Sirolimus reduces vasculopathy but exacerbates proteinuria in association with inhibition of VEGF and VEGFR in a rat kidney model of chronic allograft dysfunction

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Abstract
Background. Use of the mTOR inhibitor (mTORi) sirolimus to replace calcineurin inhibitors in kidney transplantation has been associated with improved renal function but, in a proportion of cases, also with de novo or exacerbated proteinuria. Experimental deficiency of vascular endothelial growth factor (VEGF) induces proteinuria and mTOR is required for VEGF production and signalling. We therefore explored the impact of sirolimus on the development of chronic allograft dysfunction (CAD) in the rat, with a focus on VEGF biology.

Methods. Lewis rats received F344 kidney allografts and were treated with 24 weeks of cyclosporine or sirolimus. Controls included allografts treated with cyclosporine for 10 days only and isografts treated with cyclosporine or sirolimus for 24 weeks. Kidney injury (proteinuria and histology) and expression of VEGF and VEGF-receptor (VEGFR; immunohistochemistry, laser capture micro-dissection and quantitative RT–PCR) were assessed.

Results. Allograft controls developed proteinuria, tubulointerstitial fibrosis and atrophy, glomerulosclerosis, vasculopathy and leucocyte accumulation. Proteinuria was significantly reduced in both treatment groups but significantly more in cyclosporine treated animals. Tubulointerstitial damage, glomerulosclerosis and leucocyte accumulation were significantly attenuated in both treatment groups; however, vasculopathy was reduced only by sirolimus. Significantly diminished expression of VEGF and VEGF mRNA and protein was evident in the sirolimus group. In vitro, sirolimus reduced VEGF production by podocytes (P<0.05) and inhibited VEGF-induced proliferation of podocytes, endothelial and mesangial cells.

Conclusions. Cyclosporine and sirolimus retard development of CAD in this rat model. Sirolimus exhibits greater protection against vasculopathy but induces proteinuria; effects are likely to be related to inhibition of VEGF signalling.

Keywords: chronic allograft dysfunction; cyclosporin A; kidney transplantation; sirolimus; VEGF/VEGFR

Introduction
Sirolimus is a lipophilic macrolide antibiotic and the first in the class of the mTOR inhibitors. When used by kidney recipients, sirolimus has been found to be free from calcineurin inhibitor-type nephrotoxicity and switch from cyclosporine-based to sirolimus-based therapy has produced superior kidney function and diminished interstitial fibrosis and tubular atrophy on biopsy in selected patients [1–5]. Anti-angiogenic and anti-proliferative properties of mTOR inhibitors have also underpinned their successful usage in clinical heart transplantation to retard the development of cardiac allograft vasculopathy [6]. Sirolimus use has been complicated by the development or exacerbation of proteinuria in a proportion of recipients, the mechanisms of which are complex and incompletely understood [7, 8]. The pathogenesis of proteinuria is likely multi-factorial and may involve tubular and glomerular contributions. Recent data derived from biopsy sub-studies of clinical trials which compared cyclosporine with sirolimus demonstrated that sirolimus use is associated with tubular damage and tubular proteinuria [9]. In the glomerular compartment, others have demonstrated reduced nephrin expression [10] and reduced VEGF, particularly in patients with significant proteinuria [5, 11].

Sirolimus is a specific inhibitor of the mammalian target of rapamycin (mTOR), a ubiquitous effector protein which plays a central role in mitogenic signalling pathways. Sirolimus inhibits both DNA and protein synthesis in response to cytokines and mitogens, thereby inhibiting lymphocyte progression through the G1 phase of the cell cycle and the production of cytokines important in allograft rejection. Thus, sirolimus inhibits proliferation of activated T lymphocytes in response to IL-2 and other cytokines [12]. Given the ubiquitous nature of mTOR, it is not surprising that sirolimus has activities beyond lymphocytes [12]. Vascular endothelial growth factor (VEGF) is a mitogen critical for endothelial function in vascular, lymphatic and glomerular vessels. Sirolimus has been
demonstrated to inhibit production of VEGF in vivo and in vitro through the inhibition of mTOR [13, 14], an effect opposite to the action of cyclosporine which may increase VEGF expression [15]. Additionally, sirolimus may interrupt down-stream tyrosine kinase signalling in response to VEGF-receptor (VEGFR) activation [16].

VEGF belongs to the family of platelet-derived growth factors and is mainly produced by macrophages, endothelial cells and activated T-cells [17]. VEGF signalling is mediated by at least two high-affinity receptor-binding sites: tyrosine kinase receptor VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR). Activation of VEGFR-1 mediates migration of monocytes and endothelial cells during angiogenesis under pathological conditions and has a regulatory role in the development of inflammatory disease. VEGF signalling through VEGFR-2 induces the main mitogenic, angiogenic and vascular permeability effects of VEGF activity on endothelial cells [18]. A role for VEGF in the pathogenesis of chronic allograft dysfunction (CAD) after kidney transplantation has been suggested, in particular, in promoting fibrosis [19, 20].

Physiological levels of VEGF are pivotal for maintaining vascular homeostasis and survival of glomerular cells under both physiological and pathological conditions. VEGF also maintains vascular permeability which is a requirement for normal glomerular filtration and homeostasis [21, 22]. Proteinuria has been reported in clinical studies as a consequence of therapies inhibiting VEGF in clinical studies of cancer treatment [23] and in experimental models of kidney disease [24]. The proposed mechanism of proteinuria is defective survival and differentiation of podocytes and glomerular endothelial cells in the absence of VEGF signalling [22, 25, 26].

We formed the hypothesis that the observed effects of sirolimus in inhibiting fibrosis yet promoting proteinuria during CAD may be a consequence of VEGF inhibition. We studied a rat kidney transplant model of CAD to compare the effects of sirolimus and cyclosporine on histology, proteinuria and the VEGF pathway.

Materials and methods

Animals and kidney transplantation

Male Fisher 344 and Lewis rats were obtained from the Animal Resource Centre (Perth, Australia), were housed in climate controlled animal rooms and provided free access to regular rat chow and water. The experimental protocol was approved by the University of Sydney Animal Ethics Committee.

Orthotopic renal transplants were performed with end-to-side anastomoses between the renal artery and recipient abdominal aorta and the renal vein with the recipient inferior vena cava using continuous 8–0 nylon sutures. The transplant ureter was anastomosed to the recipient bladder using an interrupted 11–0 nylon suture [27]. All procedures were performed under general anaesthesia with isoflurane. Animals weighed 200–250 g and received either 1.5 mg/kg per day of cyclosporine (Novartis, Sydney Australia), or 1 mg/kg per day of sirolimus (Wyeth, Baulkham Hills Australia). Five groups (n = 5 in each) of animals were included in this study: allografts (Fisher 344-Lewis) treated with cyclosporine for 10 days (control group); allografts (Fisher 344-Lewis) treated with sirolimus for 24 weeks (sirolimus group); allografts (Fisher 344-Lewis) treated with cyclosporine for 24 weeks (cyclosporine group); and two isograft groups (Lewis–Lewis), receiving either cyclosporine or sirolimus for 24 weeks. All animals were sacrificed at 24 weeks. Grafts were processed for molecular, histological and immunohistochemical studies.

Proteinuria

Animals were placed in a metabolic cage for 24-h urine collection every 4 weeks from Week 12. Urinary protein was measured using the BioRad Protein Assay (Bio-Rad, Gladesville, Australia).

Histology and immunohistochemistry

Tissue for light microscopy was fixed in 4% formaldehyde, embedded in paraffin and cut into 5-μm thick sections for staining with Periodic Acid Schiff, Trichrome or Verhoeff’s. The intestinal injury score was obtained by the sum of interstitial fibrosis and tubular atrophy according to the Banff 97 categories. Glomerulosclerosis, defined as segmental or global capillary collapse with increased matrix deposition and/or adhesion to Bowman’s capsule, was scored semi-quantitatively for each glomerulus (0, 25, 50 or 100%) of 20 consecutive glomeruli for each rat. Vasculopathy was assessed by identifying vessels with an internal elastic lamina in Verhoeff-van Gieson-stained sections, then scoring vasculopathy semi-quantitatively using a scale of 1–4 according to the extent of luminal reduction caused by neo-intimal proliferation (0 = normal, 1 = 25%, 2 = 50%, 3 = 75% and 4 = 100% occlusion), as previously described [28]. All scoring was performed in a blinded manner by a single observer (H.T.K.).

For immunohistochemistry, 5-μm thick tissue sections were deparaffinized in xylene, and antigen retrieval was performed by heat treatment, boiling for 10 min in 10 mM sodium citrate buffer (pH 6.0) for VEGFR-1 and VEGFR-2, or digested with 0.5 mg/mL of protease (Sigma-Aldrich, Australia) for VEGF-A, ED1(macrophages) and CD8 (CD8 T-lymphocytes). The sections were blocked with 10% normal horse serum for 20 min and incubated with primary antibodies, mouse anti-rat ED1 1:150 (Serotec, Oxford, UK), mouse anti-rat CD8 1:150 (Serotec), rabbit anti-rat VEGF-A 1:200 (Santa Cruz Biotechnology Inc., Santa Cruz, USA), rabbit anti-rat VEGFR-1 1:25 (Abcam, Cambridge, UK) and rabbit anti-rat VEGFR-2 1:25 (Abcam) for 60 min. Sections were exposed to 3% H2O2 in methanol for 5 min to suppress endogenous peroxidase, and then incubated with the biotinylated secondary antibodies: swine anti-rabbit IgG (Dako, Kingsgrove Australia) or goat anti-mouse IgG (BD Phamingen, North Ryde Australia) for 30 min. VECTASTAIN ABC kit (Vector Laboratories, Burlingame, USA) was then applied according to the manufacturer’s instructions, followed by DAB substrate chromogen solution. The slides were counterstained with haematoxylin solution. Negative controls for immunostaining utilized an isotype antibody.

ED1+ and CD8+ cells were counted in 20 high power fields per animal. Glomerular and arterial wall staining for VEGF, VEGFR-1 and VEGFR-2 were analysed using computerized morphometrics (Image-Pro Plus 4.5, Diagnostic Instruments, USA). Images were digitally acquired with an Olympus BX40 microscope from blinded slides. The density of immunostaining was calculated as image units in whole glomerular or aterial cross-sections [29].

Laser capture microdissection

Frozen sections (10-μm) were cut on a micro cryostat at ~−15°C, adhered to a Superfrost slide (Fisher Scientific, Australia), thawed at room temperature then dehydrated through graded alcohols followed by xylene. Glomeruli and arterioles were identified by morphology and captured onto CapSure HS LCM Caps (Arcturus Bioscience Inc., Basal Switzerland) using a PixCell IIe LCM system (Arcturus Bioscience Inc.). Three hundred glomeruli and 20 arterioles were captured per animal and used for RNA extraction.

In vitro cell culture

Human glomerular epithelial cells immortalized by introducing SV40-T antigen (HGec/Podocyte) have been previously described in detail [30]. This podocyte cell line was maintained in RPMI 1640 (R-8758) medium supplemented with 20% fetal calf serum, insulin, transferrin and selenite (ITS; I-3146), and cells were used between passages 15 and 25. Rat mesangial cells (1097) isolated from the Sprague-Dawley rat have been well characterized [31]. A total of 1097 cells were maintained in RPMI medium (1640, Invitrogen, Mount Waverly, Australia) supplemented with 20% fetal calf serum and were used between passages 25 and 35. Human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection (ATCC, Manassas, VA 20110), were maintained in endothelial cell growth medium supplemented with 20% fetal calf serum and were used between passages 9 and 12. All
media were supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified 5% CO₂ incubator at 37°C. Cells were pas-saged every 4–6 days by using 0.25% trypsin, and seeded at a density of 10^4/cm². To evaluate the effect of sirolimus or CsA on VEGF pro-duction, cells were treated with LPS (Sigma-Aldrich) in fresh RPMI medium with 0.5% fetal calf serum in the presence or absence of 1 nM sirolimus (Sigma-Aldrich) or 1000 nM CsA (Novartis) for 24 h. The culture medium was removed and stored at −80°C for VEGF protein analysis. Cells were immediately lysed with Trizol and lysates were stored at −80°C for total RNA extraction.

Cell proliferation assay
Podocytes, mesangial and HUVEC cell lines were pre-incubated with 20 ng/mL VEGF-A (Perprotech, London, UK) for 6 h and incubated with sirolimus or CsA for 24 h. 3H-thymidine was added for 24 h then cells were released with trypsin/EDTA, solubilized by 0.2 N perchloric acid and harvested with a Micro96 Harvester (Molecular Devices, Australia). Incorporated radioactivity was measured using a 1450 liquid scintillation counter/ Wallac Microbeta Trilux (Perkin Elmer, Australia).

Examination of mRNA expression in kidney tissue and cultured cells
Total RNA extraction from the rat kidney tissues and cell pellets from cell culture was performed with Trizol reagent (Invitrogen, Mount Waverly, Australia) according to the manufacturer’s protocol. cDNA was syn-thesized using 2-µg of total RNA with Oligo-(dT) primers (Applied Bio-systems, Scoresby Australia) following a standard protocol. Probe and primer sequences for real-time PCR were designed using Primer Express Software (Applied Biosystems) and synthesized by Sigma-Aldrich. Primer-5′-CTT CTT TAC TGC TGT ACC T3′, Reverse primer-5′-GTG CTG GCC TTG GTG AGG3′, Probe-5′-TCC CCT CGC TCC CAT CAC CAT GCA TAT GCC GAT CAA (TAMRA)3′. Probe and primer for GAPDH control were used as previously described [32]. cDNA from samples were ampli-fied in 1X Taqman Universal Master Mix (Applied Biosystems) with gene-specific primers and probe on the ABI Prism 7700 Sequence Detec-tion System (Applied Biosystems), according to the manufacturer’s in-struction. Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. All the samples were normalized to the housekeeping gene (GAPDH). All PCR reactions were repeated three times.

Statistical analysis
All results are expressed as mean ± SEM. Graphpad Prism (Version 4) was used for all statistical analysis. Comparisons between two groups of values (non-parametric data) were made with the Mann–Whitney U-test.

**Results**

**Proteinuria**
Allograft controls developed significant proteinuria (97.2 mg/day at 24 weeks; Figure 1). Proteinuria was prevented in the cyclosporine group, with values not different to iso-graft controls (29.7 ± 6.9, P < 0.05). The sirolimus group exhibited a significant reduction in proteinuria compared with controls, however, this was partial and significantly less pronounced than for cyclosporine (41.7 ± 4.1 versus 29.7 ± 6.9, P < 0.05). Isografts treated with sirolimus for 24 weeks exhibited similar proteinuria to isografts treated with cyclosporine (36.4 ± 5.4 versus 30.8 ± 5.2, P > 0.05).

**Kidney histology**
Allograft controls developed proteinuria, tubulointerstitial fibrosis and atrophy, glomerulosclerosis, vasculopathy and leucocyte accumulation (Figure 2). By comparison, both cyclosporine and sirolimus groups were significantly protected against the development of tubulointerstitial damage and glomerulosclerosis, however, only the sirolimus group was protected against vasculopathy (0.50 ± 0.16 versus 1.37 ± 0.34; Figure 2A).

**Immune cell accumulation**
Significant accumulation of both CD8+ T cells and ED1+ macrophages was evident within the renal cortex in the control group when compared with isografts. Accumulation was abrogated by treatment with either cyclosporine or sirolimus (Figure 2B).

**VEGF and VEGFR expression**
There was no significant difference in VEGF-A and VEGFR-1 mRNA expression in whole kidney between the control group and the cyclosporine or sirolimus treat-ment groups. However, the sirolimus group exhibited de-increased VEGFR-2 mRNA (Figure 3). By use of laser-capture microscopy, RNA isolated from glomeruli revealed decreased expression of VEGF-A, VEGFR-1 and VEGFR-2 in the sirolimus group compared with control and cyclosporine groups (Figure 4A). There were no sig-nificant differences in expression found between isografts treated with either sirolimus or CsA (data not shown).

Immunostaining for VEGF-A in whole cortex revealed no difference between sirolimus and cyclosporine groups (Figure 3A). However, staining for VEGF-A, VEGFR-1 and VEGFR-2 within glomeruli showed substantially decreased expression in the sirolimus group compared with the cyclosporine group (Figure 4B).

The expression of VEGF-A and VEGFR-1 mRNA and protein in arteries was substantially reduced in the siroli-mus group compared with cyclosporine (Figure 5A and B). Notably, neither mRNA nor protein expression of VEGFR-2 could be detected in the arteries. Within isografts treated with either cyclosporine or sirolimus, the
level of expression of VEGF-A and VEGFR-1 proteins was similar to the sirolimus allograft group (data not shown).

**Effect of sirolimus on production of VEGF-A by kidney cells in vitro**

The addition of sirolimus to LPS-stimulated podocytes in vitro resulted in a significant reduction in VEGF-A mRNA and protein expression in podocytes, but not in similarly stimulated endothelial or mesangial cells (Figure 6A). In contrast, the addition of cyclosporine increased VEGF-A expression in all cell lines compared with LPS alone. Thus, sirolimus compared with cyclosporine resulted in significantly lower levels of VEGF-A expression in all three cell types at both mRNA and protein levels (Figure 6A).

**Effect of sirolimus on VEGF-A-stimulated kidney cell proliferation in vitro**

VEGF-A induced significant proliferation of podocytes, mesangial cells and endothelial cells after 6 h incubation. Proliferation of all three lines was significantly inhibited by sirolimus (all $P < 0.05$) but not by cyclosporine (Figure 6B).

**Discussion**

We used a rat model of chronic kidney allograft damage, which shares several clinical and histological features in
common with CAD seen in human kidney transplant recipients [33, 34], to compare the effects of sirolimus and cyclosporine on proteinuria, histology and VEGF/VEGFR expression. Compared with control rats who received short-term cyclosporine only, long-term maintenance cyclosporine or sirolimus both significantly attenuated interstitial fibrosis and inflammation, tubular atrophy and glomerulosclerosis. However, rats on maintenance cyclosporine were significantly better protected from proteinuria than those maintained on sirolimus, despite similar degrees of protection from glomerulosclerosis and tubular damage between the two groups and the attenuation of vasculopathy by sirolimus. VEGF and VEGFR mRNA and protein expression in arterioles and within glomeruli were markedly reduced with sirolimus when compared with cyclosporine. Together with the in vitro findings that sirolimus, when compared with cyclosporin, inhibited both VEGF production by podocytes and the VEGF-induced proliferation of podocytes, mesangial and epithelial cells, these data support existing clinical evidence to suggest that interference with the VEGF-signalling pathway within glomeruli may be an important mechanism of sirolimus-induced proteinuria [13, 14, 24].

The beneficial impact of sirolimus, when compared with cyclosporine, on long-term kidney function and histology after transplantation has been partly attributed to the elimination of calcineurin–inhibitor toxicity [1, 2], and partly to direct actions of sirolimus including inhibition of VEGF pathways [35]. Potential pathological roles for VEGF include promotion of inflammation [36], fibrosis and vasculopathy [20]. However, VEGF is important in glomerular development and homeostasis, as shown by defective glomerular development and function in animals with glomerular-specific genetic deficiency of VEGF-A [37], likely due to the importance of VEGF in maintaining glomerular epithelial cell structure and function [22, 25]. Blockade of VEGF-A has also been shown to cause dysregulation of foot-process proteins nephrin and synaptopodin, both of which are essential in retarding proteinuria [38]. The use of mTOR inhibitors in several experimental models of kidney disease has resulted in increased proteinuria [11, 39] or impaired kidney repair [40], underlining the importance of VEGF in maintaining the glomerular protein-filtration barrier. Taken together, this suggests the balance of adverse versus beneficial effects of VEGF may be disease and timing dependent.

Our work suggests sirolimus may interrupt VEGF signalling at two sites. First, sirolimus administration in vivo was associated with decreased VEGF mRNA and protein expression within glomeruli and arterioles within the graft. This is consistent with the known role of mTOR in AKT signalling cascade activation, which is required for VEGF production [14]. Secondly, we found evidence of diminished glomerular and vascular expression of VEGF receptors in animals treated with sirolimus. Specific reduction of the VEGFR-2 within glomeruli may be critical, as this receptor is required for podocyte homeostasis [25]. Our related finding of the ability of sirolimus, but not cyclosporine, to inhibit podocyte, mesangial and glomerular endothelial cell proliferation in vitro provides

Fig. 3. Expression of VEGF and its receptors in whole kidney grafts. (A) There was no difference in the expression of VEGF mRNA or protein in whole allograft kidney tissue between sirolimus and cyclosporine groups. (B) For VEGF receptors, only VEGFR-2 mRNA production was reduced in the sirolimus group when compared with cyclosporine (P < 0.05).
further support that sirolimus may inhibit the VEGF pathway through receptor down regulation within the glomerulus. The combination of VEGF and VEGFR inhibition by sirolimus is likely to be additive in depriving glomerular cells of the action of this important growth factor.

Kidney tissue retrieved from isografted animals treated with sirolimus for 24 weeks showed similar levels of VEGF-A and VEGFR1 mRNA expression to allografts treated with sirolimus, however, the isografts did not develop significant proteinuria. Two likely explanations
exist: that sirolimus directly inhibits repair of previously damaged glomerular endothelium leading to proteinuria, or that the degree of reduction in VEGF signalling and the extent of underlying kidney damage together determine the magnitude of proteinuria. Either mechanism may go some way towards explaining why conversion to sirolimus is...
more likely to cause proteinuria in kidney recipients with significant pre-existing graft damage [37, 41].

Our studies have significant limitations. The model examined does not replicate all features of human CAD and the cell lines we used may differ in function from human kidney cell types and thus any extrapolation of our data to human kidney transplantation should be done with caution.

We found administration of sirolimus to be associated with progressive proteinuria and depletion of VEGF and VEGFRs from glomeruli and vessels when compared with cyclosporine, but have not proven that depletion of VEGF is mechanistically responsible for the proteinuria.

Although our study focused primarily on the effects of sirolimus, the results of cyclosporine treatment are also of interest. Cyclosporin did not inhibit expression of VEGF in vivo and clearly increased VEGF expression in endothelial and mesangial cells in vitro. A recent report suggests cyclosporin markedly induces VEGF transcriptional activation through the protein kinase C-signalling pathway, leading to increased tumour size and also enhanced tumour angiogenesis [15]. In addition, increased expression of VEGF has been described in experimental [42] and human [19] cases of cyclosporin nephrotoxicity. The contribution of cyclosporine to enhanced VEGF signalling in CAD warrants further research.

In conclusion, we found that whilst both sirolimus and cyclosporine provided some protection against inflammation and fibrosis in this rat model of chronic kidney
allograft dysfunction, sirolimus provided additional benefit in attenuating vasculopathy at the expense of proteinuria. Our observations of decreased expression of VEGF and VEGFR in glomeruli and vessels, plus inhibition of VEGF-stimulated proliferation of glomerular cells by sirolimus in vitro, suggest inhibition of VEGF signalling may be a key mechanism of both vascular protection and proteinuria in kidney transplant recipients.

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