Vascular endothelial growth factor expression and glomerular endothelial cell loss in the remnant kidney model

Darren J. Kelly, Claire Hepper, Leonard L. Wu, Alison J. Cox and Richard E. Gilbert

Department of Medicine, University of Melbourne, St Vincent’s Hospital, Fitzroy, Australia

Abstract

Background. Vascular endothelial growth factor (VEGF) is constitutively expressed in the glomerulus where it may have a role in the maintenance of capillary endothelial cell integrity. The present study sought to examine changes in VEGF expression in a model of progressive renal disease and to assess the effects of angiotensin converting enzyme (ACE) inhibition.

Methods. Subtotal nephrectomized (STNx) rats were randomly assigned to receive vehicle (n = 10) or the ACE inhibitor perindopril (8 mg/l drinking water) for 12 weeks duration (n = 10). Sham-operated rats were used as controls (n = 10). Glomerular capillary endothelial cell density was evaluated by immunostaining for the pan-endothelial cell marker RECA-1 and VEGF expression was assessed by quantitative in situ hybridization.

Results. In STNx rats glomerular capillary endothelial cell density was reduced to 19% that of sham rats (P < 0.01) with a concomitant reduction in glomerular VEGF expression, also to 19% of sham rats (P < 0.01). Perindopril treatment was associated with normalization of both capillary endothelial cell density and glomerular VEGF mRNA.

Conclusions. Reduction in glomerular VEGF expression is a feature of the renal pathology that follows subtotal nephrectomy. In the context of the known functions of this growth factor, these findings suggest that diminution in VEGF may contribute to the demonstrated loss of glomerular endothelium that develops in this model of progressive renal disease.

Keywords: angiotensin converting enzyme; endothelium; glomerulosclerosis; renin–angiotensin system; vascular endothelial growth factor

Introduction

The integrity of the glomerular capillary tuft is vital to the kidney’s primary function of plasma filtration.
two-thirds of the left kidney by selective ligation of two of three to four extrarenal branches of the left renal artery. Animals were then randomly assigned to two groups: STNx alone or STNx with the ACE inhibitor perindopril (8 mg/l drinking water, Servier, Neuilly, France). Rats were housed in a temperature (22°C) controlled room with ad libitum access to commercial standard rat chow (Norco Co-Operative Ltd., Lismore, NSW, Australia) and water during the entire study. Rats from each group were sacrificed at 12 weeks post-surgery. At death the remnant (left) kidney was then sliced sagittally and one half immersion fixed in 4% paraformaldehyde (0.1 M phosphate buffer, pH 7.4) for immunohistochemistry. All tissues were subsequently embedded in paraffin. All experiments adhered to the guidelines of the Animal Welfare and Ethics Committee of St Vincent’s Hospital and the National Health and Medical Research Foundation of Australia.

Renal function

Body weight was measured weekly. Plasma urea and creatinine were measured by autoanalyser (Beckman Instruments, Palo Alto, CA) at the beginning and end of the study. Glomerular filtration rate (GFR) was measured prior to death by a single shot Tc99m-DTPA clearance. Systolic blood pressure was measured in conscious rats using an occlusive tail-cuff plethysmograph attached to a pneumatic pulse transducer (Narco Bio-system Inc., Houston, TX). Before death, rats were housed in metabolic cages for 24 h for subsequent measurement of urinary protein excretion using Coomassie Brilliant Blue method.

Renal structure

The glomerulus was considered as the area internal to and including Bowman’s capsule. In 3 µm kidney sections stained with PAS, 50–80 glomeruli from rats were examined in a masked protocol. The degree of sclerosis in each glomerulus was subjectively graded on a scale of 0 to 4; Grade 0, normal; Grade 1, sclerotic area up to 25% (minimal); Grade 2, sclerotic area 25–50% (moderate); Grade 3, sclerotic area 50–75% (moderate to severe) and Grade 4, sclerotic area 75–100% (severe). Glomerulosclerosis was graded using the formula:

\[ GSI = \sum_{i=0}^{4} Fi(i) \]

where \( Fi \) is the per cent of glomeruli in the rat with a given score (i).

Immunohistochemistry

Glomerular capillary endothelial cell density was evaluated by immunostaining with RECA-1, a pan-endothelial cell-specific monoclonal antibody. Three micron sections were placed into histosol to remove the paraffin wax, hydrated in graded ethanol and immersed in tap water before being incubated for 20 min with normal goat serum (NGS) diluted 1:10 with 0.1 M PBS at pH 7.4. Sections were then incubated for 18 h at 4°C with RECA-1 (Sertotec, Oxford, UK). Sections incubated with 1:10 NGS instead of the primary antiserum served as the negative control. After thorough washing with PBS (3 × 5 min changes), the sections were flooded with a solution of 5% hydrogen peroxide, rinsed with PBS (2 × 5 min) and incubated with biotinylated goat antimouse IgG (Dakopatts, Glostrup, Denmark) diluted 1:200 with PBS. Sections were rinsed with PBS (2 × 5 min) and incubated with an avidin–biotin peroxidase complex (Vector, Burlingame, CA) diluted 1:200 with PBS. Following rinsing with PBS (2 × 5 min), sections were incubated with 0.05% diaminobenzidine and 0.05% hydrogen peroxide (Pierce, Rockford, IL) in PBS at pH 7.6 for 1–3 min, rinsed in tap water for 5 min, counterstained in Mayer’s haematoxylin, differentiated in Scott’s tap water, dehydrated, cleared and mounted in Depex.

The magnitude of RECA-1 immunostaining was quantified using image analysis as described previously [10]. Briefly, the glomerulus, as defined previously, was outlined by interactive tracing. Images were then captured using a BX50 Olympus microscope attached to a Fujix HC-2000 digital camera and a Pentium III IBM computer. The colour range for RECA-1 positive cells (brown on immunoperoxidase labelled sections) were selected and image analysis was performed using a chromogen-separating technique [10]. The proportional glomerular area showing positive RECA-1 immunostaining was measured from three sections per rat (n = 6/group), providing in excess of 50 glomeruli/treatment group.

In situ hybridization

In situ hybridization was performed using a cDNA encoding mouse VEGF164 (gift of Dr Steven Stacker, Ludwig Institute for Cancer Research, Melbourne, Australia). The fragment containing the entire open reading frame of VEGF was cloned into pGEM 4Z (Promega, Maddison, WI) and linearized with HindIII to produce an antisense riboprobe using T7 RNA polymerase. In situ hybridization was then performed on 4 µm paraffin sections using 32P-labelled antisense riboprobe. In brief, tissue sections were dewaxed in histosol, hydrated through graded ethanol and immersed in distilled water. Sections were then washed in 0.1 M PBS, pH 7.4 and hybridized with 32P-labelled antisense and sense-specific probes (5 × 104 c.p.m./25 ml hybridization buffer) which were added to hybridization buffer (300 mM NaCl, 10 mM Tris–HCl, pH 7.5, 10 mM Na2HPO4, pH 6.8. 5 mM EDTA, pH 8.0, 1 × Denhardt’s solution, 0.8 mg yeast RNA/ml, 50% deionized formamide and 10% dextran sulphate), heated to 85°C and 25 ml added to the sections. Coverslips were placed on the sections and the slides incubated in a humidified chamber (50% formamide) at 60°C for 14–16 h. Slides were then washed in 2 × SSC (0.3 M NaCl, 0.33 M Na2C6H4O2⋅2H2O) containing 50% formamide at 50°C to remove the coverslips. The slides were again washed with 2 × SSC, 50% formamide for a further 1 h at 55°C. Sections were then rinsed three times in RNase buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, pH 8.0, 0.5 M NaCl) at 37°C and treated with 150 mg RNase A/ml in RNase buffer for a further 1 h at 37°C, then washed with 2 × SSC at 55°C for 45 min. Finally, sections were dehydrated through graded ethanol, air dried and exposed to Kodak Biomax MR Autoradiography film for 4 days at room temperature. Slides were coated with Ilford K5 emulsion (Ilford, 1:1 with distilled water), stored with desiccant at room temperature for 21 days, developed in Ilford phenisol, fixed in Ilford Hypam and stained with H&E.
Quantitative autoradiography

Quantitative in situ hybridization, which permits the assessment of gene expression equivalent to northern blot analysis was used to determine the magnitude of gene expression. Quantification was performed using two methods: autoradiographic film densitometry and grain counting of emulsion-coated sections using established methods, as reported previously by our group [11].

Film densitometry of autoradiographic images obtained by in situ hybridization was performed by computer-assisted image analysis as described previously [12] using a Micro Computer Imaging Device (MCID, Imaging Research, St Catherine’s, Ontario, Canada). With this method quantification of transcript is based on the changes in X-ray film density that follows exposure to the radioactive emissions of radiolabelled VEGF mRNA. In situ autoradiographic images were placed on a uniformly illuminating fluorescent light box (Northern Light Precision Luminator Model C60, Image Research, Ontario, Canada) and captured using a video camera (Sony Video Camera Module CCD, Japan) connected to an IBM AT computer with a 512 × 512 pixel array imaging board with 256 grey levels. Following appropriate calibration by constructing a curve of optical density vs radioactivity quantification of digitalized autoradiographic images was performed using MCID software. Data were expressed as optical density per cm² relative to control kidneys (Relative Optical Density, ROD).

In addition to film densitometry, grain counting of emulsion-coated sections was also used to quantify gene expression and to explore the cell-specific sources of transcript within the kidney. With this method exposure of the photographic emulsion to radiolabelled VEGF mRNA leads to the formation of silver grains that can then be quantified. In brief, light microscopic images viewed through a 20 × objective lens were captured and digitized using a Fujix HC-2000 digital camera (Fuji, Tokyo, Japan). The outline of 50 glomeruli as defined by interactive tracing was used to assess glomerular gene expression in each kidney section. Regional gene expression was quantitatively measured to determine the proportion of each area occupied by autoradiographic grains as described previously [12], using computerized image analysis (Analytical Imaging Station, Imaging Research Inc.).

All sections were cut in a uniform manner in the mid-sagittal plane, hybridized to their respective probes in the same experiment and analysed in duplicate under identical conditions. All analyses were performed with the observer masked to the animal study group.

Table 1. Clinical characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>STNx</th>
<th>STNx + perindopril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>488 ± 13</td>
<td>419 ± 13*</td>
<td>428 ± 24*</td>
</tr>
<tr>
<td>Kidney weight/body weight</td>
<td>0.27 ± 0.01</td>
<td>0.42 ± 0.08*</td>
<td>0.41 ± 0.03*</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>127 ± 2</td>
<td>186 ± 12*</td>
<td>124 ± 11*</td>
</tr>
<tr>
<td>Proteinuria (mg/d)</td>
<td>21 ×/+1.1</td>
<td>328 ×/+1.2*</td>
<td>172 ×/+0.9*</td>
</tr>
<tr>
<td>Plasma creat (µM)</td>
<td>50 ± 1</td>
<td>80 ± 0.7*</td>
<td>80 ± 1*</td>
</tr>
<tr>
<td>GFR (mL/min)</td>
<td>3.3 ± 0.3</td>
<td>0.7 ± 0.1*</td>
<td>1.5 ± 0.2*</td>
</tr>
<tr>
<td>Glomerulosclerosis</td>
<td>0.1 (0-0.5)</td>
<td>3.5 (1-4)*</td>
<td>1 (0-3)*</td>
</tr>
</tbody>
</table>

Proteinuria expressed as geometric mean ×/+ tolerance factor. Glomerulosclerosis index expressed as median (range).

* P < 0.05.
** P < 0.01 vs control.
*** P < 0.05.
**** P < 0.01 vs STNx.

Statistics

Data are expressed as means ± SEM unless otherwise stated. Statistical significance was determined by ANOVA with a Fishers post-hoc comparison. Because of its skewed distribution, proteinuria was analysed using log transformed data and are represented as geometric means ×/+ tolerance factors. Data derived from the glomerulosclerosis index were not normally distributed and were expressed as median (range) and analysed using the Kruskal–Wallis test. Analyses were performed using Statview II + Graphics package (Abacus Concepts, Berkeley, CA) on an Apple Macintosh G4 computer (Apple Computer, Inc., Cupertino, CA). The correlation between VEGF and glomerulosclerosis was assessed non-parametrically using Spearman’s rho. A P-value < 0.05 was regarded as statistically significant.

Results

Renal functional and biochemical studies

Rats that underwent STNtx surgery became hypertensive and developed heavy proteinuria, elevated serum creatinine and glomerulosclerosis. Each of these parameters was reduced with perindopril treatment (Table 1).

RECA-1 expression

In sham rats there was intense localization of RECA-1 to the endothelial cells of glomerular capillary loops. RECA-1 immunolabelling was reduced to 19% of control levels in the glomeruli of STNtx rats (P < 0.01, Figures 1 and 2). This decrease in RECA-1 immunolabelling was attenuated by perindopril to levels similar to that in sham animals (Figures 1 and 2). Sections stained with normal IgG showed no staining (data not shown).

VEGF expression

In situ hybridization autoradiography revealed punctate cortical expression of VEGF mRNA consistent with localization of transcript to glomeruli (Figure 3). This pattern of distribution was not detected in
autoradiographs from kidney sections of STNx animals but present in animals that were treated with perindopril following renal mass reduction. In emulsion-dipped in situ hybridization, sections showed that VEGF mRNA was localized to the glomerular visceral epithelial cells of sham and perindopril-treated STNx rats. While detectable and still confined to podocytes, the magnitude of its expression was substantially decreased in STNx rats (Figures 4 and 5). Sections labelled with sense probe (negative control) showed no hybridization (data not shown).

Densitometric analysis of autoradiographic images confirmed a significant reduction in renal VEGF expression in STNx rats, which was attenuated by perindopril (Figure 5). Similarly, quantification of emulsion-dipped sections also showed that treatment of STNx rats with perindopril was associated with restoration of renal VEGF levels, similar to those of sham animals (Figures 4 and 5). A close inverse correlation between the degree of glomerulosclerosis and the magnitude of VEGF expression was noted (Figure 6).

Discussion

In the present study of progressive renal disease, renal mass reduction was accompanied by a diminution in glomerular VEGF expression and a decrease in glomerular endothelial cell density. In the context of the known role of VEGF in the maintenance of capillary endothelium these findings suggest that reduction in
Fig. 3. *In situ* hybridization autoradiographs for VEGF mRNA in sham kidneys (A) and following subtotal nephrectomy in rats treated with either placebo (B) or perindopril (C). Punctate cortical expression in sham and perindopril-treated rats, consistent with glomerular expression.

Fig. 4. Photomicrographs of glomeruli labelled *in situ* with antisense riboprobe to VEGF from sham kidneys (A) and following subtotal nephrectomy in rats treated with either placebo (B) or perindopril (C). Sections show abundant VEGF mRNA in glomerular podocytes (arrow) from control and perindopril-treated STNx rats compared with untreated STNx. Magnification 570×.
this cytokine may contribute to the endothelial cell loss that develops in progressive renal disease.

Subtotal nephrectomy provides a well-characterized model of non-inflammatory proteinuric renal disease, frequently used to examine the pathogenesis of progressive kidney disease. Following renal mass reduction, endothelial cell loss due to apoptotic cell death occurs as an early and progressive feature, commensurate with the development of glomerulosclerosis [13]. Such endothelial cell loss has clear implications for the maintenance of blood flow within the glomerulus. However, in addition, the injured endothelium may also initiate platelet activation and the coagulation cascade, both also implicated in the pathogenesis of glomerulosclerosis [14]. In the present study, substantial glomerular capillary loss was noted in animals that had undergone subtotal nephrectomy. The cause of this endothelial cell loss cannot be directly determined from this study. However, given the important role of VEGF in endothelial cell survival and repair [3,15] these findings do infer that the demonstrated reduction in VEGF may be contributory.

The interaction between the renin–angiotensin system and VEGF expression has been a matter of controversy. For instance, in cultured vascular smooth muscle cells and in mesangial cells, angiotensin II induces VEGF expression [8,16]. Furthermore, renin gene transfer has also been shown to restore VEGF expression to skeletal muscle [17]. However, the opposite effect has been reported in tubular epithelial cells where angiotensin II leads to a diminution in VEGF expression [9]. Similarly, divergent effects have also been reported with blockade of the RAS in the in vivo setting. For instance, in the retina, ACE inhibition has been shown to reduce VEGF expression [18], contrasting the findings of the present study in which ACE inhibition was associated with an increase in VEGF mRNA. Together, these in vitro and in vivo studies, suggest that the effects of angiotensin II on VEGF expression are cell type-specific. A similar cell specificity may also apply to the relationship between blood pressure and VEGF where hypertension is associated with increased VEGF in the retina [19] contrasting the decrease in its renal expression demonstrated in the present study.

Like many other growth factors, VEGF is a multifunctional cytokine, initially noted for its permeability enhancing actions, leading to its implication in the pathogenesis of proteinuric renal disease [20]. However, in the present study, heavy proteinuria was instead accompanied by a reduction in VEGF expression suggesting that it is unlikely to have a major role in the pathogenesis of proteinuria in this model.

In addition to its angiogenic and permeability enhancing properties, VEGF also induces collagen synthesis in cultured mesangial cells [21], possibly via transforming growth factor-β (TGF-β)-dependent mechanisms [22]. However, in the present study, an inverse correlation between VEGF expression and the extent of glomerular matrix deposition was found. Furthermore, previous reports of increased TGF-β
following subtotal nephrectomy model and its attenuation with RAS blockade [4], suggest that VEGF is unlikely to directly contribute to the matrix accumulation and consequent sclerosis that develop in this animal model.

In summary, the present study indicates that progressive glomerulosclerosis in the remnant kidney model is associated with diminution in VEGF expression and reduced glomerular capillary endothelial cell density. These findings were both attenuated by treatment with the ACE inhibitor, perindopril. Whether these changes are due to a primary effect of ACE inhibition on VEGF expression, or whether they are a consequence of other renoprotective effects of this class of drug cannot be determined form the present study.

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References


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