Sirolimus interacts with pathways essential for podocyte integrity

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Abstract

Background. The specific mTOR inhibitor sirolimus has been implicated in the pathogenesis of renal glomerular lesions and nephrotic syndrome appearance after transplantation. Podocyte injury and focal segmental glomerulosclerosis have been related to sirolimus therapy in some patients but the pathways underlying these lesions remain hypothetical.

Methods. To go further in the comprehension of these mechanisms, primary cultures of human podocytes were exposed to therapeutic-range concentrations of sirolimus.

Results. Cell viability was not affected after 2 days’ exposure to the drug but changes in cell phenotype and cytoskeleton reorganization were observed. We also evidenced that vascular endothelial growth factor (VEGF) synthesis and Akt phosphorylation were decreased by sirolimus addition. We did not observe any loss of podocyte differentiation markers with the notable exception of WT1, a transcription factor essential for maintaining podocyte integrity. WT1 gene and protein expression in podocytes were decreased in a dose-dependent manner after incubation with sirolimus.

Conclusion. Taken together, these data suggest that sirolimus could impair pathways essential for podocyte integrity and therefore predispose to glomerular injury.

Keywords: podocyte; sirolimus; toxicity; VEGF; WT1

Introduction

Sirolimus, a specific inhibitor of mammalian target of rapamycin (mTOR), has been used for one decade in renal transplantation. Since 2003, it has appeared as evidence that sirolimus therapy is associated with a high incidence of proteinuria, and sometimes nephrotic syndrome [1]. Because proteinuria is a major predictive factor of graft loss, a growing literature focused on this adverse event [2–9]. The development of proteinuria after pancreatic islet transplantation showed that proteinuria is not restricted to the field of kidney transplantation and imputable to sirolimus but rather than a consequence of chronic allograft nephropathy [10]. Whether proteinuria is of tubular or glomerular origin has been debated but the latter seems much more likely [11,12]. We recently reported that sirolimus could induce nephrotic-range proteinuria and an early onset of focal segmental glomerulosclerosis (FSGS) in renal transplant recipients who never received calcineurin inhibitors (CNI) and with high sirolimus trough levels, around 20 nM [13]. Immunohistochemistry experiments showed a typical pattern of podocyte dedifferentiation in some glomeruli. However, FSGS and nephrotic syndrome do not occur in all sirolimus-treated patients, suggesting that sirolimus toxicity is dose related and/or that a second hit is necessary to induce podocyte injury. Moreover, data from experimental studies are conflicting. On the one hand, sirolimus is detrimental in some experimental models of glomerular diseases [14,15]. On the other hand, sirolimus has been associated with a reduction of proteinuria in rat models [16–18]. Dose of sirolimus and time of administration might be determinant to explain these differences, especially in the anti-Thy1 nephritis model [14,18]. Since we have observed podocyte injury and dedifferentiation in renal biopsies of sirolimus-treated patients, we first determined whether human podocyte viability, differentiation or cytoskeletal organization would be affected in vitro after exposure to therapeutic-range sirolimus concentrations. Moreover, we assessed whether the Akt pathway, which is essential for epithelial cell differentiation and adhesion, was affected by sirolimus in vitro. At last, since vascular endothelial growth factor (VEGF) isoforms A and C are synthesized by podocytes and have emerged during the past years as essential autocrine/paracrine factors that promote the survival of both endothelial cells and podocytes, and increase the podocin/CD2AP interaction which is essential for podocyte signalling [19,20], we hypothesized that VEGF synthesis by podocytes could be impaired by mTOR inhibition. To perform these experiments, we tried to limit in vitro artefacts by using primary cultures
Material and methods

Preparation of isolated glomeruli and cultured cells

Decapsulated glomeruli devoid of afferent and efferent arterioles were prepared by sieving methods from normal human kidneys unsuitable for transplantation for vascular or urological reasons in accordance with the current French legislation and as previously described [21]. Isolated glomeruli were digested by collagenase type IV (Sigma Aldrich, St. Louis, MO, USA) for 30 min at 37°C. Detached glomerular epithelial cells were then separated by sieving and cultured in 100-mm plastic dishes and grown in an RPNI 1640 medium (Gibco BRL, Invitrogen corporation, Rockville, MD, USA), supplemented with 10% fetal calf serum (FCS), 10 mM Hepes, 2 mM glutamine, 100 U/mL penicillin and 100 U/mL streptomycin under a 5% CO2 and 95% air atmosphere at 37°C. We checked by immunofluorescence microscopy that these cells expressed the following podocyte markers: nephrin, podocin, WT-1 and synaptopodin. Cells were used at the third or fourth subculture in all experiments and were starved from FCS 24 h before all experiments.

Cell viability studies

A solution of 10 mM sirolimus (Wyeth) in ethanol was kindly provided by Dr N. Pallet (René Descartes University, Paris).

Confluent-cultured podocytes plated on 24-well culture dishes (Nunc, Wiesbaden, Germany) were starved from FCS for 24 h and exposed to sirolimus (20–100 nM) or vehicle (ethanol 10−5 mL/medium mL) for 48 h.

Cell viability was assessed by measuring the exclusion of trypan blue (Gibco BRL, Invitrogen corporation, Rockville, MD, USA). Moreover, LDH concentration in supernatant reflecting cell death was quantified by using a cytotoxicity detection kit (Roche Science Applied Biosystems, Meylan, France) according to the manufacturer’s guidelines.

Akt signalling

The role of sirolimus on Akt1 signalling cascade was assessed by measuring Akt phosphorylation (Ser473) in 96-well grown cells with the FACE-activated cell-based ELISA kit (Active motif, Rixensart, Belgium) after 24-h exposure to sirolimus (20–100 nM) or vehicle according to the manufacturer’s instructions. Briefly, cells were fixed in each duplicate well after exposure to sirolimus or vehicle for 24 h and a primary antibody specific for either Akt or the phosphorylated Akt was added to corresponding wells. A secondary antibody coupled to peroxidase was added a second time and revealed by photometry after addition of the adapted substrate. The optical density (OD) corresponding to total Akt or phosphorylated Akt expression was indexed to crystal violet staining OD. The ratio phosphorylated Akt OD/Akt OD was then analysed to determine the relative Akt phosphorylation.

VEGF synthesis

VEGF165 concentration was measured in the culture medium of podocytes exposed for 24 h in 24 wells to sirolimus (10–100 nM) or vehicle with a Human VEGF Immunoassay Quantikine kit (R&D, Minneapolis, MN, USA) according to the manufacturer’s guidelines. Briefly, 50 µL of cell supernatant was deposited in a 96-well plate previously coated with an anti-human VEGF165 antibody. After incubation with a secondary antibody and addition of the adapted substrate, VEGF quantification was assessed by photometry.

Immunofluorescence microscopy

Third subculture podocytes were grown on multiwell glass slides previously coated with human placenta collagen IV (50 µg/mL overnight at 4°C, Calbiochem, San Diego, CA, USA) and exposed to 20 nM sirolimus or vehicle alone for 48 h. At the end of the experiments, the podocytes were fixed with acetone. They were incubated for 30 min with the following primary antibodies (Ab): rabbit polyclonal anti-WT1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1/200, monoclonal anti-VEGF-A Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1/100, goat polyclonal anti-nephrin Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1/200, monoclonal anti-synaptopodin Ab (Biogen and Biotechnik, Heidelberg, Germany) at 1/10 and monoclonal anti-cytokeratin Ab cocktail C2562 (Sigma Aldrich, St. Louis, MO, USA) at 1/100. The biotinylated anti-mouse, anti-rabbit or anti-goat secondary Ab (Dako, Glostrup, Denmark) and cyanin 2-labelled streptavidin (Amersham Biosciences, Piscataway, NJ, USA) were used as previously described [22]. The slides were observed with a fluorescence microscope and the images were acquired using a digital camera set at constant acquisition parameters in order to compare the cell labelling under both conditions (control versus sirolimus). Immunofluorescence staining of actin cytoskeleton and focal adhesion plaques was performed on cells fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, using both the actin cytoskeleton and focal adhesion staining kit (Chemicon international, Temecula, USA). Confocal microscopy was performed using a Leica TCS laser scanning confocal microscope (Leica Lasertechnik, Heidelberg, Germany).

Immunohistochemistry

The renal biopsies were fixed in alcoholic Bouin’s solution and embedded in paraffin for histology and immunohistochemistry. Immunohistochemistry procedures were performed as previously described [15]. An antibody anti-WT1 C19 pAb was used (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Electron microscopy

The podocytes were grown on Nunc filter plates. At the end of the experiments, they were fixed in 2.5% glutaraldehyde
Fig. 1. Sirolimus does not modify cell viability (Trypan blue and LDH). **Trypan blue**: percentage of podocytes exposed to 20–100 nM sirolimus or vehicle for 48 h that did not exclude the trypan blue dye. The count was made with a Malassez slide at ×200 magnification. Values represent the mean ± SEM of three independent experiments. Chi-square test: $P =$ non-significant for all conditions compared to vehicle. **LDH**: quantification of LDH in the supernatant of podocytes exposed to 20–100 nM sirolimus or vehicle for 48 h. Values represent the mean ± SEM of six independent experiments. Bilateral student $t$-test: $P =$ non–significant for all conditions compared to vehicle.

for 6 h, and rinsed in PBS. The filters covered by podocytes were cut in 2 mm large stripes with a razor blade. The stripes were post-fixed in osmium tetroxide and processed according to a standard transmission electron microscopy technique.

**Western blot analysis**

Podocytes were grown in 100-mm dishes and exposed to 10–100 nM sirolimus or vehicle under serum-free conditions for 48 h. For western blot analysis, cell lysates were prepared by scraping cells into an ice-cold protease inhibitory buffer. Protein quantification was performed according to the standard Bradford technique. Twenty-five microgram proteins were separated by electrophoresis Novex BisTris 7.5% gels using an XCell SureLock™ Mini-Cell (NuPAGE, Invitrogen, San Diego, CA, USA) as described by the manufacturer and transferred onto a nitrocellulose membrane (Immobilon-P, Millipore, Billerica, MA, USA) prior to detection of the WT-1 protein with a specific primary Ab (dilution 1/400) and a peroxidase-labelled anti-IgG secondary Ab (dilution 1/4000). Thereafter, the membrane was developed with the ECL plus detection reagent (Amersham Biosciences, Piscataway, NJ, USA).

**Real-time polymerase chain reaction (real-time PCR) analysis**

Podocytes grown in a six-well plate were starved and exposed to vehicle, 10 or 20 nM sirolimus for 48 h. Total RNA was extracted from podocytes using RNeasy Micro Kit columns (QIAGEN, Hilden, Germany). By using a reverse transcriptase (QIAGEN, Hilden, Germany), cDNA was obtained from RNA and then amplified in a thermocycler (ABI Prism 7000) as follows: 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 45 s and 60°C for 1 min, by using SYBR Green (QIAGEN, Hilden, Germany) and specific primers for WT1: F: AGAATACACACGCAAGGTGTCT and R: GATGCACCCGTACAGAGTC, and hypoxanthine phosphoribosyltransferase (HPRT): F: GACCAGTCAACAGGGAAT and R: AACACTTCGTGGGGTCTTTTC. HPRT is the housekeeping gene used for normalization. Results are expressed as $2^{-\Delta\Delta Ct}$.

**Statistical analyses**

Results are expressed as mean ± SEM. Comparisons between groups of values were made with the chi-square test and bilateral $t$-test for unrelated groups. A difference between groups of $P < 0.05$ was considered significant.
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**Results**

**Absence of sirolimus-induced cytotoxicity in vitro**

Trypan blue and LDH assay provided no evidence for an increase in cell death after 48-h exposure to therapeutic-range or even 100 nM sirolimus concentrations (Figure 1). In addition, we performed electron microscopy in podocytes grown on polycarbonate filters that showed no evidence for mitochondrial changes after exposure to sirolimus, as it can be observed when cellular toxicity occurs (data not shown). FCS starvation was limited to 48 h during sirolimus exposure since preliminary data showed that a longer starvation was associated with an increase in spontaneous cell mortality and phenotypic changes under all conditions.

**Absence of sirolimus-induced podocyte dedifferentiation**

The expression of podocyte differentiation markers (nephrin, synaptopodin, WT-1) and dedifferentiation markers (cytokeratin) was assessed by immunofluorescence microscopy in podocytes grown on the collagen IV matrix and treated by 20 nM sirolimus or vehicle for 48 h (Figure 2). We did not evidence any change in antigen expression after exposure to the mTor inhibitor, with the notable exception of WT-1: its expression was dramatically reduced in podocytes exposed to 20 nM sirolimus (Figure 2).

**Decrease of WT1 mRNA and protein by sirolimus**

Western blots confirmed that exposure to sirolimus for 48 h decreased WT1 expression in a dose-dependent manner in comparison to vehicle (Figure 2). Real-time PCR results showed that mTOR inhibited WT1 gene expression as well (Figure 2). WT1 expression was also assessed by immunohistochemistry in human renal transplant kidney biopsies from patients who received sirolimus (Figure 3). In patients who developed FSGS lesions, a decrease in WT1 expression was observed in podocytes involved in FSGS lesions whereas WT1 expression was preserved in adjacent podocytes. In contrast, WT1 expression was not decreased in kidney biopsies from patients who received sirolimus and did not develop proteinuria and FSGS lesions.

**Modifications of podocyte cytoskeleton due to sirolimus**

An immunofluorescence study of vehicle-treated podocytes grown on glass slides revealed actin cytoskeleton fibres inserted on basal focal adhesions that were evidenced by vinculin staining (Figure 4). We did not evidence major phenotypic changes in podocytes exposed to 20 nM for 48 h but cells seemed more round-shaped and cytoskeleton reorganized: focal adhesions persisted but their pattern of distribution was more peripheral and adopted a coronal distribution. Actin fibres tended to be localized under the...
Fig. 3. WT1 immunohistochemistry. Panel A–C: renal biopsies from sirolimus-treated patients with proteinuria and FSGS. (A and B) Loss of WT1 expression in the podocyte nuclei of a cellular subtype FSGS lesion (arrowheads), whereas WT1 is normally expressed in visceral and parietal podocytes (arrows) (A: ×300 and B: ×600); (C) loss of WT1 expression in the area of a tuft to capsule adhesion in a not otherwise specified (NOS) subtype FSGS lesion (arrowheads), whereas WT1 is normally expressed in visceral and parietal podocytes (arrows) (C: ×400); panel D: renal biopsy from a rapamycin-treated patient without proteinuria or FSGS: normal WT1 expression in the podocytes (D: ×300). See online supplementary material for a colour version of this figure.

Fig. 4. Sirolimus modifies cytoskeleton organization. Morphology of control podocytes (A, B, C and D) and podocytes that were exposed for 48 h to 20 nM sirolimus (E, F, G, H). Actin fibres (A and E) and the focal adhesion protein vinculin (B and F) were visualized by confocal microscopy after staining with rhodamine-conjugated phalloidin and anti-vinculin antibody/FITC-conjugated secondary antibody, respectively. Focal adhesions are more peripheral and actin fibres tend to be localized under the cytoplasmic membrane after sirolimus exposure (merge actin + vinculin + DAPI Blue, C and G). Magnification ×630. Electron microscopy: no ultrastructural changes are observed in the podocytes under the sirolimus condition versus control condition (H and D, respectively). Note that the mitochondria (arrows) are unchanged under both conditions. Magnification ×12 500. See online supplementary material for a colour version of this figure.
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Decrease in Akt phosphorylation in podocytes after exposure to sirolimus

Since Akt/PKB is essential for cell adhesion and cytoskeleton organization, we analysed whether this pathway was involved in sirolimus-induced modifications of podocyte.

Activation of the Akt pathway results in a relative increase of the Akt phosphorylated form. A significant decrease in the phosphorylated Akt (Ser 473)/total Akt ratio was observed even for a moderate sirolimus concentration (20 nM, Figure 5). This ratio remained in a similar range with higher sirolimus concentrations (50 or 100 nM).

Decrease in VEGF synthesis by podocytes after exposure to sirolimus

VEGF is a growth factor involved in the maintenance of podocyte integrity [19,20]. Immunofluorescence staining
showed that VEGF expression was decreased in podocytes after exposure to 20 nM sirolimus (Figure 2). In addition, assessment of VEGF165 excretion in podocyte supernatant by ELISA provided evidence that sirolimus reduced VEGF synthesis even at moderate concentrations (Figure 6).

**Discussion**

Massive urinary protein excretion has been observed after switching from CNI to sirolimus in patients with chronic allograft nephropathy. Whether proteinuria was due to sirolimus or only a consequence of CNI withdrawal remained unsolved until high-range proteinuria was observed during sirolimus therapy in islet transplantation or in the absence of CNI withdrawal. Moreover, we recently described that sirolimus glomerular toxicity could occur within months following renal transplantation in patients treated de novo by sirolimus who never received CNI [13].

The study was designed to assess whether sirolimus promotes podocyte dedifferentiation and/or death. In addition, we focused on two critical pathways essential for epithelial cell survival: VEGF synthesis and Akt signalling. First, VEGF is a growth factor acting in an autocrine manner that is essential for podocyte survival. Second, the Akt pathway is critical for epithelial cell differentiation, adhesion and survival and is involved in podocyte intracellular signalling [19,20,23]. We observed de novo FSGS lesions in renal transplant recipients with high sirolimus trough levels, around 20 nM, suggesting a dose-related toxicity. However, sirolimus peak dosage was not determined. We estimated that sirolimus concentrations ranking from 10 to 100 nM in vitro could be relevant to in vivo conditions. However, a first limitation of this in vitro study was the duration of sirolimus exposure. In vivo, podocyte injury was indeed symptomatic after a few weeks of sirolimus therapy. In vitro, serum deprivation was needed to obtain well-differentiated podocytes but 72-h serum deprivation or more impaired cell survival under all conditions, so we limited experiments to 48-h serum deprivation and sirolimus exposure. This limitation may partly explain why we did not observe an increase in cell death after 48-h inhibition of mTOR. Another hypothesis consistent with the prevalence and the variability of sirolimus-induced proteinuria is that sirolimus might predispose to podocyte injury by interfering with survival pathways but is not sufficient to induce cell death by itself. It is noteworthy that many patients who receive sirolimus do not develop proteinuria.

However, we identified that sirolimus therapeutic levels decrease vascular VEGF synthesis and interfere with the Akt pathway in podocytes.

Sirolimus has antiangiogenic activities linked to a decrease in VEGF production and to an inhibited response of vascular endothelial cells to stimulation by VEGF [24]. In clinical settings, a decrease in intra-glomeruli VEGF associated with thrombotic microangiopathy has been described in renal transplant recipients who received sirolimus [25]. A recent report also showed that sirolimus could reduce VEGF synthesis in vitro in glomerular endothelial cells [26]. In addition, in the remnant kidney model in the rat, mTor inhibitor everolimus increased proteinuria and glomerulosclerosis and was associated with reduced glomerular VEGF mRNA and protein [16]. VEGF-C and A isoforms are synthesized by podocytes and exert a pro-survival effect in an autocrine and paracrine manner [19,20]. VEGF-A enhances podocyte survival through VEGF-R2, increases podocyte differentiation and promotes the podocin–CD2AP interaction [20]. We observed that the decrease in VEGF synthesis occurred even with low sirolimus concentrations in human podocyte supernatant and could therefore participate in the development of podocyte lesions.

Akt/PKB is a key regulator of cell adhesion and it can be hypothesized that the slight cytoskeleton changes observed in sirolimus-treated podocytes may be related to the interaction with the Akt pathway. The complex mTorC2 (containing mTor, rictor, mLST8 and Sin1) phosphorylates Ser473 and therefore activates Akt/PKB. It has been reported that sirolimus inhibits the assembly of mTORC2 and that prolonged rapamycin treatment reduces the levels of mTORC2 below those needed to maintain Akt/PKB signalling [27]. Consistent with that work, we observed a decrease of Akt Ser473 phosphorylation in podocytes with moderate sirolimus concentrations. It is noteworthy that the Akt pathway is activated by VEGF and that conversely the Akt pathway promotes VEGF synthesis [28,29]. Taken together, these data suggest the existence of complex interactions between mTOR, VEGF and Akt signalling pathways, thereby having the potential to affect podocyte adhesion and survival (Figure 7).

Since we observed dedifferentiation of human podocytes in vivo, we tried to evidence sirolimus-induced dedifferentiation by immunofluorescence but failed to show any increase of dedifferentiation marker or loss of differentiation marker, with the notable exception of WT1, a protein expressed in podocytes only in the mature kidney. Immunohistochemistry performed in human kidney transplant recipients who received sirolimus and developed FSGS lesions revealed that WT1 expression was decreased only in FSGS lesions, similar to other differentiation markers or VEGF. However, these in vivo data did not allow us to determine whether a decrease in WT1 expression is necessary for the onset of FSGS lesions or only results from podocyte dedifferentiation. The finding that WT1 gene and protein expression decreases in parallel to mTor inhibition by sirolimus points to a role of WT1 gene in the pathways involved in FSGS lesions in vivo. WT1 expression could be decreased by sirolimus by interfering with survival pathways or by interfering with the development of WT1 gene expression.
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Fig. 7. Sirolimus and podocytes: hypothetical mechanisms. mTor complexed to Rictor phosphorylates Akt at Ser473. Therefore, sirolimus may inhibit the Akt pathway that is essential for cellular adhesion and survival. Sirolimus also inhibits mTor-induced VEGF expression, a growth factor critical for podocyte survival. VEGF binding to the VEGF receptor may activate the Akt pathway. At last, we identified that mTor inhibition decreases WT1 expression. See online supplementary material for a colour version of this figure.

syndrome and Denys-Drash syndrome. WT1+/R394W heterozygous mice develop a progressive glomerulosclerosis and podocyte lesions that recapitulate Denys Drash syndrome [30]. Truncated forms and post-transcriptional modifications of WT1 lead to many isoforms responsible for distinct cellular functions and the WT1 protein can act as a transcriptional activator or repressor depending on the cellular and chromosomal context [31]. Moreover, WT1 over-expression occurs in most cases of acute lymphoblastic leukaemia and chronic myelogenous leukaemia and in many solid cancers: an oncogenic role of WT1 is suspected in such contexts and high WT1 expression levels have been correlated to a poor prognosis in acute leukaemia [32].

No relationship has been previously described between mTOR and WT1. Since sirolimus may be efficient as an anti-tumoural agent in leukaemia and cancers, it would be of interest to determine if WT1 expression levels are correlated to mTOR activity in cancer cells [33].

In conclusion, short-term exposure of human podocytes to sirolimus at ‘therapeutic’ concentrations results in an interaction with the Akt pathway and VEGF synthesis in vitro. These pathways are involved in podocyte survival and sirolimus might therefore predispose to the FSGS lesions observed in vivo when given at high doses or when an additional ‘hit’ occurs. Moreover, the relationship between mTOR and WT1 might be of interest beyond the field of transplantation if confirmed in other human cell lines or cancers.

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Conflict of interest statement. None declared.

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.
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