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VEGF induces proliferation, migration, and TGF-β1 expression in mouse glomerular endothelial cells via mitogen-activated protein kinase and phosphatidylinositol 3-kinase

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Abstract

The role of glomerular endothelial cells in kidney fibrosis remains incompletely understood. While endothelia are indispensable for repair of acute damage, they can produce extracellular matrix proteins and profibrogenic cytokines that promote fibrogenesis. We used a murine cell line with all features of glomerular endothelial cells (glEND.2), which dissected the effects of vascular endothelial growth factor (VEGF) on cell migration, proliferation, and profibrogenic cytokine production. VEGF dose-dependently induced glEND.2 cell migration and proliferation, accompanied by up-regulation of VEGFR-2 phosphorylation and mRNA expression. VEGF induced a profibrogenic gene expression profile, including up-regulation of TGF-β1 mRNA, enhanced TGF-β1 secretion, and bioactivity. VEGF-induced endothelial cell migration and TGF-β1 induction were mediated by the phosphatidyl-inositol-3 kinase pathway, while proliferation was dependent on the Erk1/2 MAP kinase pathway. This suggests that differential modulation of glomerular angiogenesis by selective inhibition of the two identified VEGF-induced signaling pathways could be a therapeutic approach to treat kidney fibrosis.

Keywords: Vascular endothelial growth factor; Transforming growth factor-β1; Endothelial cells; Migration; Proliferation; Cytokine; Angiogenesis; Fibrosis; Kidney; MMP; Signal transduction

Kidney fibrosis is a common feature of many forms of chronic renal disease and frequently leads to a relentless and inevitable decline in renal function [1]. Its pathomechanisms are multifactorial and not fully understood. The renal glomerulum is composed of three different cellular components: mesangial, endothelial, and epithelial (podocytes) cells. Among them, mesangial cells have received primary attention as effector cells of glomerulosclerosis [2]. In contrast, much less is known about the role of glomerular endothelial cells in renal fibrosis. They have long been recognized as key mediators of hemodynamic changes in chronic renal disease [3–5]. However, they also influence the disease course due to modulation of cell proliferation, cytokine production, and extracellular matrix composition. For example, glomerular endothelial cells express higher levels of collagen IV [6], a major component of the glomerular basement membrane [7], than do mesangial cells. Moreover, during the early course of renal fibrosis due to renal allograft reaction, there is increased endothelial cell proliferation with a concomitant increase in the number of glomerular capillaries, suggesting stimulated angiogenesis [8].

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Vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells. Its expression is induced by, among others, hypoxia, glucose, several cytokines, and growth factors including transforming growth factor-β1 (TGF-β1) and fibroblast growth factor [9]. In diabetes, VEGF mediates neoangiogenesis associated with proliferative retinopathy [10] and can induce expression of the profibrogenic cytokine connective tissue growth factor (CTGF) in retinal endothelial cells [11]. An in vivo study showed that VEGF was up-regulated in glomerular epithelial and tubular epithelial cells in the early phases of diabetic nephropathy [12]. Also, there is an early and transient up-regulation of renal VEGF receptor-2 (VEGFR-2) in experimental diabetes [13]. In diabetic rats, administration of a neutralizing VEGF monoclonal antibody decreases hyperfiltration, albuminuria, and glomerular hypertrophy during the early phases of diabetic nephropathy [14]. On the other hand, it was demonstrated that insufficient angiogenesis is a crucial factor for the failure of regeneration of the ageing kidney [15], in thrombotic microangiopathy [16], in cyclosporine-induced nephropathy [17], and after severe renal volume reduction [18] (5/6 nephrectomy). In the latter three models, administration of VEGF had a clear therapeutic benefit, slowing the development of renal fibrosis [17,19,20].

These diverging data show that it is not entirely clear if—or under what circumstances—a stimulation of angiogenesis prevents or promotes fibrosis in progressive kidney disease, i.e., whether the proliferative response is merely insufficient to counteract injury [21,22] or if it may even perpetuate it, triggering profibrogenic gene expression with resultant development of fibrotic lesions [23,24]. A uniformly antifibrogenic effect of angiogenesis stimulation in chronic kidney disease is unlikely. Such a model would be at odds with findings that implicate up-regulated angiogenesis as profibrogenic during long-term peritoneal dialysis [24–26] and liver fibrogenesis [27]. Also, such a model could not explain numerous in vitro observations describing proangiogenic activities of cytokines with well-described profibrogenic properties such as leptin [28] and CTGF [29,30], and vice versa [11].

TGF-β1 is the key profibrogenic cytokine involved in tissue fibrosis, including glomerulosclerosis [31–33]. Studies have shown that all three glomerular cell types express TGF-β1, with the highest levels found in glomerular endothelial cells [34]. Therefore, we investigated the response of glomerular endothelial cells to VEGF with respect to migration, proliferation, and synthesis of TGF-β1 as well as of other molecules relevant to fibrosis. The signal pathways involved in these processes were also investigated, since differential inhibition of signal cascades resulting from angiogenesis stimulation might offer therapeutic potential.

Materials and methods

Cell culture. A representative murine glomerular endothelial cell line (glEND.2) was established with the same methodology as previously described for the cell lines mlEND.1 and bEND.3 [35,36]. Briefly, mouse glomerular endothelial cells were immortalized by retroviral infection with the pym T oncogene. They are identifiable as endothelial cells on the basis of the following established criteria [37]: cobblestone morphology, uptake of acetylated-LDL, and positivity for the cell-surface adhesion molecules CD34, CD31/PECAM-1, CD105, and CD144. CD62E expression was induced by IL-1 activation. These properties were confirmed during our experiments on a regular basis. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), with 450 mg/dl glucose (PAA Laboratory GmbH, Germany) supplemented with non-essential amino acids, 1 mM sodium pyruvate, 5 μM 2-mercaptoethanol (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (FCS) at 37 °C in 7.5% CO_2 . Cells were passaged every 7–10 days by light trypsinization.

Measurement of cell migration. The effects of VEGF on glEND.2 cell migration were measured in a modified 48-well Boyden chamber (Neuro Probe, Cabinohn, MD, USA). Polycarbonate membranes (pore size 8 μm, polyvinyl-pyrrolidone-free) were coated with 20 μg/ml human collagen type I (isolated from placenta as previously described [38]) in DMEM at 37 °C for 1 h. Mouse recombinant VEGF or placenta growth factor (PlGF, R&D Systems GmbH, Wiesbaden, Germany), with or without other test substances, was placed in the lower chambers, which were then covered with the membrane. Cells which had been serum-starved for 24 h were trypsinized, resuspended at 2×10^5 cells/ml in DMEM containing 0.1% bovine serum albumin (BSA), and plated into the upper chambers. Cells were then allowed to migrate through the membrane for 6 h. Afterwards, the membrane was removed, residual cells on the upper side were scraped off, and the membrane was stained with a differential stain (Diff-Quik, Baxter Diagnostics, Chicago, USA). The number of cells that had migrated towards the lower surface of the membrane was counted in three fields under an Olympus CK40 microscope at 40× magnification. Samples were analyzed in triplicate on three separate occasions.

Effects of VEGF or PlGF on migration were confirmed by an in vitro scratch assay. Cells cultured to confluence in 24-well plates were starved for 24 h. Then, a scratch was placed using a 100 μl sterile pipette tip (width about 1 mm). Cells were treated with VEGF with or without other test substances, and the reduction of the initial scratch width was measured after 16 h in three different regions of the well using a Zeiss micrometer grid eyepiece. At the end of the incubation periods, cells were examined for any toxic effects. No morphological changes were observed in the cultures treated with any of the agents and at the concentration used.

Measurement of cell proliferation. The bromodeoxyuridine (BrdU) incorporation assay was used as a measure of DNA synthesis. glEND.2 cells were made quiescent in serum-free medium for 24 h and then incubated with different concentrations of VEGF or PlGF, with or without other test substances for 48 h. BrdU was added during the last 4 h before BrdU incorporation was determined by a colorimetric ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. All experiments were done with 6–8 wells per experiment and repeated at least three times.

For direct cell counting, 1×10^4 glEND.2 cells were seeded in 24-well plates, starved in serum-free medium for 24 h, and stimulated with VEGF or PlGF for 48 h. At the end of the incubation period, cells were washed with PBS, trypsinized, and counted using a Casy 1-model TT cell counter (Schaerfe System GmbH, Reutlingen, Germany).

Real-time polymerase chain reaction (PCR). Total RNA was prepared from cell lysates using the RNApure kit (PeqLab, Erlangen, Germany) according to the manufacturer's instructions. 0.5 μg of total RNA was reverse-transcribed into first-strand cDNA with random hexaprimers using Superscript II reverse transcriptase (Invitrogen,