Inducible Overexpression of sFlt-1 in Podocytes Ameliorates Glomerulopathy in Diabetic Mice


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Abstract

**Objective:** Podocyte-specific, doxycycline (DOX)-inducible overexpression of the soluble VEGF receptor-1 (sFlt-1) in adult mice was used to investigate the role of VEGF-A/VEGF receptor (VEGFR) system in diabetic glomerulopathy.

**Research Design and Methods:** We studied non-diabetic and diabetic transgenic mice and wild-type controls, treated with vehicle (VEH) or DOX for 10-weeks. Glycemia was measured by a glucose-oxidase method and blood pressure by non-invasive technique. sFlt-1, VEGF-A, VEGFR2, and nephrin protein expression in renal cortex were determined by western immunoblotting; urine sFlt-1, urine free VEGF-A, and albuminuria by ELISA; glomerular ultrastructure by electron microscopy, VEGFR1 and VEGFR2 cellular localization with immunogold techniques.

**Results:** Non-diabetic DOX-treated transgenic mice showed a 2-fold increase in cortex sFlt-1 expression and 4-fold increase in sFlt-1 urine excretion (p<0.001). Urine free VEGF-A was decreased by 50% and cortex VEGF-A expression upregulated by 30% (p<0.04). VEGFR2 expression was unchanged while its activation reduced in DOX-treated transgenic mice (p<0.02). Albuminuria and glomerular morphology were similar among groups.

DOX-treated transgenic diabetic mice showed a 60% increase in 24h urine sFlt-1 excretion and a ~70% decrease in urine free VEGF-A when compared to VEH-treated diabetic mice (p<0.04), had lower urine albumin excretion at 10-weeks than VEH-treated diabetic mice (D-VEH vs D-DOX: 117.5[69-199] vs 43[26.8-69] μg/24h, geometric mean [95% CI], p=0.003). Diabetes-induced mesangial expansion, glomerular basement membrane thickening, podocyte foot-process fusion, and TGFβ1 expression were ameliorated in DOX-treated diabetic animals (p<0.05). Diabetes-induced VEGF-A and nephrin expression were not affected in DOX-treated mice.

**Conclusions:** Podocyte-specific sFlt-1 overexpression ameliorates diabetic glomerular injury, implicating VEGF-A in the pathogenesis of this complication.
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Vascular endothelial growth factor A (VEGF-A) is constitutively expressed in glomerular visceral cells (podocytes). Paracrine VEGF-A signaling occurs between podocytes and adjacent endothelial and mesangial cells which express the VEGF receptors (VEGFR1, VEGFR2)(1-3) and both autocrine and paracrine signaling may occur in podocytes themselves (4).

VEGF-A has been implicated in the regulation of glomerular barrier properties to protein filtration. In normal animals (5;6) and in cancer patients (7) inhibition of VEGF-A results in proteinuria, while in proteinuric conditions which are associated with glomerular VEGF-A upregulation such as diabetes, systemic inhibition of VEGF ameliorates albuminuria (8-10). This evidence suggests that a tight regulation of VEGF-A expression level is required to maintain the physiological permselective properties of the glomerular filter.

The results of previous studies conducted using either VEGF gene targeting techniques (5), or by administration of inhibitory agents of the VEGF/VEGFR system such as antibodies (6;8;9), or chemicals (10), may have been affected by potential interference with animal organ development, and lack of tissue specificity in the mechanisms of action of systemic inhibitors.

The soluble VEGF receptor-1 (sFlt-1), a splice variant of the VEGFR1, lacks the transmembrane and complete intracellular tyrosine kinase domain of VEGFR1, but binds to VEGF with the same affinity and specificity as that of the full length receptor (11;12), and has potent and selective VEGF inhibitory action (11). sFlt-1 acts in two major ways: it can sequester VEGF competing for its binding to the VEGF receptors, or can form heterodimers with the extracellular region of the membrane spanning VEGFR1 and VEGFR2, thus inhibiting the activation of downstream signaling pathways (11;12).

To target the action of the podocyte-expressed VEGF-A we developed a transgenic mouse model to overexpress sFlt-1 specifically at the podocyte level with an inducible expression system which is induced only after complete development, in the adult animal, by the administration of doxycycline (Tet-on).

The aim of this study was to investigate the role of VEGF-A upregulation in the pathogenesis of diabetic glomerulopathy by locally inhibiting podocyte-expressed VEGF-A activity.

RESEARCH DESIGN AND METHODS

All materials and chemicals were purchased from Sigma-Aldrich (Dorset, UK), restriction endonucleases were obtained from Fermentas GMBH (St.Leon-Rot, Germany), and DNA ligase kit was from Roche Applied Science (Sussex, UK).

**Generation of transgenic animals.** Construct generation (TRE construct): The human sFlt-1 cDNA was obtained from the plasmid pbb-sFlt-1 (gift of Dr. K.A. Thomas)(12) by BamHI restriction endonuclease digestion. The obtained cDNA was then cloned into the plasmid pBI-G Tet-Vector (GenBank Accession #U89933)(Takara Bio Europe/Clontech Saint-Germain-en-Laye, France). The resulting plasmid, pTRE bidirectional LacZ/sFlt-1, was studied for the presence and orientation of the insert (sFlt-1 cDNA) in the final vector by restriction endonuclease digestion. The ~8Kb AsnI-AsnI DNA fragment was utilized for microinjection and generation of transgenic animals.

**Generation of transgenic mice:** Transgenic mice were generated in the Transgenic Facility, Medical Research Council-Imperial College London. Animals were kept according to the “Guidelines on the Use of Animals in Research” and the number of utilized animals kept to a minimum. Mice were housed in a pathogen-free environment at 21°C, with 12-
hour light-dark cycle, all receiving a standard laboratory animal chow diet (Beekey Feeds) and water *ad libitum*.

*Animal genotyping:* Transgene genomic integration was initially studied using standard southern blotting technique. Subsequently, mice were genotyped by PCR as described (13). For the “pTRE bidirectional LacZ/sFlt-1” plasmid, we utilized the following set of primers recognizing the 3’-sv40 poly-A DNA sequence: sense 5’-ACCTATAAAAATAGGCGTATCAGCA-3’, antisense 5’-TGGCTGATTATGATCCTGCA-3’. The genotyping for the podocin-rtTA was also studied using PCR, as previously described (14).

**Induction of podocyte sFlt-1 overexpression in transgenic mice:** Experiments were conducted in mice obtained from the breeding of homozygous Podocin-rtTA mice (Pod/Pod) with heterozygous pTRE-LacZ/sFlt-1 (sFlt-1+/+) mice in order to obtain litters containing animals heterozygous for both constructs (Pod/sFlt-1). Expression of sFlt-1 was induced in mice by the administration of doxycycline (DOX)(2mg/ml) with the drinking water as previously shown (13). Water was supplemented with sucrose (5% w/v, and abbreviated as vehicle: VEH) to enhance palatability. Doxycycline was replaced every third day and protected from light at all time. Controls were treated with VEH alone.

**X-gal staining:** Standard histochemical detection of nuclear β-galactosidase activity was determined as previously described (13).

**sFlt-1 expression:** sFlt-1 protein levels were assessed by western immunoblotting techniques in kidney cortex lysate and by ELISA in 24h urine collection.

**Experimental design and determinations.** Experiments were conducted in 8-week old non-diabetic or diabetic mice in control (Pod/+, only positive for the Podocin-rtTA transgene) and double heterozygote Pod/sFlt-1 mice treated with either VEH or DOX for up to 10 weeks. 24-hour urine collections for each animal (in individual metabolic cages) were made at baseline (8-week old mice, before DOX administration), between 6-8 weeks and at 10 weeks thereafter. At the end of the study, mice were weighed and then killed, and cortex isolated for protein molecular determinations, and electron microscopy studies.

**Streptozotocin-induced diabetes in transgenic mice:** Diabetes was induced in ~5-week old mice with daily intraperitoneal streptozotocin injections for 5 days as previously described (15). Control mice were injected with citrate buffer only. After 2 weeks, diabetes was verified by blood glucose determination with glucose-oxidase method. Mice with a glycaemia lower than 22 mM were not included in the study.

**Blood Pressure:** Systolic blood pressure was measured from the mouse tail with the CODA non-invasive plethysmography blood pressure transducer (Kent Scientific Co., France).

**Creatinine clearance:** Blood samples were collected using heparinised tubes via cardiac puncture at the time of killing of the animals. Urine was collected as detailed above. Plasma and urine creatinine (Cr) concentration was determined by isotope dilution electro-spray mass spectrometry (ID-MS)(16). Creatinine clearance (μl/min•g) was estimated as urinary Cr x urine volume x 1440 min–1 x plasma Cr–1 x body weight (g)–1.

**Urine albumin excretion:** Urine volume was recorded and aliquots (1 ml) were stored at -80 °C for subsequent analysis. Albumin concentration in urine was measured in triplicate by ELISA using an anti-mouse albumin antibody (Bethyl Laboratories, TX, USA). Urine albumin excretion was expressed as 24h albumin excretion rate (μg/24h).
Urine free VEGF-A and sFlt-1 levels: Urine samples were collected and immediately centrifuged at 13,000g for 5 minutes, then stored at -80 °C. A commercial ELISA kit was utilized to measure Free-VEGF-A165 (R&D Systems Europe Ltd. Abingdon, UK). This assay specifically measures unbound VEGF-A, and does not cross react with VEGF-A bound to sFlt-1. Another ELISA was utilized to specifically measure sFlt-1 (RELIATech, Braunschweig, Germany)(17;18). For both determinations, assays were conducted in duplicate and results expressed as pg/24h for VEGF-A165, and ng/24h for sFlt-1.

Western blotting: The following antibodies (and dilutions) were used: rabbit, monoclonal anti sFlt-1 (1:500)(Sigma-Aldrich, Dorset, UK); rabbit, polyclonal anti nephrin intracellular domain (1:500) was donated by Dr. H. Holthofer (Helsinki University, Finland); goat, anti-mouse VEGF-A (1:250)(R&D-System, Abingdon, UK); rat anti VEGFR2 (1:250)(R&D-System, Abingdon, UK), rabbit anti VEGFR2(pY951)(1:500)(BioSource, Nivelles, Belgium); rabbit, polyclonal anti TGFβ-receptor2 (1:400)(Santa Cruz, CA, USA); rabbit polyclonal anti TGFβ1(1:400)(Santa Cruz, CA, USA), anti β-actin (1:5000)(Sigma, Dorset, UK).

Pieces of renal cortex were lysed in modified RIPA buffer containing proteases and phosphatases inhibitor cocktail (Sigma-Aldrich, Dorset, UK). Equal amounts of total protein lysate (40-200µg) were run on 7.5% or 10% SDS-polyacrylamide denaturing gels and transferred to nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK). The membranes were incubated in 5% non-fat milk or 3% bovine serum albumin, 0.1% tween in phosphate buffered saline (PBS) for 3h at room temperature for sFlt-1, VEGF-A, nephrin, VEGFR2, TGFβ-receptor2, TGFβ1, β-actin or VEGFR2(pY951) antibody respectively. Membranes were subsequently probed with specific horseradish peroxidase-conjugated secondary antibodies (1:5000-10000)(Dako, Denmark) for 1h at room temperature. Bands were revealed using an enhanced chemiluminescence kit (Amersham Biosciences, Little Chalfont, UK). Quantification of the immunoreactive bands was performed using densitometric scanning using image software (Image J)(National Institute of Health, Bethesda, USA).

Glomerular morphology: Mesangial volume fraction, as an index of glomerular extracellular matrix deposition, GBM thickening, podocyte foot process width (FPW), and glomerular capillaries endothelial fenestrae were studied with electron microscopy techniques as previously described (19).

Glomerular cells VEG receptors expression: Immunogold electron microscopy technique was used to study the presence of VEGF receptors on glomerular cells in both non-diabetic and diabetic mice. One mm3 pieces of cortical tissue were fixed in 2% paraformaldehyde for 4h, dehydrated in alcohol and embedded in LR-white resin (TAAB). Ultrathin sections were taken and mounted on carbon-coated nickel grids. The grids were first immersed in 80 mM ammonium chloride in PBS (10 minutes), then PBS containing 0.2M glycine and 0.5% bovine serum albumin (BSA)(10 minutes), followed by 10% normal rat serum for 30 minutes, all at room temperature. The grids were then incubated with primary antibody (rabbit anti-mouse VEGFR1 and VEGFR2, ABCAM, UK) diluted 1:100 in PBS/0.5% bovine serum albumin (BSA)(10 minutes), followed by 10% normal rat serum for 30 minutes, all at room temperature. The grids were then incubated with primary antibody (rabbit anti-mouse VEGFR1 and VEGFR2, ABCAM, UK) diluted 1:100 in PBS/0.5% BSA overnight at 4°C. After washing in PBS/0.5% BSA, the grids were incubated with 10 nm gold-conjugated goat anti-rabbit IgG diluted 1:20 in PBS/0.5% BSA for 1h at room temperature. First antibody was omitted in negative controls. After washing, the grids were stained with 2% aqueous uranyl acetate.
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and examined by transmission electron microscopy.

Statistics. Results are expressed as mean±SEM unless otherwise stated. Data for albuminuria were log transformed before analysis. Among group comparisons were performed by ANOVA followed by post hoc analysis for between group differences using LSD test. A value for p ≤ 0.05 was considered statistically significant.

RESULTS

Generation of transgenic mice: We obtained 8 positive founders for the pTRE bidirectional LacZ/sFlt-1 construct. Seven of 8 founders transmitted the transgene to the offspring. Mice derived from the F1 generation were then bred with FVB-N-mice homozygous for the construct podocin-rtTA (gift from Dr J Kopp, Bethesda, Maryland, USA), expressing the rtTA specifically in podocytes (14). First generation offspring carrying either both transgenes, or podocin rtTA only (mixed background BL6-CBA/FVB-N, abbreviated to Pod/sFlt1 and Pod/+ as controls) were studied for doxycycline-inducible transgene expression. Of these 7 remaining lines, one showed a doxycycline-inducible LacZ (nuclear localization) expression in podocytes with no basal leaky expression of the transgene and was subsequently used in all experiments.

Role of podocyte sFlt-1 overexpression in adult control, non-diabetic, mice: Eight week old adult double transgenic mice (Pod/sFlt-1) or single transgenic mice (Pod+/+) were treated with DOX or VEH for up to 10 weeks (both sexes showed a similar phenotype and were analyzed together). X-gal staining revealed no signal in Pod/+ DOX and VEH kidneys, confirming that the staining protocol did not detect endogenous galactosidase activity (not shown). Furthermore, Pod/sFlt-1 mice exposed to VEH also failed to give a positive signal (Fig. 1A), excluding ‘leakage’ of LacZ expression in non-induced mice. In contrast, kidneys from adult Pod/sFlt-1 mice exposed to DOX showed positive X-gal staining (Fig. 1A), consistent with podocyte expression of the transgene (13;14). X-gal staining was analyzed at different time points after DOX administration (10 days, 5-10 weeks) and the results were superimposable (not shown). After 10 weeks treatment with DOX, Pod/sFlt-1 animals showed a significant inducible sFlt-1 overexpression (100% increase) when assessed in kidney cortex lysate (Fig. 1B). The upregulation of sFlt-1 was associated with an increase in 24h urine sFlt-1 levels which was 4-5 fold higher in DOX-treated Pod/sFlt-1 mice when compared to Pod/sFlt-1 mice on VEH or Pod/+ control mice (p<0.001)(Fig. 2A). This was paralleled by a reduction in urine free VEGF-A in Pod/sFlt-1 DOX treated mice suggesting a sFlt-1 mediated “sequestration/ binding” of VEGF-A (p<0.04)(Fig. 2B). Renal cortex VEGF-A was upregulated in Pod/sFlt-1 DOX mice when compared to the other groups (p<0.03)(Fig. 2C). No difference was observed in renal cortex expression levels of VEGFR2 among the groups of mice studied (Pod/+ VEH: 1.5±0.3; Pod/+ DOX: 1.7±0.3; Pod/sFlt-1 VEH: 1.7±0.3; Pod/sFlt-1 DOX: 1.5±0.2 VEGFR2/ β-actin arbitrary units, n=5-6/ group), VEGFR2 activation, determined by phosphorylation of VEGFR2 on Tyr951 (20;21) was reduced by approximately 50%, but not abolished, in Pod/sFlt-1 treated with DOX (Fig. 2D).

VEGFR expression in glomerular cells: Immunogold staining showed localisation of VEGFR1 and VEGFR2 in endothelial cells as previously reported. We detected expression of VEGFR2, but not VEGFR1, in podocyte cell body and foot processes in vivo (Fig. 3).

Clinical and biochemical features: There were no difference in creatinine clearance at 10 weeks (Pod/+ VEH: 9.2±1.5; Pod/+ DOX: 10.5±2.2; Pod/sFlt-1 VEH: 9.3±1.6; Pod/sFlt-1 DOX: 10.6±1.7 µl/min·g body weight, n=6-9/ group), and albuminuria throughout the
study (geometric mean [95% CI] µg/24h, n=10-12/group - baseline: Pod/+ VEH: 22.3 [11.9-41.9]; Pod/+ DOX: 23.9 [16.7-34.1] ; Pod/sFlt-1 VEH: 33.3 [17.1-65.0]; Pod/sFlt-1 DOX: 28.1 [18.8-42.1]; 10 weeks: Pod/+ VEH: 20.6 [14-30.4]; Pod/+ DOX: 22.7 [15.7-32.9]; Pod/sFlt-1 VEH: 28.4 [18.6-43]; Pod/sFlt-1 DOX: 22.2 [14.1-34.8]). Glomerular morphology was similar among the four groups of animals (not shown). DOX administration did not affect mice behaviors or body weight which was similar in all groups (not shown).

**Role of podocyte sFlt-1 overexpression in diabetic mice:** In these experiments only Pod/sFlt-1 mice were studied. Mice were made diabetic at 5 weeks of age. Treatment with VEH or DOX was started in 8-week old adult non-diabetic (C) and diabetic (D) Pod/sFlt-1 mice and continued for up to 10 weeks (18 weeks of age)(sexes were pooled for analysis because they showed a similar phenotype).

**Clinical and biochemical features:** By the end of 10-week DOX treatment, diabetic animals were lighter, had raised blood pressure, and higher creatinine clearance compared to control animals. DOX did not affect any of these variables in either the non diabetic or diabetic mice (Table 1).

Albumin excretion rate was similar at baseline in the two diabetic groups (D-VEH vs D-DOX geometric mean [95% CI]: 28 [21-38] vs 23 [14-36] µg/24h). By week 10 it had risen to 117.5 [69-199] µg/24h in D-VEH and this rise was significantly blunted in the D-DOX group overexpressing the sFlt-1 (D-DOX: 43 [26.8-69] µg/24h)(p=0.003).

To obtain insights in the role of primary podocytes’ sFlt-1 overexpression in diabetes, we studied urine sFlt-1 and free-VEGF-A excretion, VEGF-A protein expression and VEGFR2 expression and phosphorylation in the renal cortex lysate.

**sFlt-1, free VEGF-A 24h urine excretion:** Urine sFlt-1 excretion was higher in VEH-treated diabetic mice when compared to VEH-treated non diabetic animals (p=0.02). In the diabetic group urine sFlt-1 excretion was significantly higher in DOX-treated Pod/sFlt-1 mice compared to VEH-treated animals (D-VEH vs D-DOX: 19.9±6.3 vs 35±4.9 ng/24h, p=0.03)(Fig. 4A), a pattern similar to that seen in control non diabetic animals (Fig. 2A). Urine free VEGF-A 24h excretion was significantly reduced after 10 weeks in DOX-treated diabetic mice when compared to VEH-treated diabetic mice (p=0.04)(Fig. 4B).

**VEGF-A expression, VEGFR2 expression and phosphorylation in renal cortex:** Renal cortex VEGF-A protein expression was significantly upregulated as expected in diabetic VEH-treated mice when compared to the non-diabetic VEH-treated group (C-VEH vs D-VEH, p=0.02); DOX treatment did not induce any change in VEGF-A expression in diabetic mice (Fig. 5A).

VEGFR2 expression was upregulated in diabetic mice by approximately 80% (C-VEH vs D-VEH: 100±16.7 vs 178±11.7, VEGF-R2/β-actin % change, n=5-6, p=0.01); DOX treatment did not affect VEGFR2 expression in diabetic mice (not shown). Similarly VEGFR2 activation, determined as ratio of phosphorylation of VEGFR2 on Tyr951 over total VEGFR2 was increased by approximately 60% in diabetic mice (C-VEH vs D-VEH: 100±15 vs 158±10, VEGFR2Tyr951/VEGFR2tot % change, n=5-6, p=0.01); DOX treatment was accompanied by a non-significant decrease in VEGFR2 phosphorylation in diabetic mice (Fig. 5B).

To investigate further the changes in albuminuria and to examine the interaction between the VEGF-A/VEGF receptor system and the TGFβ1 pathway in diabetic mice treated with DOX we studied nephrin, TGFβ1, and TGFβ-receptor2 proteins expression in renal cortex protein lysate with Western immunoblotting.
Nephrin, TGFβ1, and TGFβ-receptor2 proteins expression in renal cortex: Analysis of nephrin protein expression in total renal cortex protein lysate resulted in two bands at 196 and 200 KDa representing different degrees of nephrin glycosylation (Fig. 6A)(22). Diabetic VEH-treated animals had a higher level of nephrin expression than non-diabetic control VEH-treated mice (p=0.002). Treatment with DOX did not alter the expression of nephrin in either the diabetic or the non diabetic control mice.

TGFβ1 expression was upregulated in diabetic mice by 46% (C-VEH vs D-VEH: 100±5.4 vs 146±9.8 TGFβ1/β-actin % change, n=4-5, p=0.007). In diabetic mice treatment with DOX significantly reduced TGFβ1 expression to virtually control levels (D-VEH vs D-DOX: 146±9.8 vs 104.7±4.5 TGFβ1/β-actin % change, n=4-5, p=0.001). DOX treatment did not change TGFβ1 expression in non diabetic control mice (Fig. 6B).

TGFβ-receptor2 protein expression was slightly but not significantly increased in diabetic mice; DOX treatment did not affect its expression either in non diabetic or in diabetic animals (not shown).

Mesangial area expansion: Mesangial volume fraction (VvMes) was increased in VEH-treated diabetic mice compared to VEH-treated control animals (C-VEH vs D-VEH: 0.16±0.1 vs 0.22±0.01, p=0.003). Administration of DOX significantly reduced VvMes in the diabetic mice (D-VEH vs D-DOX: 0.22±0.01 vs 0.18±0.01, p=0.04) but had no effect in the control mice (C-VEH vs C-DOX: 0.16±0.01 vs 0.17±0.01)(Fig. 7A,B).

GBM thickening, podocytes foot processes width (FPW), and endothelial fenestrae: GBM thickness was significantly increased in diabetic mice compared to VEH-treated non-diabetic mice (C-VEH vs D-VEH: 176±8 vs 219±8, nm, p=0.001). Treatment with DOX ameliorated GBM thickening in diabetic mice (DVEH vs DDOX: 219±8 vs 197±7, p=0.05) but had no significant impact in non-diabetic control animals (Fig. 8A,D).

FPW was increased in VEH-treated diabetic mice compared to VEH-treated non diabetic control animals (C-VEH vs D-VEH: 197±10 vs 241±11, nm, p=0.005). Treatment with DOX inducing sFlt-1 overexpression was paralleled by a reduction in foot process width in diabetic mice (D-VEH vs D-DOX: 241±11 vs 210±8, p=0.03) but had no effect in non diabetic control animals (Fig. 8B,D).

No differences were observed in percentage of fenestrated endothelium occupying the endothelial surface, which was approximately 53-57%, and similar in the four groups of mice studied (Fig. 8C,D).

DISCUSSION

VEGF-A appears critical for the maintenance of capillary integrity particularly in the glomerulus. In normal animals, systemic blockade of VEGF-A action is associated with endothelial cell damage, reduced nephrin expression and proteinuria (6).

Moreover constitutive genetically-mediated downregulation of VEGF-A expression in podocytes leads to severe impairment of glomerular function and glomerular endothelial damage (5). Recent elegant studies in mice using an inducible podocyte-specific system for site specific VEGF ablation resulted in glomerular barrier disruption, proteinuria and glomerular thrombotic microangiopathy mimicking the proteinuria and glomerular damage seen in patients receiving VEGF-inhibitors, such as bevacizumab, for the treatment of neoplastic disease (23). Concordantly in pre-eclampsia in humans the greatly elevated circulating levels of sFlt-1 are associated with proteinuria and glomerular injury suggesting that binding of sFlt-1 to VEGF may impede its vasoprotective action at the capillary level (18;24).

On the other hand there are pathological conditions, such as diabetes, in
which the development of proteinuria and glomerular pathology is associated with VEGF-A overexpression in the podocytes. In the experimental diabetic animal systemic administration of VEGF inhibitors, such as antibodies or chemicals, ameliorates proteinuria and glomerular damage (8-10).

These contrasting situations suggest that both deficiency and excess of VEGF may be detrimental to the physiological integrity of glomerular capillaries.

In this work we used an inducible expression system which triggers VEGF-A inhibition after complete kidney development (in the adult mouse). To achieve this we overexpressed, specifically in podocytes, the powerful VEGF inhibitor sFlt-1 (11), thus avoiding systemic elevation of sFlt-1 levels which, as seen in pre-eclampsia, may disrupt the capillary wall (18). The system of podocyte-specific conditional inhibition of VEGF-A thus circumvents the potentially important confounding effects on renal glomerular development and aspecific VEGF inhibition in other tissues which may have occurred with previous experimental approaches.

Our findings demonstrated that in diabetes tissue specific inhibition of VEGF-A by excess sFlt-1 led to amelioration of albuminuria and glomerulopathy presumably by blocking the molecular events mediated by diabetes-induced VEGF overexpression.

The sFlt-1 is a 110 KDa molecular weight protein which, under physiological conditions is not filtered through the glomerular barrier. The podocyte-specific overexpression of sFlt-1 which was induced in our system by the administration of doxycycline (see Fig. 1) would lead to a very localized action of this molecule which within the podocyte would sequester VEGF-A and inhibit its local autocrine effect as well as its migration towards the endothelium and the mesangium.

The contention of paracrine/autocrine action of podocyte secreted VEGF is supported by the presence of VEGF receptors both in endothelial and mesangial cells, as previously reported (3;25), and by the detection for the first time in this work, of VEGFR2 in mouse podocytes in vivo. Both VEGFR1 And VEGFR2 are expressed in mouse podocyte in vitro (4) but we were unable to detect VEGFR1 in vivo; the discrepancy between the in vivo and in vitro observations is probably attributable to the artificial setting of the in vitro culture system, which can trigger the induction of molecules normally not expressed in vivo (3).

Supplementary proof of sFlt-1 podocyte overproduction was provided by its significantly increased 24h urine excretion and clear, though indirect, evidence of sFlt-1 inhibitory action by the marked reduction in urine free VEGF-A excretion. This was true, though in different degrees, for both non-diabetic and diabetic mice treated with doxycycline suggesting sequestration of VEGF-A.

In non diabetic control mice, sFlt-1 overexpression-induced inhibition of VEGF-A resulted in no glomerular functional or morphological abnormalities. We believe that the absence of structural and functional alterations was probably explained by the upregulation of VEGF-A which occurred in the doxycycline treated animals. This upregulation may represent a compensatory response to interrupted VEGF-A signalling because of sFlt-1 overexpression and sequestration of VEGF-A. This might have mitigated the effect of sFlt-1 mediated inhibition, and indeed free VEGF-A in the urine, though reduced by approximately 50%, was still present in substantial amount, and was paralleled by a still present, although reduced, VEGFR2 activation/phosphorylation. This interpretation is supported by recent evidence which suggests that in the adult mice full ablation of VEGF is
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required to induce definite glomerular pathological changes (23;26).

Diabetic mice developed the typical renal functional and structural abnormalities of proteinuria, mesangial expansion, GBM thickening and podocyte foot process fusion. These changes were associated with upregulation of VEGF-A and VEGFR2 expression in renal cortex as previously described (25). Similarly to non-diabetic mice, doxycycline-induced sFlt-1 overexpression resulted in raised 24h urine excretion of sFlt-1 and significant reduction (though not abolition) of urine free VEGF-A excretion, suggesting again “sequestration” of functional VEGF-A. Of interest 24h urine sFlt-1 excretion was higher in the diabetic mice treated with VEH compared to that of the control non-diabetic mice receiving VEH, mimicking similar observations described in diabetic patients (17).

DOX treated diabetic mice showed a modest non-significant reduction in VEGFR2 phosphorylation compared with VEH-treated animals. The higher degree of glomerulosclerosis observed in VEH versus DOX-treated diabetic mice might explain this observation, as it has been reported that VEGF receptor activation is inversely related to the degree of glomerular injury (27). We focused our work on VEGFR2 expression and phosphorylation as VEGFR2, rather than VEGFR1, is involved in the biological action of VEGF-A in adult animals (28;29), and is overexpressed in experimental diabetes (25).

Inhibition of VEGF-A at the podocyte level in diabetic mice improved the albuminuria and the morphological changes of the glomerular filtration barrier by mitigating GBM thickening and preventing foot process fusion, but also by markedly reducing mesangial matrix deposition. This suggests the presence of a podocyte-podocyte as well as podocyte-mesangium cellular network, where podocyte-specific gene expression can modulate the biology of other subset of glomerular cells as previously suggested for other podocyte secreted cytokines (13;23;26). Indeed the sFlt-1 overexpression-induced amelioration of GBM thickening and of mesangial extracellular matrix volume is likely to result from an inhibition of either the prosclerotic action of VEGF-A (30;31) or the reduction of TGFβ1 expression (32) or both. A potential relationship between VEGF-A and TGFβ1 is suggested by studies reporting a VEGF-A-mediated TGFβ1 expression in mouse glomerular endothelial cells (33).

Interestingly, in the diabetes sFlt-1 overexpressing mice, no further upregulation of the already overexpressed VEGF-A occurred, suggesting a “ceiling” for renal cortex VEGF-A upregulation in the context of diabetes.

In this mouse model neither diabetes nor the overexpression of sFlt-1 altered the number of endothelial fenestrae in the glomerular capillary.

Nephrin is a slit diaphragm protein whose deletion or expression downregulation has been implicated in the pathogenesis of proteinuria in diseases such as the congenital nephrotic syndrome of the Finnish type or in minimal change nephrotic syndrome (34-36). In experimental animal model of diabetes nephrin expression has been reported to be either upregulated or downregulated (10;37-39). In our diabetic mice nephrin was upregulated when compared to control non-diabetic animals and the podocyte overexpression of sFlt-1 did not affect this upregulation. These findings, in line with previous reports (10) suggest that nephrin expression downregulation, though it may be sufficient, is not necessary for the development of proteinuria and concur with the concept that albuminuria may occur by a variety of mechanisms such as redistribution of nephrin at the ultrastructural level (10;40;41).
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Doxycycline, which was used in this study to induce sFlt-1 upregulation, is a non-selective inhibitor of metalloproteinases (42;43), enzymes which regulate extracellular matrix deposition, and may have confounded some of our findings. However our present results in the non diabetic control mice and previous observations in normal animals (13) and diabetic rodents (44), suggest no direct doxycycline-mediated effects on albuminuria and extracellular matrix at the dose used in this study. Moreover doxycycline-induced inhibition of metalloproteinases would favor matrix accumulation and would result, if anything, in an underestimate of the improvement in glomerular extracellular matrix deposition in the podocyte sFlt-1 overexpressing diabetic mice.

Our findings provide insight into the direct, local (autocrine/paracrine) role of VEGF-A in diabetes-induced glomerular injury although the experimental design of this work makes our results more relevant to the prevention rather than treatment of diabetic glomerular damage. Together with work by others (23) these findings support the notion that in order to preserve vascular integrity, a fine balance in the regulation of VEGF-A expression is required in as much as either too little or too much of this cytokine would result in capillary vascular pathology. These considerations highlight the potential pitfalls of using systemic therapies to tackle tissue specific-changes.

ACKNOWLEDGEMENTS
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Reference List


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Table legend

Table 1: Clinical and biochemical features in Pod/sFlt-1 non diabetic control and diabetic mice treated with VEH or DOX for 10 weeks.
Diabetic mice were lighter than control mice. Systolic blood pressure and creatinine clearance were raised in the diabetic mice. None of these variables were affected by DOX administration within either the non-diabetic or diabetic group (n=7-10 per group).

**Table 1**

<table>
<thead>
<tr>
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<th>Control VEH</th>
<th>Control DOX</th>
<th>Diabetic VEH</th>
<th>Diabetic DOX</th>
</tr>
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<tbody>
<tr>
<td>Fed blood glucose (mmol/L)</td>
<td>7.1 ± 1.1</td>
<td>6.2 ± 1.0</td>
<td>26.08 ± 1.6</td>
<td>27.4 ± 1.8</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>28.4 ± 2.3</td>
<td>28.0 ± 1.8</td>
<td>21.1 ± 2.3</td>
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<tr>
<td>Creatinine clearance (μl/min•g)</td>
<td>9.3 ± 1.6</td>
<td>10.6 ± 1.7</td>
<td>14.9 ± 2.9</td>
<td>14.5 ± 3.6</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>106.1 ± 5.0</td>
<td>107.1 ± 4.8</td>
<td>122.8 ± 4.9</td>
<td>124.0 ± 4.9</td>
</tr>
</tbody>
</table>
Figure legends

Fig. 1: Podocyte inducible overexpression of LacZ/sFlt-1.

(A) Nuclear LacZ staining of glomerular podocytes was detected in DOX treated but not in VEH treated double transgenic mice (10 weeks treatment). No staining was observed in Pod/+ administered with VEH or DOX (not shown). Densitometry quantitative analysis of sFlt-1 expression (as a ratio with β-actin) (B) in total kidney cortex lysate after 10 weeks treatment with VEH or DOX.

Data are expressed as mean ± SEM, n=4-8 per group, Pod/sFlt-1 DOX vs all other groups (*p<0.01), Pod/+ VEH vs Pod/+ DOX (ns).
Fig. 2: sFlt-1 and VEGF-A 24h urine excretion, VEGF-A expression and VEGFR2 phosphorylation in renal cortex in podocyte-specific sFlt-1 overexpressing mice after 10 weeks treatment with VEH or DOX.

(A) Urine sFlt-1 excretion was significantly increased in Pod/sFlt-1 DOX treated mice (Pod/sFlt-1 DOX vs all other groups, *p<0.001, Pod/+ VEH vs Pod/+ DOX (ns), n=6-10). (B) 24h urine free VEGF-A excretion was significantly reduced in Pod/sFlt-1 DOX treated mice (Pod/sFlt-1 DOX vs all other groups, *p<0.04, Pod/+ VEH vs Pod/+ DOX (ns), n=5-10 per group). (C) By densitometry, quantitative analysis of renal cortex VEGF-A protein expression was significantly upregulated in Pod/sFlt-1 DOX treated mice (Pod/sFlt-1 DOX vs all other groups, *p<0.03, Pod/+ VEH vs Pod/+ DOX (ns), n=5-6 per group), and (D) ratio of phosphorylated (Tyr^{951}) VEGFR2 over total VEGFR2 was downregulated in Pod/sFlt-1 DOX treated mice (Pod/sFlt-1 DOX vs all other groups, *p<0.01, Pod/+ VEH vs Pod/+ DOX (ns), n=4-8 per group)
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Fig. 3: Expression of VEGFR1 and VEGFR2 in mouse glomerular cells.
Representative transmission EM image of VEGFR2 (A), VEGFR1 (B), and negative control (C) immunogold staining (arrows on black dots) in mouse glomerular capillaries. Positive immunogold staining is seen, as expected, in endothelial cells (EC)(A, B) and, for the first time, for VEGFR2 in podocytes cell body (P) and foot processes (Pfp)(A). GBM=glomerular basement membrane. Scale bars correspond to 0.5 μm.

Fig. 4: 24-hours urine sFlt-1 and free VEGF-A excretion in diabetic podocyte-specific sFlt-1 overexpressing mice after 10 weeks treatment with VEH or DOX.
(A) VEH-treated diabetic mice had higher urine sFlt-1 excretion than VEH-treated non-diabetic control mice (CVEH vs DVEH, n=6-10/ group, *p=0.02). Urine sFlt-1 excretion was significantly higher in diabetic DOX-treated mice than in diabetic VEH-treated animals (D-VEH vs D-DOX, #p=0.03, n=7-10 per group).
(B) In diabetic mice urine free VEGF-A excretion was significantly reduced in DOX-treated vs VEH-treated animals (D-VEH vs D-DOX, #p=0.04, n=6-10 per group.)
Fig. 5: Renal cortex VEGF-A protein expression and VEGFR2 phosphorylation, in podocyte-specific sFlt-1 overexpressing diabetic mice after 10 weeks treatment with VEH or DOX. Non-diabetic control mice treated with VEH are reported for comparison.  
(A) Representative western immunoblotting and densitometry quantitative analysis of renal cortex VEGF-A expression expressed as % change over non-diabetic VEH-treated mice. VEGF-A protein was significantly upregulated in diabetic VEH-treated mice by 30% over VEH-treated control animals (C-VEH vs D-VEH, *p=0.02). No differences in VEGF-A expression were observed between VEH or DOX-treated diabetic mice (n=5-9 per group).  
(B) Densitometry quantitative analysis of phosphorylated (Tyr^{951}) VEGFR2 over total VEGFR2 in kidney cortex lysate expressed as % change over non-diabetic VEH-treated mice. VEGFR2 phosphorylation was significantly raised in diabetic VEH-treated mice by 58% over VEH-treated control animals (C-VEH vs D-VEH, *p=0.01), no differences was observed between VEH or DOX-treated diabetic mice (n=5-6 per group).
Fig. 6: Renal cortex nephrin and TGFβ1 expression in podocyte sFlt-1 overexpressing non-diabetic control and diabetic mice after 10 weeks treatment with VEH or DOX.
Renal cortex nephrin (A) and TGFβ1 (B) expression were upregulated respectively by ~4 and 1.5 fold in VEH-treated diabetic versus VEH-treated non-diabetic control mice (C-VEH vs D-VEH, *p<0.007). DOX administration did not affect nephrin expression in either diabetic or non-diabetic control mice. Conversely DOX administration was paralleled by downregulation of TGFβ1 expression levels in diabetic mice (D-VEH vs D-DOX, #p=0.001). Representative Western immunoblotting and quantitation of multiple experiments expressed as % change over non-diabetic VEH-treated mice are shown for both nephrin and TGFβ1 (n=4-8, per group).
Fig. 7: Mesangial expansion in podocyte sFlt-1 overexpressing control non-diabetic and diabetic mice after 10 weeks treatment with VEH or DOX

(A) Quantitative electron microscopy of mesangial volume fraction (VvMes) in control and diabetic mice treated with VEH or DOX (n=6-8 per group). VvMes was increased in VEH-treated non diabetic control mice (C-VEH vs D-VEH, *p=0.003). Treatment with DOX reduced significantly VvMes in diabetic mice (D-VEH vs D-DOX, #p=0.04) but had no effect in non-diabetic control animals. (B) Representative images on transmission electron microscopy show amelioration of glomerular mesangial expansion in DOX-treated diabetic mice compared to VEH-treated diabetic animals (scale bar: 5 μm).
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Fig. 8: GBM thickening, foot processes width, and endothelial fenestrae in podocyte sFlt-1 overexpressing control non-diabetic and diabetic mice after 10 weeks treatment with VEH or DOX.

Quantitative electron microscopy determination of GBM thickening (A), podocytes foot processes width (B), and endothelial fenestrae (C), in control and diabetic mice treated with VEH or DOX (n=7-8 per group).

GBM thickness (A) was higher in VEH-treated diabetic mice versus VEH-treated non-diabetic control animals (C-VEH vs D-VEH, *p=0.001). Administration of DOX reduced GBM thickness in diabetic mice (D-VEH vs D-DOX, #p=0.05), but had no effect in non-diabetic mice. Foot process width (B) was increased in VEH-treated diabetic mice versus VEH-treated non-diabetic control animals (C-VEH vs D-VEH, *p=0.005). DOX administration ameliorated foot process fusion in diabetic mice (D-VEH vs D-DOX, #p=0.03), but no effect was seen in non-diabetic mice. The endothelial surface covered by endothelial fenestrae was not changed within the four groups of animal studied (C). Representative images on transmission electron microscopy show amelioration of GBM thickening and foot process width in diabetic DOX treated mice (scale bar: 2 μm).