Everolimus inhibits glomerular endothelial cell proliferation and VEGF, but not long-term recovery in experimental thrombotic microangiopathy

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Abstract

Background. Everolimus is a potent immunosuppressant used in renal transplant therapy, but its effects on renal endothelial cell regeneration after injury are unknown. The effects of an everolimus therapy were investigated in a model of renal thrombotic microangiopathy (TMA) with specific endothelial cell (EC) injury in the rat in vivo as well as in glomerular ECs in vitro.

Methods. During the early regenerative phase (day 3) of the renal microvascular injury model in vivo, everolimus inhibited glomerular EC proliferation by up to 60% compared with vehicle-treated rats, whereas apoptosis was not different in these groups. This decreased EC proliferation was associated with an enhanced deposition of fibrin in everolimus treated animals on day 3. In cultured glomerular endothelial cells, everolimus effectively and dose dependently inhibited cellular proliferation. This anti-proliferative effect was associated with a reduced phosphorylation of the p70S6 kinase and reduction of the pro-angiogenic factor VEGF in glomeruli in vivo and in cultured podocytes in vitro.

Results. Despite the prolonged EC repair and in contrast to the anti-Thy1 nephritis model, everolimus therapy did not disturb the long-term repair reaction in this thrombotic microangiopathy model.

Conclusion. Everolimus is anti-proliferative for glomerular EC in vitro and in vivo and does not seem to have detrimental long-term effects in experimental renal TMA, when only the glomerular endothelium, but not the mesangium is severely injured.

Keywords: glomerular endothelial cell proliferation; glomerulosclerosis microvascular injury; rapamycin derivative everolimus; VEGF

Introduction

Due to the relative lack of nephrotoxicity, the novel mammalian target of rapamycin (mTOR) inhibitors sirolimus or its derivative everolimus are potent novel immunosuppressants increasingly used in renal transplant therapy. Nevertheless, adverse effects of mTOR inhibitors have been described in human renal allografts [1–3] and glomerulonephritis [4] as well as experimental kidney disease [5,6]. The mTOR inhibitors have a distinctive mechanism of action, where inhibition of a multifunctional serine–threonine kinase, mTOR—inhibits both co-stimulatory activation and growth factor-mediated cell proliferation of T-lymphocytes, but also of other cell types such as vascular smooth muscle cells (VSMC) [7], endothelial cells (EC) [8] and mesangial cells (MC) [9]. This relatively general anti-proliferative effect of mTOR inhibitors seem to be worrying especially during acute repair phases after severe (renal or other) injury, but so far is not really predictable in humans possibly due to the lack of standardization of the human situation.

Hereby, investigation of standardized experimental model systems with a highly reproducible and well-characterized time course and the possibility of repeated histological examination may help to understand clinical observations. Recently, we have described the specific conditions and potential mechanisms leading to adverse actions of everolimus in a reversible mesangial proliferative glomerulonephritis model, the anti-Thy1 model [5]. In this model, with combined injury of the glomerular mesangium and...
endothelium, everolimus treatment caused dramatic adverse effects with a high mortality rate as well as progressive apoptosis, crescent formation and glomerulosclerosis. Hereby, the development of adverse everolimus effects was strikingly linked to its marked inhibition of endothelial cell proliferation/repair, but not necessarily to its inhibition of mesangial cell proliferation.

In this current study, we were interested whether everolimus-mediated inhibition of capillary repair is specifically critical only in the situation of a combined MC and EC injury with the occurrence of complex microaneurysms (as in the anti-Thy1 model) or also in the situation of a specific glomerular endothelial lesion. Therefore, we investigated whether the mTOR inhibitor everolimus would also adversely affect the time course of disease and repair in a well-established rat model of thrombotic microangiopathy (TMA) with exclusive damage of the renal endothelium [10]. This model is induced by selective renal artery perfusion with a specific anti-EC antibody involving renal capillaries. Glomerular endothelial cell (GEN) injury in this model peaks on day 1 and is followed by GEN activation and repair due to cellular proliferation within the next 6 days (peak on day 3) after disease induction [10]. Although this experimental situation of everolimus therapy in TMA is not completely and directly transferable to the human situation, exclusive or predominant renal microvascular injury is also a prominent feature of many forms of human renal disease including haemolytic-uraemic syndrome, malignant hypertension, scleroderma, pre-eclampsia, vasculitis and acute antibody-mediated rejection after renal transplantation [11]. In particular, haemolytic uraemic syndrome [12] or acute humoral transplant rejection may either occur during mTOR therapy [13–15] or during calcineurin inhibitor-based immunosuppression, and therefore in the latter situation, a switch towards an mTOR inhibitor-based therapy may be especially considered/required [16] in transplant patients. In addition, mTOR inhibitors have also been considered to be promising drugs in the treatment of glomerulonephritis with various lesions, but preliminary results were surprisingly discouraging [4].

Methods

In vitro studies

Cultured mouse GEN were immortalized by transfection with polyoma middle T-antigen as described previously [17]. Cells were characterized by immunocytochemistry via positive staining for the typical EC markers MECA-32, CD31 and the lack of staining for MC markers such as positive staining for the typical EC markers MECA-32, CD31 and the lack of staining for MC markers such as WT-1 and cytokeratin, and maintained in Diblecco’s Modified Eagles Medium (DMEM) containing 10% fetal calf serum (FCS), 1 mM pyruvate, penicillin/streptomycin, non-essential amino acids and 5 mM mercaptoethanol at 37°C and 7.5% CO2 [18].

Mouse podocytes isolated from kidneys of ImmortoMouse® mice (Charles River, St Louis, MO, USA), carrying a temperature-sensitive mutant of the immortalizing SV40 large T antigen under control of the interferon-γ (INF-γ)-inducible H-2Kb promoter were grown in RPMI 1640 containing 10% FCS, penicillin/streptomycin and γ-interferon at 33°C. For cultivation of differentiated podocytes, cells were seeded on collagen type I without γ-interferon at 37°C for at least 10 days as described previously [19].

The vascular endothelial growth factor (VEGF) expression was investigated using differentiated podocytes treated with 20 or 100 nM everolimus or solvent (ethanol) for 24 and 48 h. Toxicity of everolimus on cultured cells was excluded by lactate dehydrogenase measurements using a LDH cytotoxicity detection kit (MoBiTec, Göttingen, Germany) following manufacturer’s instructions.

Cell proliferation assay. The effect of soluble everolimus (at concentrations between 2.4 nM and 0.3 μM) regarding cellular proliferation were quantified using a REDOX indicator proliferation assay following manufacturer’s instructions (Alamar blue SM Assay, Serotec, Düsseldorf, Germany) [20]. Mouse GEN (2.5 × 10^5/well) were seeded in 96-well tissue culture plates, growth arrested for 24 h in 0.1% FCS, and stimulated with 3% FCS compared with non-stimulated serum-free GEN. Everolimus was added to cells stimulated with 3% FCS. Proliferation was assessed after growth for 72 h.

Western blot analysis. The proteins were extracted from cultured mouse GEN or podocytes using 50 mM Tris, 1% (v/v) Nonident P-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na3VO4, 1 mM NaF, and proteinase inhibitor cocktail (Complete, Boehringer, Mannheim, Germany) as extraction buffer. For a better solubility, extracts were sonified for 30 s at 50°C power and 50% duty cycle using a Sonoplus HD70 (Bandelin, Berlin, Germany). Thirty micrograms of protein were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis using gels containing 10% acrylamide for analysing p70S6 kinase and 15% acrylamide for the analysis of VEGF. The mouse monoclonal antibodies against p70S6 kinase (sc-8418), P-p70S6 kinase (sc-8416) and VEGF-A were all purchased from Santa Cruz Biotechnology (Santa Cruz, UK) as detection antibody as described [10]. Microemulsion formulations

In vivo studies

Animal model. The animal studies were performed in accordance with the Internal Animal Review Board (Regierung von Mittelfranken: 621-2531.31-04/03). Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) weighing 180–220 g were used for all the studies. The animals were fed standard rat chow (Altromin 1324, Spezialfutterwerke GmbH, Lage, Germany) and tap water ad libitum.

Experimental renal TMA was induced by selective renal artery perfusion of the right kidney through superior mesenteric artery with purified IgG of the anti-GEN antibody as described [10]. Microemulsion formulations
Experimental design. All rats were randomly allocated to six different groups with anti-GEN nephritis (n = 6–8), where groups 2, 4 and 6 were vehicle-, while groups 1, 3 and 5 were everolimus-treated by gavage on a daily basis. In groups 1 and 2, renal survival biopsies were obtained on day 3 and rats were sacrificed on day 6. To differentiate, whether long-term effects of everolimus therapy are dependent or independent of its anti-proliferative effect restricted to the early phase of disease, rats in groups 3 and 4 were treated daily only until day 10 in contrast to groups 5 and 6 with continuous daily treatment. In groups 3–6, rats were sacrificed after 42 days (Figure 1). A serum sample and a 24 h urine collection for measuring proteinuria, serum creatinine and urea were done before obtaining the survival or sacrificial biopsies.

Renal morphology and immunohistochemistry. Tissue for light microscopy was fixed in methyl Carnoy’s solution, embedded in paraffin, and cut into 3 μm sections for periodic acid Schiff reagent (PAS) or indirect immunoperoxidase staining as described elsewhere [10,21]. For each biopsy, 40–60 cortical glomerular cross-sections (400-fold magnification) were evaluated in a blinded fashion. For determination of basic levels of p70S6 kinase, P-p70S6 kinase, VEGF and OX-7, four kidney biopsies from healthy rats were investigated each.

Glomerulosclerosis was defined as segmental or global capillary collapse with increased matrix deposition and/or adhesions with the Bowman’s capsule on a scale of 0–4 (0 = normal, 1 = one small focus, 2 = <25%, 3 = 25–50%, 4 = >50% of glomerular surface area is involved).

The following antibodies were used in this study:

(i) a murine IgG1 monoclonal antibody (mAb) against the human proliferating cell nuclear antigen (PCNA, clone PC10), cross-reactive with rat (DAKO, Glostrup, Denmark) [10,21].
(ii) a murine monoclonal IgG1 mAb to a rat cytoplasmic antigen present in monocytes, macrophages and dendritic cells (clone ED-1) (Linaris, Wertheim-Bettingen, Germany) [10,21].
(iii) a murine IgG1 mAb specific for rat MC (clone OX-7) (Serotec Ltd, Oxford, UK) [22,23].
(iv) α-SM-actin (1A4) a murine IgG2a mAb to human α-SMA detecting activated MC from mouse, rat and human (DAKO) [22,23].
(v) a rabbit polyclonal antibody to human VEGF-A cross-reactive with rat VEGF (sc-152, Santa Cruz Biotechnology, Santa Cruz, USA) [24].
(vi) a goat polyclonal antibody to human collagen IV cross-reactive with rat (Southern Biotechnology Associates, Inc., Birmingham, UK) [22,23].
(vii) a murine IgG1 mAb against human fibrinogen and fibrin cross-reactive with rat (American Diagnostic Inc., Greenwich, USA) [10].
(viii) a murine IgG1 mAb against mouse MCP-1 cross-reactive with rat (sc28879, Santa Cruz Biotechnology) [25].
(ix) a murine IgG mAb specific for rat aminopeptidase P on endothelial cells (clone JG-12) [26,27].
(x) a murine IgG mAb specific for the rat endothelial cell antigen (RECA-1) (Serotec Ltd, Oxford, UK) [21,28].
(xi) a mouse monoclonal antibody against rat p70S6 kinase (sc-8418; Santa Cruz Biotechnology, Santa Cruz, USA) [29].
(xii) a mouse monoclonal antibody to phosphorylated p70S6 kinase recommended for the detection in rats (sc-8416; Santa Cruz Biotechnology, Santa Cruz, USA) [29].

Negative controls for immunostaining included either deleting the primary antibody or substitution of the primary antibody with equivalent concentrations of an irrelevant murine mAb or pre-immune rabbit IgG.

Glomerular expression of MCP-1, VEGF and α-SMA actin was graded semiquantitatively [10,21,30] and reflected changes in the area and intensity of glomerular staining: 0 = very weak or absent staining; 1+ = weak staining with <25% of the glomerular tuft showing focally increased staining; 2+ = 25–49% of the glomerular tuft with focally increased staining; 3+ = 50–75% of the glomerular tuft demonstrating increased staining; and 4+ = >75% of the glomerular tuft stained strongly. It was previously shown that this scoring system is reproducible between different observers and in very good agreement with results obtained by computerized morphometry as demonstrated earlier.
and in this study for collagen IV and OX-7. Collagen IV and OX-7 expression was quantified by measuring the percentage of stained glomerular area using the Metavue Imaging System (Visitron Systems GmbH, Puchheim, Germany).

Glomeruli with intact endothelium were defined when strong JG-12 staining was present in over 75% of the glomerular tuft [28]. Peritubular capillary loss and/or regeneration was assessed using a rarefaction index as described by Kim et al. [28].

**Immunohistochemical double staining.** To determine the number of proliferating GEN and mesangial cells double immunostaining for PCNA, a marker of cell proliferation, and RECA-1 or JG-12 as EC-specific markers or OX-7, a MC marker, were performed as previously described [21,24,28]. As secondary antibody we used a rat adsorbed, biotinylated horse anti-mouse IgG (Vector, Burlingame, USA) which was detected by horseradish peroxidase conjugated avidin D (Vector, Burlingame, USA). To avoid non-specific staining a Biotin/Avidin blocking kit (Vector, Burlingame, USA) was used before incubation with the second primary antibody. The peroxidase substrate 3,3’-diaminobenzidine (DAB, substrate kit, Vector) was used with nickel resulting in a black nuclear staining (PCNA) and without nickel leading to brown cellular staining (RECA, JG12 or OX-7).

**TUNEL assay.** Apoptotic cells were detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) assay as described previously [32]. DNAse-treated slides were used as positive controls. Cells were regarded as TUNEL-positive per glomerular cross-section, if their nuclei were stained black and displayed typical apoptotic morphology with chromatin condensation.

**Miscellaneous measurements.** Urinary protein was measured using the BioRad Protein Assay (BioRad, München, Germany) and BSA (Pierce, Bonn, Germany) in concentrations from 10–500 mg/ml as standards following manufacturer’s instructions.

**Statistical analysis.** All values are expressed as mean±SD. Statistical significance (defined as \( P < 0.05 \)) was evaluated using the Student’s \( t \)-test.

**Results**

**Everolimus inhibits glomerular endothelial cell recovery in TMA**

Typical histological changes after induction of the TMA model as shown by PAS-staining (Figure 2A–C) demonstrate a marked (day 3) and ceasing (day 6) endothelial repair reaction that is finished in the second week after disease induction and leads to an intact glomerular architecture on day 42. Clearly, everolimus therapy impaired recovery of the glomerular endothelium. In agreement with previous studies, glomerular proliferative activity in this TMA model consisted mainly of EC, peaked on day 3 and ceased around day 6 [10]. Everolimus therapy inhibited glomerular cell proliferation by 60% as identified by PCNA-positive cells (Figure 3B) and resulted in a reduction of...
Fig. 3. Everolimus therapy inhibits EC regeneration in a model of TMA. In experimental TMA, cell number per glomerular cross-section was counted on days 3 ($P = 0.008$), 6 ($P = 0.015$) and on day 42 after continuous (con) or everolimus treatment during the proliferative phase (pro) (A). Glomerular cell proliferation as assessed by the average number of PCNA-positive cells (B) was mainly due to GEN proliferation as demonstrated by PCNA/RECA-1 (C) positive cells per glomerular cross-section. (D) shows double immunostaining for PCNA (black nuclei) and RECA-1 (dark grey cytoplasmic staining of the endothelium), where several proliferating GEN are marked by arrows (magnification 400×). Apoptosis, as assessed by TUNEL staining, was not different in everolimus- and placebo-treated animals (E). Everolimus therapy inhibited repair of the glomerular endothelium as indicated by the increased percentage of glomeruli with <75% of an intact endothelium (F). Glomerular accumulation of fibrin (as an indirect measure of endothelial cell stress/activation) was evaluated by immunostaining (G). Everolimus effects on GEN proliferation in vitro was assessed by the Alamar blue proliferation assay (H).
glomerular cell number on days 3 and 6 (Figure 3A). Using double staining techniques (RECA-1 or JG-12 as EC markers and PCNA as a marker for proliferating cells), most of the proliferating glomerular cells during TMA disease were confirmed as GEN (Figure 3C and D). Evaluating this double staining technique, everolimus inhibited GEN proliferation during the recovery phase of disease on days 3 and 6 by up to 62.57% (Figure 3C). In contrast to cellular proliferation, everolimus therapy did not influence glomerular cell death as assessed by TUNEL staining (Figure 3E). The percentage of glomeruli with positive staining for ECs was markedly decreased in everolimus-treated rats vs vehicle controls until day 3 (52 vs 70%) (Figure 2G–I), but hardly different on day 6 (77 vs 85%) and equivalent at the end of the experiment on day 42 (87 vs 88%) reaching values hardly different from healthy control rats (Figure 3F).

In agreement with a delayed glomerular EC recovery during everolimus treatment, glomerular accumulation of fibrin (as an indirect measure of endothelial cell stress/activation) was transiently increased up to day 3 vs vehicle control (Figures 2D–F and 3G). Nevertheless on day 6, no significant difference was observed anymore (Figure 3G) and after 42 days no fibrin was detected in either group (data not shown).

GEN proliferation was also inhibited in vitro using cultured glomerular ECs. Representative results are shown in Figure 3H demonstrating an equivalent, dose-dependent inhibition of GEN proliferation by everolimus after 72 h. A significant inhibitory effect started at concentrations of 2 nM. Maximal inhibitory effect of everolimus was observed at concentrations of about 20 nM. However, the remaining proliferative activity was higher than that in non-stimulated cells. No difference in morphology was observed comparing both control and everolimus-treated cultures. GEN-viability as tested by trypan blue exclusion was not affected by everolimus at the concentrations used in this study.

Severe injury in the TMA model is restricted to the endothelium

In the TMA model, the mesangium remained intact as assessed on day 3 compared with healthy kidneys by detection of OX-7 positivity (MC marker) within the glomeruli (Figure 4A). Nevertheless indicating some MC activation, the number of proliferating MC was slightly increased from $0.29 \pm 0.05$ in healthy control rats to $1.49 \pm 0.77$ PCNA/OX-7 positive cells per glomerular cross-section on day 3 in the placebo-treated group and significantly reduced in everolimus-treated animals (Figure 4B). Although GEN injury and recovery is the predominant glomerular feature in this...
model, this indirect MC activation is also indicated by de novo induction of glomerular α-SMA, a marker for activated MC. Everolimus therapy transiently inhibited de novo induction of glomerular α-SMA in rats with TMA (Figure 4C). Despite de novo expression of α-SMA as one part of the ‘myofibroblastic’ phenotype of activated MC, collagen I as the other phenotypic part was induced only at a very low level without significant differences between everolimus-treated and placebo-treated TMA rats (Figure 4D).

Everolimus inhibits phosphorylation of the p70S6 kinase and VEGF during TMA and in cultured podocytes in vitro

Parallel to regulation of the proliferative activity of glomerular ECs in the TMA model (Figure 3C), the glomerular number of positive cells for total and for the phosphorylated form of the p70S6 kinase was regulated during TMA. While the number of positive cells for total p70S6 kinase was doubled on day 3 and decreased to nearly control levels on day 6 (Figure 5B), the number of cells for the phosphorylated form of p70S6 kinase was more than doubled on both days 3 and 6. Everolimus treatment significantly reduced cells positive for the phosphorylated form of mTOR target p70S6 kinase on days 3 and 6 (Figure 5A), but did not affect the number of cells positive for total p70S6 kinase. Furthermore, glomerular VEGF protein was up-regulated in the renal TMA model in parallel to the proliferative activity of the glomerular endothelium peaking on day 3 and ceasing on day 6 (Figure 4C). Everolimus therapy significantly reduced glomerular VEGF in vivo on days 3 and 6 by 30 ± 14% and 36 ± 15%, respectively as assessed by immunostaining (Figure 5C and D). Since VEGF was predominantly expressed by podocytes during TMA (Figure 5D, arrows), the effect of everolimus on VEGF expression was also investigated in cultured podocytes (Figure 6). Cultured differentiated podocytes were either treated with 20 or 100 nM everolimus for 24 and 48 h and the amount of VEGF, phosphorylated and non-phosphorylated p70S6 kinase was compared with
- tubulin or β-actin using western blot analysis. In accordance with the in vivo study, the phosphorylated p70S6 kinase was reduced by 40–60%, 24 and 48 h after everolimus treatment (Figure 6A), while total p70S6 kinase was not changed by everolimus treatment after 24 h and to a lower extent after 48 h (maximum 48%) (Figure 6B). VEGF expression was markedly reduced to 13 and 10% of control when cells were incubated with 20 and 100 nM everolimus for 24 h (Figure 6C). Treatment for 48 h with everolimus resulted in a further reduction to 3% of control when everolimus was used in a concentration of 100 nM (Figure 6C). The results of one representative experiment is demonstrated in Figure 6, while all in vitro experiments showed similar results.

**Everolimus long-term therapy is not harmful in TMA**

Since inhibition of GEN repair may be problematic for the long-term recovery of glomeruli from TMA, we investigated, whether everolimus therapy would affect glomerulosclerosis 42 days after disease induction either given daily continuously (con) or if restricted to the first 10 days during active endothelial proliferation (pro). Although most glomeruli recover completely from GEN injury, some increased glomerulosclerosis and matrix expansion can still be observed 42 days after disease induction (Figure 7A). Considering that this model is not really proteinuric, and both the diseased and the non-diseased kidney contribute to urine secretion, proteinuria was not significantly changed by everolimus treatment (Figure 7B).

Continuous everolimus therapy for 42 days (con) clearly did not affect the recovery from renal disease adversely as indicated by glomerulosclerosis scoring or glomerular matrix accumulation, as assessed by a semiquantitative scoring system (glomerulosclerosis) on day 42 (Figure 7A) or by computer-assisted quantitation of immunostaining for collagen IV, which was even slightly improved ($P = 0.002$, Figure 7C) vs vehicle-treated animals.

To examine the importance of the early anti-proliferative effects from potential long-term (adverse) effects of everolimus therapy in TMA, we added two additional groups where either everolimus or vehicle was given only for the first 10 days (glomerular proliferation ceases after 6 days) and all parameters were evaluated 42 days after disease induction as before. Again no adverse long-term effect of everolimus treatment was seen. In contrast to the continuous everolimus therapy, the early 10 day everolimus therapy did not show any trend towards an even beneficial effect after 42 days (Figure 7).

In addition, the number of glomerular ED-1-positive monocytes/macrophages was slightly reduced after continuous everolimus therapy on day 42 (0.83 ± 0.38 ED-1-positive cells vs 1.55 ± 0.37 in the vehicle-treated group; $P = 0.008$). Nevertheless, no influence of everolimus on the glomerular expression of the major macrophage chemoattracting protein, MCP-1 was found at any time point during TMA disease (MCP-1 score: 0.61 ± 0.38 vs 1.06 ± 0.44 on day 3 and 0.08 ± 0.08 vs 0.16 ± 0.2 on day 6 comparing placebo group with RAD-treated animals).

**Discussion**

Due to the relative lack of nephrotoxicity, the novel mTOR inhibitors sirolimus or its derivative everolimus are potent novel immunosuppressants increasingly...
used in renal transplant therapy. Nevertheless, unexplained adverse effects of mTOR inhibitors such as renal deterioration or proteinuria have been described in a fraction of patients with glomerulonephritis [4] or with renal allografts after conversion from calcineurin inhibitors [1–3]. The successful use of an mTOR inhibitor therapy seems to be dependent on the clinical situation and potentially on the underlying renal lesion or disease, but no specific pattern or pathomechanism can be provided in these human studies. Since the development of calcineurin-inhibitor-induced TMA occurs in 2–10% of renal transplant patients and a medication switch to the novel immunosuppressants sirolimus or its derivative everolimus is more frequently considered in these patients, we investigated whether the mTOR inhibitor everolimus might adversely affect the healing of established renal TMA in a well-characterized experimental model in the rat.

This question regarding a specifically problematic glomerular target cell or lesion for an mTOR inhibitor therapy is in particular relevant, since everolimus seems to be detrimental in the anti-Thy1 model with both EC and MC injury [5,6], but even beneficial in experimental renal disease models with a predominant podocytic injury and little affection of the general glomerular architecture [6,33]. GEN injury and death in our experimental renal TMA model is associated with increased glomerular capillary rarefaction and is followed by GEN repair due to cellular proliferation within the next 6 days (peak on day 3). Since the study design was chosen to determine whether everolimus therapy could impair short- and long-term recovery after established endothelial injury, treatment was not started until 24 h after disease induction and short-term (3 and 6 days) as well as long-term effects (after 42 days) of this therapy were investigated.

The first major finding was that everolimus treatment caused a delay but not a complete blockade of endothelial recovery. Consistent with its marked anti-proliferative activity, everolimus treatment inhibited GEN proliferation by up to 60% on days 3 and 6 resulting in a transient relative hypocellularity (no change in the rate of apoptotic cells) and an increased endothelial rarefaction index compared with vehicle-treated rats. This everolimus-induced anti-proliferative effect was also confirmed in GEN in vitro, showing that everolimus blocks proliferation of glomerular ECs incompletely to values found in non-stimulated cells. The delay of the endothelial repair and transient glomerular hypocellularity during TMA was associated with increased glomerular fibrin indicating short-term disease aggravation.

Fig. 7. Everolimus long-term therapy does not impair glomerulosclerosis, matrix accumulation and proteinuria in TMA. Glomerulosclerosis (score 0–4) was almost significantly reduced on day 42 after continuous treatment with everolimus (A, \( P = 0.07 \)). Proteinuria was slightly but not significantly reduced by everolimus treatment on days 3/42 (B). Matrix accumulation as assessed by glomerular collagen IV immunostaining was investigated in rats continuously treated with everolimus (con, \( P = 0.002 \)) for 42 days and in animals treated only during proliferative phase (pro) using computer-assisted image analysis (C).
long-term adverse effects in experimental TMA, since neither an increased death rate nor an increased glomerular injury index with increased sclerosis nor impairment of renal functional parameters were observed after 42 days. Nevertheless, this study clearly demonstrates that everolimus has the potential to exacerbate the acute phase of glomerular endothelial injury, which is likely to occur in humans as well. While this experimental model is characterized by a single-timed endothelial injury, endothelial injury during human disease is usually ongoing for a longer period of time. Therefore, treatment with mTOR inhibitors should probably be used with caution during active microangiopathy or acute humoral rejection, but appears to be relatively safe once acute injury has subsided.

Besides its potential implication for the human situation, these results are also interesting in a more general fashion regarding the pathophysiology of glomerular healing. Therefore, we asked why inhibition of glomerular regeneration in case of the anti-Thy1 model leads to adverse long-term consequences [5,6], but unexpectedly not in case of our TMA model. The everolimus doses used were identical in both nephritis models. In contrast to the TMA model, the severe anti-Thy1 model is characterized by a combined injury of the glomerular mesangium and endothelium with capillary disruption and the appearance of glomerular microaneurysms that require the coordinated repair of the glomerular endothelium followed by the mesangium via a proliferative and migratory response [5,21,23,36]. When we recently defined the specific conditions and potential mechanisms in the anti-Thy1 model that caused an everolimus-induced faulty repair with the development of focal segmental glomerulosclerosis like lesions in about 30% of all the glomeruli [5], adverse everolimus effects were strikingly linked to a marked inhibition of EC proliferation/repair, but not to its inhibition of mesangial cell proliferation. Hereby, the extent of glomerular EC injury does not seem to be the critical factor, since EC injury as indicated by the number of the responding proliferating ECs is clearly higher in the TMA model than in the anti-Thy1 model. Rather, the type of glomerular lesion appears to be important. In the anti-Thy1 model marked mesangiolysis combined with GEN injury leads to capillary disruption with the formation of microaneurysms, and the requirement of a timely and coordinated repair reaction, where the mesangium follows the endothelium. This situation may make the glomerulus especially vulnerable regarding a delayed EC (and the following MC) repair induced by everolimus. Also, the repair rate during everolimus therapy is much higher in the TMA model than in the anti-Thy1 model. In the TMA model, glomerular cell count increased by 20% despite of everolimus treatment during days 3–6 in contrast to the anti-Thy1 model where cell count was not significantly changed between days 4 and 7 after model induction in animals treated with the same everolimus dose. In experimental TMA, glomerular injury is almost completely restricted to the endothelium, while no microaneurysm occurs and the mesangium is completely intact as indicated by an unchanged number of OX-7-positive MCs [10,28]. Nevertheless, some minor MC activation to a ‘myofibroblastic phenotype’ also occurs in the TMA model as demonstrated by de novo induction of glomerular γ-SMA early in disease [10,22] and is inhibited by everolimus. This difference regarding the exclusiveness of the target of injury (endothelium) may keep the glomerular architecture, stability and structural support much better intact than in the anti-Thy1 model and may still allow a final healing reaction of the glomerulus even after a delayed repair reaction with transient disease aggravation as demonstrated in the TMA model. The long-time frame of treatment for 42 days may have helped to level out differences in the glomerular capillary repair that were still visible earlier and even accompanied by increased fibrin accumulation (such as on day 3).

The second major finding of this study was that everolimus not just blocked phosphorylation of the p70S6 kinase, but also reduced glomerular VEGF suggesting this as a potential additional mechanism for its growth inhibitory effect on the GEN during experimental TMA. The relevance of rapamycin-induced down-regulation of VEGF was supported by a recent study of rapamycin-induced TMA cases showing an association of sirolimus-induced TMA with decreased expression of VEGF in human transplanted kidneys [15]. Therefore, Sartelet and colleagues [15] suggested down-regulation of VEGF in podocytes as a potential mechanism for increased risk of renal TMA after sirolimus therapy. The pro-angiogenic vascular endothelial growth factor (VEGF) is important for endothelial cell regeneration and glomerular function. Specific blockade of the angiogenic factor VEGF by aptamers selectively inhibited early GEN regeneration/proliferation in the reversible anti-Thy1 model [37]. Infusion of VEGF in a chronic progressive nephritis model induced by injection of both, anti-Thy1 antibody and Habu snake venom, enhanced glomerular capillary repair/proliferation [38]. In mice, both glomerular-selective deletion and overexpression of VEGF resulted in the development of glomerular disease [39]. Furthermore, mice with podocyte-specific haploinsufficiency for all VEGF isoforms develop glomerular endotheliosis, the renal lesion seen in preeclampsia [40]. Considering this ongoing requirement for tight regulation of VEGF signalling between the podocyte and the glomerular endothelium, it is important to note that VEGF expression in the TMA model was significantly reduced, but not completely blocked by everolimus. Nevertheless, in vitro experiments using cultured podocytes showed a very pronounced reduction of cellular VEGF by everolimus. These novel data in glomerular cells are consistent with in vitro experiments in human renal cancer cells [41] and breast carcinoma cells [42], where sirolimus treatment also resulted in a reduction of VEGF. In addition, in a mouse tumour model, sirolimus also demonstrated anti-angiogenic activities
linked to decreased VEGF and to an inhibited response of vascular ECs after stimulation by VEGF [43]. Decreased VEGF expression levels were also described in a recent study in patients with trough levels between 4.4 and 14.6 ng/ml who developed a sirolimus-induced TMA [15].

Although matrix accumulation is not a predominant feature of this TMA model, the third finding of this study was that everolimus therapy inhibited glomerular matrix expansion as well as macrophage accumulation independently of its anti-proliferative effect. Since everolimus therapy did not affect glomerular macrophage accumulation at earlier time points (days 3 and 6), the late decrease may be more likely the consequence than the cause of decreased glomerular fibrosis. Other studies reported anti-fibrotic effects by rapamycin therapy in an in vivo model of liver fibrosis [44], renal ischaemia reperfusion injury model [45] or thoracic-aorta to abdominal-aorta allograft model [46]. In these studies, the anti-fibrotic effect was linked to a parallel occurring anti-proliferative effect, which makes it hard to differentiate between cause and result regarding cytokine responses [44,45]. In our current study, this anti-fibrotic effect was diminished, when everolimus was given only during the proliferative phase of disease for the first 10 days (glomerular proliferation ceases after 6 days), being consistent with an independent anti-fibrotic effect. This interpretation is supported by a study, where long-term everolimus therapy for several months markedly inhibited renal matrix accumulation in ageing rats without any additional injury [47].

We conclude that the potent mTOR inhibitor everolimus specifically inhibited GEN proliferation in vitro and in vivo in a model of TMA, which was transiently aggravated. Despite a delayed glomerular capillary repair, everolimus therapy did not adversely affect the final healing as seen in the anti-Thy1 nephritis model. The recovery of the glomerular architecture in the TMA model is likely due to the preserved mesangium as structural support and the lack of capillary disruption with microaneurysms in contrast to the anti-Thy1 model and also to a sufficient remaining proliferative activity due to partial VEGF blockage and partial inhibition of EC proliferation. These findings support the concept that treatment with mTOR inhibitors should probably be used with caution during active microangiopathy, acute hemoraul rejection or other situations of renal disease with predominant EC injury, but appears to be relatively safe once acute injury has subsided.

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