldite (Polysciences). For EM morphology, ultrathin araldite sections were mounted on naked 400 mesh grids, stained with uranyl acetate and lead citrate, and coated with carbon. For EM immunohistochemistry, ultrathin LR-white sections of approximately 60 nm were mounted on 200 mesh nickel grids, coated with formvar film, and impregnated with carbon. The sections were treated with 0.5 Tween 20 (blocking buffer) for 15 min, labeled with polyclonal anti-VEGF (Santa Cruz Biochemicals, Santa Cruz, CA) diluted 1:50 in blocking buffer for 2 h, rinsed five times in PBS, and incubated for 1 h with goat anti-rabbit IgG conjugated to 15 nm gold (Biocell, England) diluted 1:50 in blocking buffer. This was followed by five rinses in PBS, a rinse with a stream of distilled water, and staining for 5 min with saturated uranyl acetate in 50% ethanol. Examination of all sections was carried out using a JEOL-100B electron microscope at 80 KV.

Relative Quantitative Reverse Transcription-PCR (RT-PCR)

Oligonucleotide primers were designed to amplify VEGF mRNA from GEC and RPTEC cells to identify unique VEGF isoforms. The sequence of the sense primer was 5'-GGACATCTTCCAGGAGTACC-3', and the sequence of the antisense primer was 5'-GTTCCCGAAACCCTGAGG-3'. Total RNA was isolated from GEC and Clone 8 cells with Trizol total RNA isolation reagent (Life Technologies BRL), and the mRNA was then reverse transcribed to cDNA with AMV reverse transcriptase and amplified with expand high fidelity enzyme mix, which were provided in the Titan One Tube RT-PCR System (Boehringer Mannheim, Indianapolis, IN). Equal amounts of mRNA (0.5 μg) from different samples were used in one 25-μl reaction containing 1 $\times$ RT-PCR reaction buffer, 0.2 mM dNTP, 5 mM DTT, and 0.4 uM each primer. The RT-PCR profile consisted of a 30-min incubation at 50°C and 2-min denaturation at 94°C and was then followed by 10 s of denaturation at 94°C, 10 s of annealing at 55°C, and 20 s of elongation at 68°C. 18S mRNA internal control was also co-amplified by incorporate 18S/18S competition mRNA primer (Ambison, Austin, TX) in an another reaction tube. The appropriate 18S:18S Competimer ratio and optimal PCR cycles were selected for each cell line according to manufacture-suggested procedure. Products were analyzed by running 10% of the reaction mixture on a 2% agarose gel with ethidium bromide staining. The ratios of VEGF to 18 sRNA were obtained in at least four independent experiments. The sequences of primers of other VEGF which we used are as follows:

VEGF-B

1) sense, 5'-TTATGCACGTGCCACATGCCAGC-3'; antisense, 5'-GGCGTCTGCAGCGGCAACGGCAG-3' (for GEC)

2) sense, 5'-CAGCTGCGTGACTGTGCAGCGCTG-3'; antisense, 5'-TCAGGGCGCTGGTGGTGCTG-3' (for RPTEC)

VEGF-C: sense, 5'-CCACCATGCAC TTGCTGTGCTTCTTGTC-3'; antisense, 5'-CAATACTTTTCAGGATCTCTGTG-3'

VEGF-D: sense, 5'-CTGGAGAATGCCTTTTGCAACA-3'; antisense, 5'-CCACAGCTTCCAGT CCTCAGAGT-3'

RT-PCR amplification of mRNA encoding for VEGF receptors 1 and 2 was performed following the above protocol. Sense and antisense primers used were as follows:

VEGF-R-1: sense, 5'-GTGGAAGAAATGGCAAACAA-3'; antisense, 5'-ACAGGTCAGAAGCCCTATTT-3'

VEGF-R-2: sense, 5'-GAAAGTACACTGGAGCCTAC-3'; antisense 5'-GAAATGACACTGGAGCCTAC-3'

Immunoprecipitation

After exposure to different culture condition, media from both cell lines were collected and centrifuged for 5 min at 10,000 rpm to remove cell debris. Cells were harvested and lysed in lysis buffer (RIPA buffer containing 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.01 M Tris, pH 8.0, 0.14 M NaCl) containing proteinase cocktail (Roche, Mannheim, Germany) for 30 min at 4°C. The cell lysates were centrifuged at 10,000 rpm for 15 min, and supernatant fractions were collected. For detecting VEGF, media and cell lysates were subjected to immunoprecipitation. Briefly, protein concentration in each group of cell lysates was balanced according to the amount of protein in cell lysates. Protein-balanced medium and cell lysates were kept overnight at 4°C on a rocker with addition of 1 μg/ml rabbit anti-human VEGF polyclonal antibody (Santa Cruz Biochemicals). Next, 15 μl of GammaBind plus sepharose beads (Pharmacia, Uppsala, Sweden) were added for another 2 h. The sepharose beads were collected by centrifugation, washed two times in RIPA buffer and one time in TSA buffer (0.01 M Tris, 0.14 M NaCl, pH 8.0), and one additional wash was performed with 0.05 M Tris, pH 8.0. After boiling for 5 min in SDS gel loading buffer, supernatants were collected and divided into two portions: with and without addition of 2.5% 2-mercaptoethanol. Twenty-microliter samples were electrophoresed on a 4 to 20% SDS polyacrylamide gel. Separated proteins were blotted onto PVDF membranes (Millipore, Bedford, MA), blocked in PBS containing 1% casein for 60 min and incubated overnight at 4°C in 1:3000 dilution of primary mouse anti-human VEGF monoclonal antibodies (Santa Cruz Biochemicals). After intense washing, the membranes were incubated with 1:5000 dilution of secondary horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Life Science, Arlington Heights, IL) for 1 h at room temperature. The membranes were thereafter washed once again and incubated in enhanced chemiluminescence substrate reagent (Amersham) for 1 min. The blots were exposed to x-ray film for 1 to 5 min, and the molecular weight of immunodetected bands was compared with molecular weight standards (Norvex, CA). To further confirm the balanced loading of media and cell lysate for immunoprecipitation, Western blot analyses of β-tubulin was performed. Results were quantitated by scanning films and determining band density by using Scion-image beta-3b software.

Results

VEGF is Immunodetectable in Podocytes and Proximal Tubular Epithelial Cells

Immunoelectron microscopy of rat renal cortex using antibodies against VEGF revealed that immunogold labeling was prominent in glomerular epithelial cells and in the glomerular basement membrane, in accord with our previous study (15) (Figure 1A). In addition, immunogold labeling was conspicuously present in the proximal tubular epithelium and the tubular basement membrane of the rat kidney (Figure 1B). This observation could not be attributed to the trapping of the circulating VEGF by these cells because endothelial cells showed no labeling. To further analyze the hypothesis that VEGF may be produced by the proximal tubular epithelium to be deposited in the tubular basement membrane, potentially contributing to the maintenance of a branching phenotype of peritubular capillary endothelium, we (*1*) performed quantitative RT-PCR of VEGF mRNA isoforms expressed by RPTEC and (*2*) established a co-culture model of RPTEC and HUVEC.

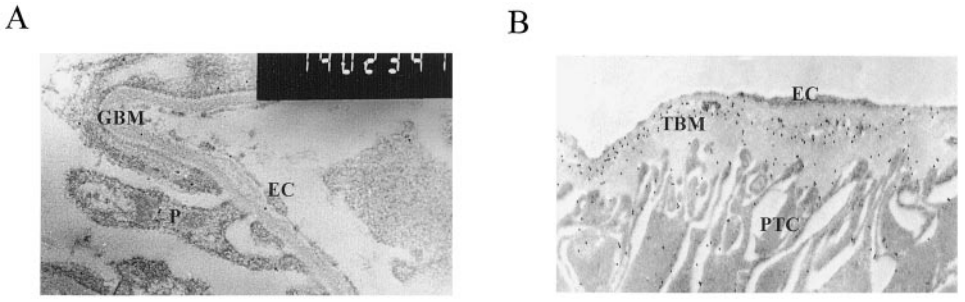

Figure 1. Immunoelectron microscopy of vascular endothelial growth factor (VEGF) distribution in the glomerular epithelial cell (A) and proximal tubule cell (B) of the rat kidney. (A) A typical image of a glomerular tuft showing podocytes, basement membrane, and endothelial cells. Gold-labeled anti-VEGF was conspicuous in podocytes and in the basement membrane. Endothelial cells showed no immunodetectable VEGF, suggesting that VEGF in the basement membrane was not blood-born but secreted by podocytes. (B) There was high-degree labeling of proximal tubular cells and tubular basement membrane with antibodies to VEGF. P, podocyte; EC, endothelial cell; GBM, glomerular basement membrane; PTC, proximal tubular epithelial cell; TBM, tubular basement membrane. Magnifications: ×14,000 in A; ×10,000 in B.

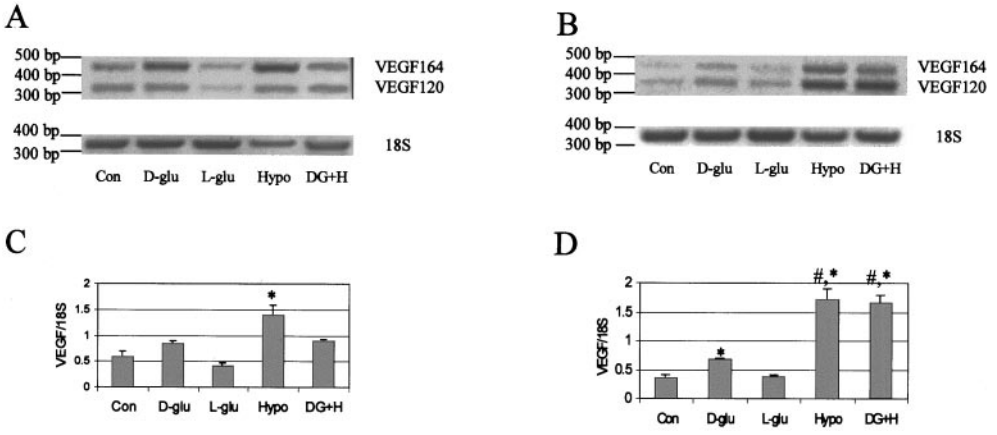

Figure 2. Relative quantitative RT-PCR for VEGF in glomerular epithelial cells (GEC) and proximal tubular epithelial cells (RPTEC). (A and B) Agarose gel electrophoresis for VEGF in GEC (A) and RPTEC (B). Both bands shown are two isoforms of VEGF, $VEGF_{164}$ and $VEGF_{120}$, respectively. The sizes expected for $VEGF_{164}$ and $VEGF_{120}$ are 438 bp and 306 bp, respectively. 18S was used as internal control. (C and D) The ratio of VEGF mRNA expression to 18S RNA in GEC (C) and RPTEC (D). Con, control group; D-glu, 30 mM D-glucose group; L-glu, 30 mM L-glucose group; Hypo, hypoxic group; DG+H, 30 mM D-glucose and hypoxic group. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the D-glucose group.

In parallel, comparative studies were performed in co-culture model of GEC and HUVEC.

VEGF is Expressed by Cultured Glomerular and Proximal Tubular Epithelial Cells

Both GEC and RPTEC expressed VEGF mRNA as detected by RT-PCR (Figure 2). The isoforms of VEGF expressed by each cell type were as follows: two isoforms of VEGF-A, $VEGF_{164}$, and $VEGF_{120}$ were expressed in both RPTEC and GEC (Figure 3). In addition to VEGF-A, GEC expressed VEGF-B (Figure 4) and VEGF-C mRNA, which were absent in RPTEC (data not shown).

Immunoprecipitation followed by immunoblot analyses of VEGF abundance revealed that GEC and RPTEC expressed VEGF protein (Figure 5). These data collectively establish that VEGF is expressed at the level of the message and the protein in GEC and RPTEC

Regulation of VEGF Expression by Hypoxia and Hyperglycemia

It has been previously demonstrated that hypoxia is a potent stimulus for VEGF production (17). In GEC, 24-h hypoxia resulted in a 2.4- and 1.6-fold increase at the level of the message and protein expression, respectively. In RPTEC, 24-h hypoxia resulted in a 4.9- and 1.9-fold increase at the level of the message and protein expression, respectively (Figures 2 and 5). $VEGF_{120}$ and $VEGF_{164}$ mRNA expression induced by hypoxia were increased 4.6- and 5.2-fold, respectively in RPTEC. In GEC, mRNA expression of $VEGF_{164}$ after 24-h hypoxia was increased 3.45-fold, but $VEGF_{120}$ was not changed significantly.

Analysis of VEGF-B showed that 24-h hypoxia resulted in an almost 50% decrease of the mRNA expression in GEC (Figure 4). There was no change of in VEGF-C mRNA expression after hypoxia in GEC (data not shown).

When GEC were exposed to 30 mM D-glucose *versus* L-

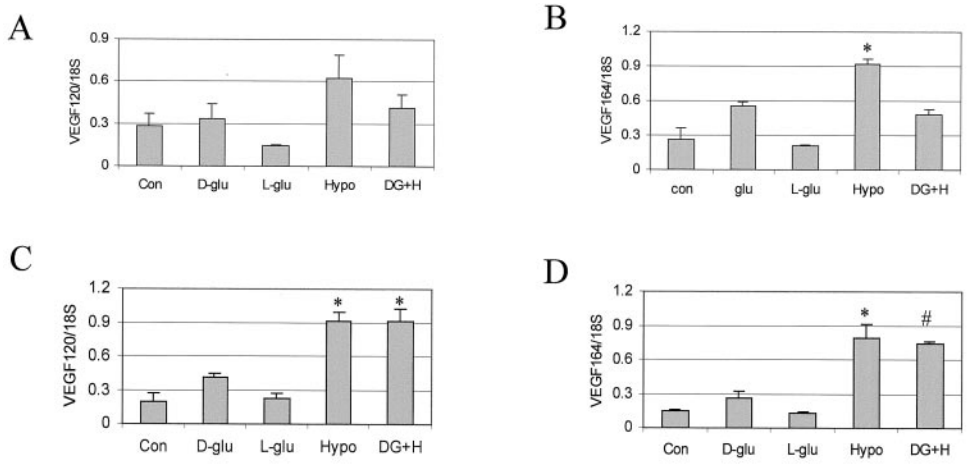

Figure 3. Relative quantitative RT-PCR for two VEGF isoforms in GEC and RPTEC. (A and B) The ratio of $VEGF_{120}$ mRNA (A) and $VEGF_{164}$ mRNA (B) expression to 18S RNA in GEC. (C and D) The ratio of $VEGF_{120}$ mRNA (C) and $VEGF_{164}$ mRNA (D) expression to 18S RNA in RPTEC. 18S RNA was used as internal control. * $P < 0.05$ compared with the control group; # $P < 0.01$ compared with the control group.

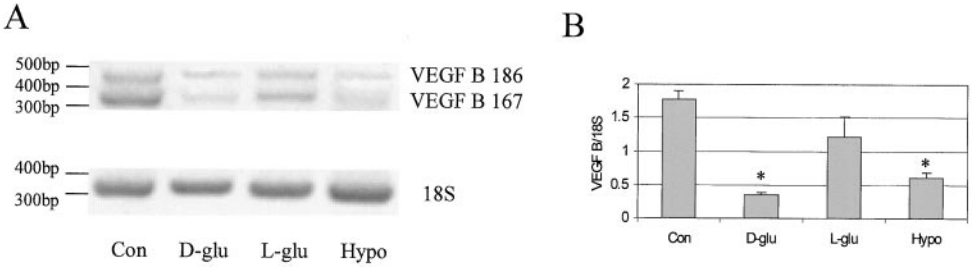

Figure 4. Relative quantitative RT-PCR for VEGF-B mRNA in GEC. (A) Agarose gel electrophoresis for VEGF-B mRNA in GEC. Both bands shown are two isoforms of VEGF, VEGF B_{186} and VEGF B_{167}, respectively. The sizes expected for VEGF B_{186} and VEGF B_{167} are 466 bp and 367 bp, respectively. 18S was used as internal control. (B) The ratio of VEGF-B mRNA expression to 18S RNA expression. * $P < 0.01$ compared with the control group.

glucose and each group compared with control cells incubated in the presence of 5 mM D-glucose, there were no significant changes of mRNA expression in GEC. However, VEGF protein expression in GEC after D-glucose was increased 1.5-fold ($P < 0.01$). In RPTEC, there was a 1.9-fold increase of VEGF mRNA expression after the exposure to high D-glucose ($P < 0.05$). $VEGF_{120}$ was increased significantly, but $VEGF_{164}$ did not change. In addition, VEGF protein expression was increased 1.45-fold ($P < 0.01$) in 30 mM D-glucose group compared with control.

In GEC subjected to combined treatment with high D-glucose and hypoxia, VEGF protein abundance showed 1.3-fold increase without significant increase in mRNA expression. In RPTEC, VEGF mRNA expression significantly increased (4.7-fold) in combined treatment group, which was higher than in the 30 mM D-glucose group, but there was no additive increase compared with hypoxic group. $VEGF_{120}$ and $VEGF_{164}$ mRNA expression were also increased 4.6- and 4.9-fold, respectively, in RPTEC. Combining 30 mM D-glucose and hypoxia resulted in a 1.4-fold increase in VEGF protein expression ($P < 0.01$).

VEGF Is a Necessary and Sufficient Growth Factor Supporting Capillary Formation under Control and Hypoxic, but Not Hyperglycemic, Conditions

To examine the functional role of VEGF produced by GEC and RPTEC, we next performed co-culture experiments using a sandwich epithelial-endothelial cell configuration separated by a growth-factor-depleted Matrigel. By the experimental design, any observed effects of epithelial cells on morphology of HUVEC in this system could be attributed to growth factors generated by the epithelium. In a separate series of experiments, neutralizing VEGF antibodies were introduced into co-cultures after 24 h to examine the role of VEGF among the secretory growth factors. Figure 6 shows representative images of capillary tube formation by HUVEC in co-culture system and grids that were used to quantify the angiogenesis score. The data demonstrated (Figures 6 and 7) that RPTEC supported capillary cord formation and sprouting of endothelial cell (HUVEC cultured alone do not survive for 24 h without serum or growth factors; not shown). This process was enhanced 1.2-fold by RPTEC exposed to hypoxia for 24 h before plating HUVEC ($P < 0.05$). When RPTEC were exposed to 30

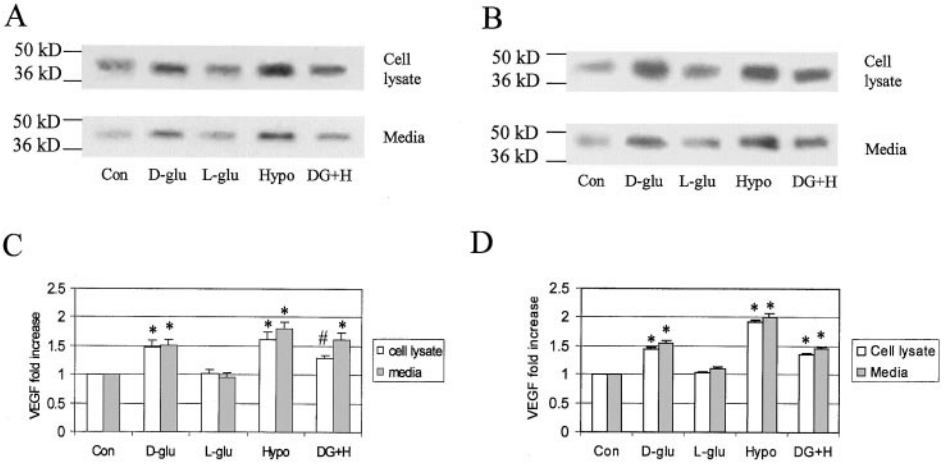

Figure 5. VEGF protein expression in GEC and RPTEC. (A and B) Immunoprecipitation for VEGF in GEC (A) and RPTEC (B). Both bands shown are VEGF protein expressions from cell lysate and media, respectively. VEGF was detected using immunoprecipitation with the polyclonal antibody followed by blotting with the monoclonal antibody. (C and D) VEGF expression in GEC (C) and RPTEC (D). * $P < 0.01$ compared with the control group; # $P < 0.05$ compared with the control group.

mM D-glucose, significant decrease of capillary tube formation was detected, compared to control group after 24 h ($P < 0.01$). Defective angiogenic response could not be accounted for by decreased expression of VEGF receptors; in fact, both hypoxia and hyperglycemia resulted in the increased abundance of mRNA encoding for VEGFR1 and VEGFR2 (Figure 8). Under the conditions of combined hypoxia and 30 mM D-glucose, capillary tube formation was significantly decreased compared with the hypoxia group but not different from the control group. After administration of neutralizing VEGF antibodies, significant regression of capillary tube formation was observed in both the control and hypoxic groups. There was no significant change in capillary tube formation in high D-glucose or L-glucose groups. Under all these conditions, administration of the neutralizing VEGF antibodies, but not the irrelevant IgG (data not shown), resulted in a dramatic regression of endothelial capillaries. The above quantitative experimental data presented in Figures 2 through 8 are summarized qualitatively in Table 1 for clarity of presentation.

Discussion

VEGF is a well-known angiogenic and survival factor for endothelial cells. So far VEGF expression in normal human and rat kidney has been known to be confined to podocytes and distal and collecting duct epithelium (10,18). GEC have been identified as the site of constitutive production of VEGF (19). Formation of fenestrae and increased permeability of glomerular endothelial cells have been attributed to VEGF produced by GEC (7), facilitating the high rate of glomerular filtration.

In the rat, normal kidney showed diffuse expression of VEGF in all tubules of the renal cortex and medulla (20). However, proximal tubules in the human kidney exhibit only faint, if any, labeling of VEGF mRNA and VEGF protein when studied by *in situ* hybridization and immunohistochemistry (19,20,21). Only one publication reported that human proximal tubular cells produced VEGF in culture (22). However, these researchers found no significant increase of VEGF mRNA expression after hypoxia. In contrast, we detected a robust constitutive VEGF expression in proximal tubular epithelium of the rat kidney using immunoelectron microscopy. We confirmed this finding of VEGF expression under normal condition in cultured human proximal tubular cells by RT-PCR and immunoprecipitation. These findings prompted us to search for the possible role of VEGF produced by proximal tubular epithelium in the maintenance of peritubular capillaries using an *in vitro* sandwich co-culture system.

As summarized in Table 1, we found that VEGF mRNA and protein expression after hypoxia were increased in both RPTEC and GEC. Hypoxia is a well-established potent stimulus for VEGF production in some cells, acting by inducing transcription of VEGF gene and stabilization of VEGF mRNA (23,24). Our results showed that $VEGF_{164}$ was increased in both RPTEC and GEC after hypoxia, whereas $VEGF_{120}$ was increased only in the RPTEC. The observed differences could be due to the cell type-specific variations in VEGF gene transcription, translation, or post-translational modifications and were beyond the scope of this study.

Previous studies showed that both the low and the high ambient glucose increase the production of VEGF in several cell types (25,26,27,28). There is little data, however, on the effect of high glucose on VEGF expression by renal epithelial cells. One study showed that high glucose induced upregulation of VEGF mRNA and protein expression in podocytes (29). In our experiments, GEC incubated in high glucose showed an increase of VEGF protein expression only, whereas there was an increase of both VEGF mRNA and protein expression in RPTEC.

When high D-glucose and hypoxia were combined, VEGF mRNA and protein expression was higher than control in both cell types (except that mRNA was unchanged in GEC) but there was no additivity of the effects of high D-glucose and hypoxia.

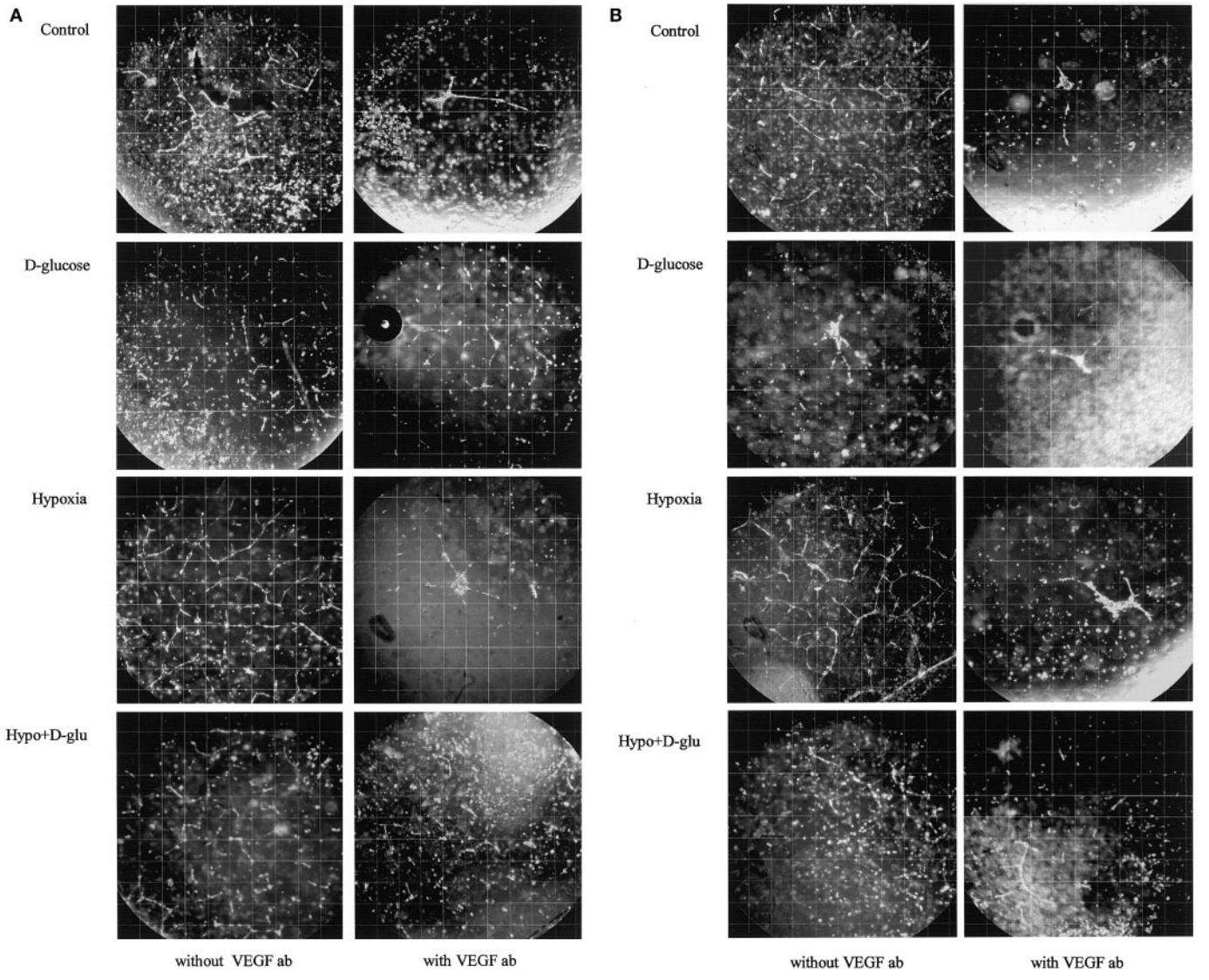

Figure 6. The capillary tube formation under different conditions in co-culture system. (A) Co-culture system with human umbilical vein endothelial cell (HUVEC) and RPTEC. (B) Co-culture system with HUVEC and GEC. Photographs shown are taken after 24 h. ab, anti-VEGF neutralizing antibody. Magnification, ×20.

These data collectively established the fact of VEGF production and hypoxic and hyperglycemic regulation of this process in proximal tubular epithelium. Its functional role was approached using a co-culture system, consisting of proximal tubular epithelial cells and endothelial cells. Our zero-hypothesis was that, if the epithelial cells produce VEGF, this growth factor could support the angiogenic phenotype of endothelial cells in the absence of any other added growth factors. We observed that capillary tube formation occurred after 24-h co-culture under normoxic conditions. When RPTEC were exposed to hypoxia before the initiation of co-culture, the capillary tube formation was significantly increased. This finding was consistent with the observed hypoxic induction of VEGF and suggested that it was this VEGF induction that enhanced capillary formation. Under both circumstances, capillary pruning occurred after application of neutralizing VEGF antibodies, further confirming the primary role of VEGF in the *in vitro* angiogenesis, when all other growth factors have been intentionally depleted from the medium.

There were no significant differences in angiogenesis of L-glucose–treated cells compared with control. In spite of the fact that VEGF production in high D-glucose–treated epithelial cells was increased, capillary tube formation was significantly reduced compared with euglycemic control. In addition, we observed that capillary tube formation was decreased in the group with combined hypoxia and high D-glucose compared with hypoxia alone. The possible causes for suppressed angiogenic competence of HUVEC in hyperglycemic environment, though not examined in this study, have been the focus of previous work (30,31). It has been demonstrated that VEGF receptor expression was depressed in patients with diabetes mellitus, inhibiting neovascularization of transplanted islets (32). This was not the case in our HUVEC, which showed a modest increase in mRNA for VEGF receptors 1 and 2, sug-

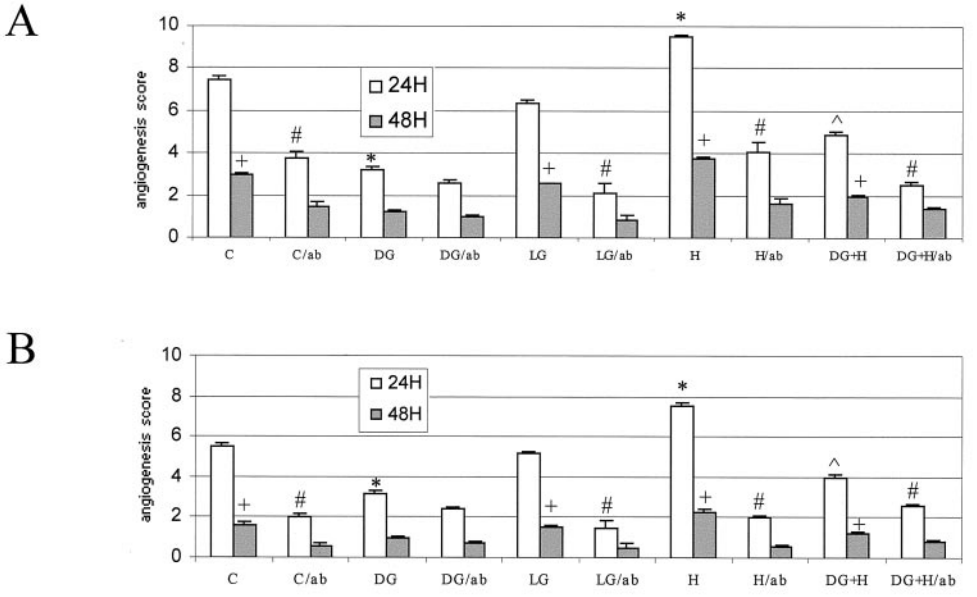

Figure 7. Angiogenesis scores in epithelial-endothelial co-culture systems. (A) Co-culture system with HUVEC and RPTEC. (B) Co-culture system with HUVEC and GEC. White bars, after 24 h; gray bars, after 48 h; * $P < 0.05$ compared with the control group; $^{\#}$ $P < 0.05$ compared with the group without antibody; $^{+}$ $P < 0.05$ compared with each 24-h group, $P < 0.05$ compared with Hypo.

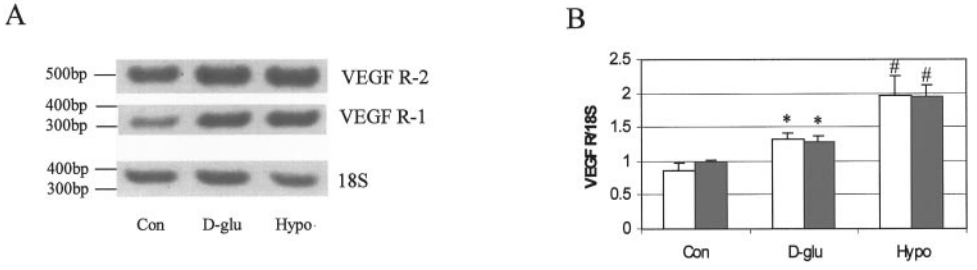

Figure 8. Relative quantitative RT-PCR for VEGF-R-1 and VEGF-R-2 in HUVEC. (A) Agarose gel electrophoresis for VEGF-R-1 and VEGF-R-2 mRNA in HUVEC. The sizes expected for VEGF-R-1 and VEGF-R-2 are 380 bp and 500 bp, respectively. 18S was used as internal control. (B) The ratio of VEGF-R mRNA expression to 18S RNA expression. White bars, VEGF-R-1; gray bars, VEGF-R-2. * $P < 0.05$ compared with the control group; $^{\#}$ $P < 0.01$ compared with the control group.

gesting that the short-term hyperglycemia does not downregulate these receptors. Furthermore, it has been reported that diabetic environment delayed angiogenic response to HGF, uPA, and uPAR (32) due to development of endothelial dysfunction (33,34). Potential changes in the expression of glucose transporters, although not studied by our group, have been examined previously (35). It has been demonstrated that endothelial cells (except retinal endothelium) are unable to downregulate the expression of glucose transporters in hyperglycemia and therefore remain continuously exposed to the elevated glucose influx and oxidant stress (36,37,38). These data collectively indicate that preexisting endothelial dysfunction may modify cell responsiveness to VEGF and that VEGF under these circumstances becomes insufficient for angiogenesis. We and others have previously demonstrated that endothelial generation of nitric oxide is a prerequisite for cell migration in response to VEGF or endothelin-1 and that responsiveness of endothelial cell to motogenic stimuli may become aberrant in hyperglycemic states or upon exposure to glycated matrix proteins (30,34,39–43). It should be emphasized, however, that any extrapolations of data from our experiments in a model system to the whole animal or humans with diabetes mellitus require most judicious precaution.

In addition to the above changes in VEGF-A mRNA and protein expression, mRNA for VEGF-B was significantly decreased after the exposure to high glucose and hypoxia compared with control. There were no detectable changes in the abundance of VEGF-C mRNA, effectively eliminating the potential contribution of these factors to our data. These results and the clear-cut effect of anti-VEGF antibodies definitively attribute the observed co-culture angiogenic phenomena to changes in VEGF-A produced by epithelial cells. Table 1 summarizes comparative characteristics of RPTEC and GEC under basal and stress conditions.

Although the function of VEGF in the glomerulus is thought to be related to the fenestration of glomerular endothelium and

Table 1. Summary of comparative analysis of VEGF synthesis and effects in RPTEC and GEC

	RPTEC	GEC
VEGF mRNA expression	$VEGF_{120}$, $VEGF_{164}$	$VEGF_{120}$, $VEGF_{164}$
VEGF-B mRNA expression	No	VEGF B_{167}, VEGF B_{186}
VEGF-C mRNA expression	No	Yes
VEGF-D mRNA expression	No	No
Response to hypoxia	VEGF mRNA: ++	VEGF mRNA: +
	VEGF protein: +	VEGF protein: +
	Angiogenesis: +	Angiogenesis: +
Response to high D-glucose	VEGF mRNA: +	VEGF mRNA: ±
	VEGF protein: +	VEGF protein: +
	Angiogenesis: −	Angiogenesis: −
Response to high glucose and hypoxia	VEGF mRNA: ++	VEGF mRNA: ±
	VEGF protein: +	VEGF protein: +
	Angiogenesis: ±	Angiogenesis: ±

VEGF, vascular endothelial growth factor; RPTEC, proximal tubular epithelial cells; GEC, glomerular epithelial cells; ++, marked increase; +, increase; ±, no change; −, decrease.

increased permeability, thus facilitating glomerular filtration, the role of VEGF produced by the proximal tubular epithelial cells has been enigmatic. Our study shows (*1*) that proximal tubular epithelial cells constitutively produce VEGF, (*2*) that its production is dramatically upregulated by hypoxia and by high glucose, (*3*) that VEGF secreted by proximal tubular epithelial cells support angiogenesis in culture, and (*4*) that hyperglycemic environment suppresses hypoxia-induced angiogenesis. We hypothesize that the regulated production of VEGF by the proximal tubular epithelium may play a profound role in the regulation of peritubular capillary network.

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