Nephrol Dial Transplant (2012) 27: 537–541
doi: 10.1093/ndt/gfr318
Advance Access publication 22 July 2011

mTOR inhibition and erythropoiesis: microcytosis or anaemia?

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Abstract

Background. Anaemia and microcytosis are common post kidney transplantation. The aim of this study was to evaluate the potential role of mammalian target of rapamycin (mTOR) inhibition in the development of anaemia and microcytosis in healthy animals and in human erythroid cultures *in vitro*.

Methods. Rats with normal kidney function were treated with sirolimus ($n = 7$) or vehicle ($n = 8$) for 15 weeks. Hemograms were determined thereafter. In the sirolimus withdrawal part of the study, rats received sirolimus (SRL) for 67 days ($n = 4$) 1 mg/kg three times per week or for 30 days ($n = 4$) and were observed until Day 120. Hemograms were performed regularly. Peripheral blood mononuclear cells from healthy controls (HC; $n = 8$), kidney transplant patients with sirolimus treatment with (SRL + MC; $n = 8$) or without microcytosis (SRL − MC; $n = 8$) were isolated and cultured in the absence or presence of SRL (5 ng/mL).

Results. SRL-treated animals had a reduced mean corpuscular volume (MCV) and elevated erythrocyte count compared with control animals after 15 weeks of treatment. This effect was evident as early as 4 weeks (MCV: 61.5 ± 1.8 versus 57 ± 1.7 fL; P = 0.0156; Red blood count 7.4 ± 0.3 × 10^9/L versus 8.6 ± 0.5 × 10^9/L; P = 0.0156) and was reversible 90 days after SRL withdrawal. SRL in the culture medium of erythroid cultures led to fewer colonies in cultures from HC as well as from kidney transplant patients (without SRL: 34.2 ± 11.4 versus with SRL: 27.5 ± 9.9 BFU-E-derived colonies P = 0.03), regardless if the cultures were derived from recipients with normocytic or with microcytic erythrocytes. The presence of tacrolimus in the culture medium had no influence on the number and size of colonies.

Conclusion. mTOR inhibition induces microcytosis and polyglobulia, but not anaemia in healthy rats. This might be caused by growth inhibition of erythroid precursor cells.

Keywords: anaemia; microcytosis; mTOR inhibitor; rapamycin; sirolimus

Introduction

Anaemia post kidney transplantation is common and has been associated with cardiovascular complications. Moreover, this problem seems to be under recognized. Shah *et al.* performed a study on a large post-transplantation population from three centres in the UK to elucidate the prevalence of post-transplant anaemia, its determinants and the use of erythropoiesis-stimulating agents (ESA) in these patients. The prevalence of anaemia was 45.6%; however, only 9.6% of patients were treated with ESA [1]. The etiology of anaemia post kidney transplantation is multifactorial. Iron deficiency, deficient erythropoietin production, chronic inflammatory disease, infection, drug toxicity and transplant dysfunction are the major causes.

In recent years, clinical targets of hemoglobin (Hb) concentrations in chronic kidney disease patients have been modified toward lower levels moving from near normal concentrations to Hb concentrations between 11 and 13 g/dL. In the Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT) study, Hb concentrations of ~13 g/dL were associated with an increased risk of stroke [2].

The application of the mammalian target of rapamycin (mTOR) inhibitor sirolimus (SRL) has been identified as a possible risk factor for the development of anaemia in kidney transplant patients [3]. Ekberg *et al.* [4] showed that low-dose SRL treatment was associated with a higher incidence of anaemia.

Friend *et al.* [5], however, observed that the development of anaemia in kidney transplant patients treated with SRL depended on the extent of allograft dysfunction, i.e. SRL-treated patients with a high glomerular filtration rate (GFR) after kidney transplantation were less likely to develop anaemia than SRL-treated patients with a low GFR.

The aim of this study was to evaluate the potential role of mTOR inhibition in the development of anaemia and

microcytosis in different settings: in rats with normal kidney function in order to study the genuine effect of SRL on erythropoiesis without the interfering effect of renal insufficiency, in kidney transplant patients who were converted from a calcineurin inhibitor (CNI)-containing regimen to an SRL-based treatment and *in vitro* cultures of peripheral blood erythropoietic progenitor cells.

Materials and methods

Experimental animal studies

Male Wistar rats (Charles River Laboratories España, Barcelona, Spain) weighing 200–250 g were kept at constant temperature and humidity with a 12-h light/dark cycle. The animals had free access to standard rat chow (Harlan Interfauna Ibèrica S.L., Barcelona, Spain) and water. This study was approved by and conducted according to the guidelines of the local animal ethics committee (Local animal studies ethics committee, Decret 214/97).

SRL versus control for 4 months

Male Wistar rats were randomized to two groups according to intraperitoneal administration of either vehicle (VEH; $n = 8$) or SRL ($n = 7$) 1 mg/kg three times per week, over 15 weeks. Whole blood samples were collected in ethylenediamine tetraacetic acid vacutainer tubes at the end of the study to analyze the mean corpuscular volume (MCV), red blood count (RBC) and the Hb concentration.

SRL continuous 2- versus 1-month withdrawal

Male Wistar rats were randomized to two groups. Rats in Group 1 ($n = 4$) received SRL treatment for 67 days, 1 mg/kg three times per week. Rats in Group 2 ($n = 4$) received SRL treatment for 30 days, then SRL treatment was stopped and the animals were observed until Day 120. Whole blood samples were collected at the beginning of the study and then regularly thereafter to determine MCV, RBC and Hb.

In vitro *hematopoietic progenitor culture*

Mononuclear cells were isolated from peripheral blood samples obtained from healthy controls (HC; $n = 8$) or kidney transplant patients with sirolimus treatment with (SRL + MC; $n = 8$) or without microcytosis (SRL − MC; $n = 8$). Microcytosis was defined as MCV <81 fL. Isolation was performed by density gradient centrifugation, using Histopaque (Sigma–Aldrich, Madrid, Spain), at 600 *g* for 30 min. After washing with phosphate-buffered saline, cells were counted and seeded, in duplicate, in a methylcellulose medium, containing Agar Leukocyte Conditioned Media (Agar-LCM) as a source of colony-stimulating factors (MethoCult® 4531; Stem Cell technologies, Grenoble, France), in the presence of 3 U/mL of erythropoietin (NeoRecormon®; Roche, Barcelona, Spain) and in the absence or presence of SRL (5 ng/mL) for 14 days.

The cultures were performed at 37°C under 5% of CO_2 and extra humidity (Nuaire Incubator, Inc. Laboratory Equipment, Plymouth, MN). After 15 days of incubation, erythroid colony-forming units (E-CFU) were visualized and counted using a ×4 objective in an inverted microscope (Leica Microsystems, Wetzlar, Germany) considering that each colony consists of >40 cells. Images were captured by a video camera (ProgRes MF; Infaimon S.L., Barcelona, Spain) and transferred to a personal computer for morphometric evaluation with an automated image analysis system (SigmaScan; Systat Software Inc.).

Statistical analysis

Statistical analysis was performed using the SPSS 14.0 statistics package. Values are given as mean ± SD. The Wilcoxon or Mann–Whitney *U*-tests were used as applicable.

Results

SRL versus control for 4 months

Fifteen weeks of sirolimus treatment resulted in a significant reduction of MCV (Figure 1a). This was accompanied by an increase of the erythrocyte count (Figure 1b). Moreover, after 15 weeks of treatment, the Hb concentration was significantly higher in the SRL-treated animals (Figure 1c).

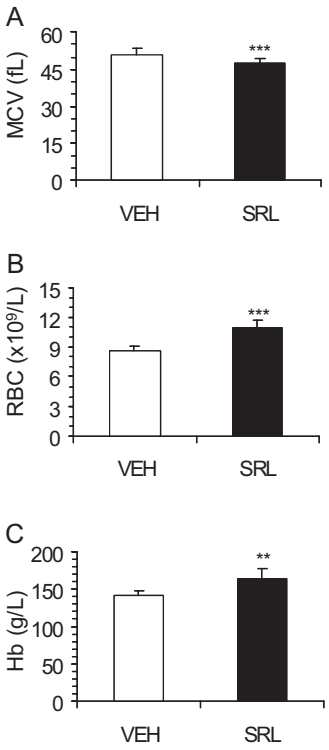

Fig. 1. Effect of sirolimus treatment in healthy rats (1 mg/kg three times per week, over 15 weeks) on **(A)** MCV; **(B)** RBC and **(C)** Hb concentration. *Significantly different compared to vehicle (VEH)-treated group. **P < 0.01; ***P < 0.001.

SRL continuous 2- versus 1-month withdrawal

Four weeks of SRL treatment led to a decrease of MCV (61.5 ± 1.8 to 57 ± 1.7 fL; P = 0.0156). Rats which remained on SRL treatment (Group 1) showed an MCV of 56.3 ± 1.7 fL after 67 days of treatment. Rats in Group 2 (SRL withdrawal after 4 weeks) had only a slight increase of their MCV at 9 weeks (58.0 ± 0.8 fL; P = not significant compared with Group 1) and reached an MCV near the original value 90 days after cessation of SRL treatment (60.4 ± 0.5 fL at the end of the study; P = not significant compared with the beginning of the study). The RBC was 7.4 ± 0.3 × 10^9/L at the beginning of SRL treatment, increased to 8.6 ± 0.5 × 10^9/L (P = 0.0156) and stayed at this concentration in Group 1 during the SRL treatment (8.8 ± 0.9 × 10^9/L) period of 67 days, whereas the RBC only returned to the original value 90 days after cessation of treatment in Group 2 (7.4 ± 0.3 × 10^9/L; P = not significant compared with Day 0). A similar pattern could be observed for the Hb concentration (increase from 14.5 ± 0.33 g/dL to 16.5 ± 0.51 g/L after 28 days; P = 0.0156). The Hb concentration then remained stable during the treatment period of 67 days in Group 1 (16.1 ± 1.2 g/dL), and the full period of observation (90 days after cessation) was needed in Group 2 in order to reach the original range (13.7 ± 0.42 g/dL) (Figure 2).

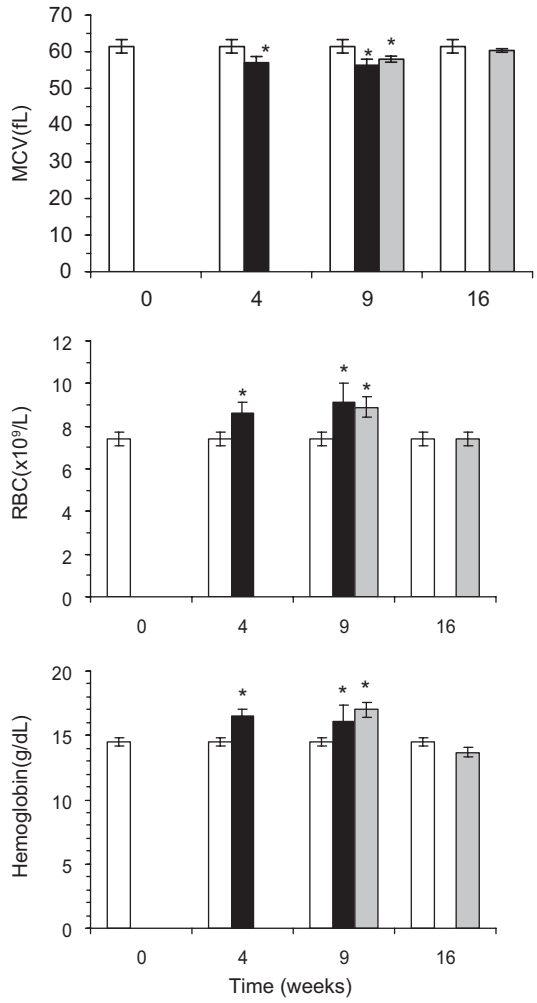

Fig. 2. Comparison of the effect of sirolimus treatment on MCV, RBC and Hb concentration over 9 weeks (black bars), sirolimus treatment over 9 weeks with subsequent withdrawal and observation for further 7 weeks (gray bars) compared with vehicle treatment (white bars). *P < 0.05

Hematopoietic progenitor cell culture experiments

The Hb concentration was 13.1 g/dL in patients with SRL treatment and microcytosis (SRL + MC) ($n = 8$; age 54.9 ± 15; 5.1 ± 4 years after transplantation) and 13.5 g/dL in patients with SRL treatment and without microcytosis (SRL − MC) ($n = 8$; age 56.8 ± 15; 5.2 ± 2 years after transplantation) (P = not significant). There were no significant demographic differences nor differences in terms of renal function, SRL dosing or iron concentrations between patients with and without microcytosis. The RBC was 5.1 and 4.7 × 10^9/L in SRL + MC and SRL − MC, respectively (P = 0.034). MCV was 76 fL in SRL + MC and 87 fL in SRL − MC (P < 0.0001). SRL in the culture medium led to fewer colonies in HC and kidney transplant patients (without SRL: 34.2 ± 11.4 versus with SRL: 27.5 ± 9.9 BFU-E-derived colonies P = 0.03). The same difference was seen if the three groups were analyzed separately (HC, SRL − MC, SRL + MC) (Figure 3). The presence of tacrolimus in the culture medium had no influence on the number of colonies. SRL but not TAC treatment reduced

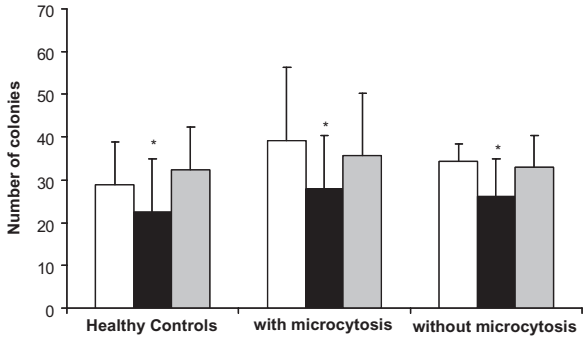

Fig. 3. Effect on the number of colonies by adding erythropoietin (EPO) alone (open bars) or EPO and SRL (closed bars) or EPO and tacrolimus (gray bars) to cultures derived from erythroid progenitor cells. *Significantly different compared to EPO treatment, P < 0.05.

the size of the colonies in HC (Figure 4) and in kidney transplant patients with and without microcytosis.

Discussion

Anaemia post kidney transplantation is a clinical problem that is underrecognized. This is the first study evaluating the influence of mTOR inhibition on erythropoiesis independently of renal insufficiency in an animal model and in cell culture experiments.

Our results demonstrate that mTOR inhibition alone does not induce anaemia in a rat model of mTOR inhibition with normal renal function. The most prominent effect was microcytosis, which could be seen as early as 2 weeks after initiation of SRL treatment. Microcytosis in the animal model was compensated by an increase of the erythrocyte concentration. Moreover, the effect on MCV and RBC was reversible after withdrawal of SRL. However, >90 days off treatment were required to reach the original values again. The life span of erythrocytes in Wistar rats is ~60 days [6], i.e. more than a whole life span in addition to the time of erythrocyte maturation of 14 days is needed to reverse the effect of mTOR inhibition.

Kim *et al.* [7] first described microcytosis under SRL treatment in kidney transplant patients. They compared SRL-based with Cyclosporin A (CsA)-based immunosuppression in a randomized trial and found that the Hb concentrations were not significantly different between the two treatment arms in spite of the existence of microcytosis in the SRL arm. We provide further support for these observations in conversion patients. Our patients demonstrated a lower MCV (some of them reaching MCV values below the lower normal limit), but similar Hb concentrations at 1 year after the conversion [8]. The lower MCV seemed to be compensated by a higher RBC. In contrast to the animal model, however, not all of the patients were able to fully compensate this effect by a higher erythrocyte count in order to keep the same Hb concentration without ESA treatment. The incidence of ESA treatment in the studied conversion patients was >60%. Therefore, one could argue that mTOR inhibition causes at least a relative erythropoietin resistance. Probably, other factors such as the inhibtion of erythropoiesis by severe renal insufficiency or treatment with mycophenolate may contribute to the induction of

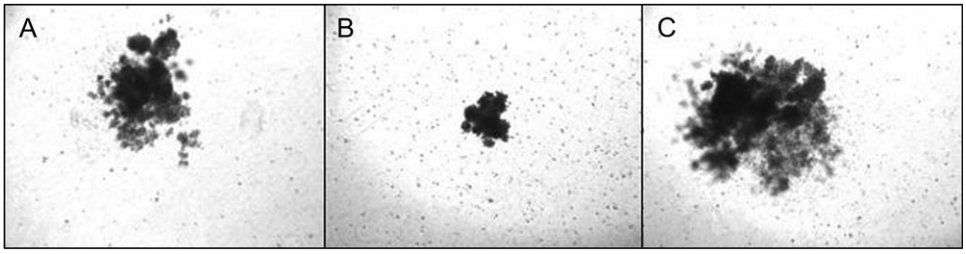

Fig. 4. Effect of sirolimus treatment on size of colonies derived from erythroid progenitor cells of HC. Erythropoieitin only was added to the culture (**A**). Erythropoietin and sirolimus (**B**) or erythropoietin and tacrolimus (**C**) were added. Magnifications, ×40.

anaemia in these patients. This hypothesis is supported by the study of Friend *et al.* [5], who observed that the effect of SRL on anaemia was dependent on the SRL dose and on renal function.

Moreover, in a randomized controlled trial of psoriasis patients with normal or near normal kidney function comparing SRL-containing protocols [SRL alone (3 mg/m^2 body surface area) or SRL in combination with low-dose CsA] with full-dose CsA treatment, anaemia was not observed in spite of high doses of SRL which induced leukocytopenia and thrombocytopenia in some patients [9].

Maiorano *et al.* [10] described an interference of SRL with iron homeostasis. This suggests that iron deficiency is not the unique mechanism of microcytosis associated with mTOR inhibitor treatment. Although it might be tempting to carry out a generous iron supplementation in these patients with the aim to increase the MCV, the clinician should perform iron supplementation in patients on mTOR inhibition as cautiously as in patients on other immunosuppressants in order to avoid iron overload.

Several authors described anaemia under SRL treatment. Augustine *et al.* [3] studied anaemia in 214 kidney or kidney–pancreas transplant patients, who had either SRL-based or mycophenolate mofetil-based immunosuppressive therapy and observed anaemia to be more frequent in the SRL group (57 versus 31%). In a further study, these authors found a higher degree of erythropoietin resistance in SRL-treated patients [11]. Thaunat *et al.* [12] studied anaemia in 46 patients who were converted from a CNI-containing to an SRL-containing therapy and observed that anaemia in SRL patients correlated with the concentration of proinflammatory cytokines such as interleukin-6 and *tumor necrosis factor*-alpha but also with increased levels of CRP and fibrinogen. Sanchez-Fructuoso *et al.* [13] analyzed patients who were converted from a CNI to everolimus and observed transient anaemia with low serum iron levels, normal/high ferritin concentration and elevation in serum of inflammatory cytokines.

Sofroniadou *et al.* [14] demonstrated that SRL-based immunosuppression caused early, profound and sustained erythrocyte microcytosis which is reversed after drug discontinuation; they suggested that SRL could induce an iron-deficient state.

In order to clarify the direct effect of mTOR inhibition on erythropoiesis, we performed erythroid progenitor cell cultures *in vitro*. Interestingly, the effect of addition of SRL to the culture medium was a decrease of the number of erythroid colonies in SRL-treated patients (microcytic and non-microcytic) and HC. Thus, the effect most probably did not depend on the *in vivo* pretreatment. SRL is known to produce a cell cycle restraint and therefore to block cell growth in many cell types. Thus, a possible explanation for the effect on erythroid progenitor cells could be the antigrowth effect of SRL which prolongs the maturation of erythroid progenitor cells and consequently leads to a lower number of colonies, producing erythrocytes of decreased size when compared to normal growth.

One of the limiting factors of the study is that in this specific study, SRL blood concentrations were not measured. However, in previous studies by our group with animals of the same strain and the same weight, SRL was administered under the same conditions and at the same doses reaching 38.8 ± 7.9 ng/mL [15].

In conclusion, our study demonstrates that treatment with mTOR inhibitors primarily leads to microcytosis independently of kidney function. This seems to be compensated by an increase of the RBC. mTOR inhibition does not induce anaemia *per se*, but anaemia may occur in SRL-treated patients in the presence of additional factors that inhibit the necessary mechanism of normal or increased erythropoiesis, which is needed to compensate for erythrocytes of smaller volume. In the clinical setting, SRL-associated anaemia can be treated successfully with ESA and adequate iron supplementation, keeping in mind that microcytosis in these patients is likely to be induced by mTOR inhibition and not only by iron deficiency.

Acknowledgements. The authors would like to thank Ful Navalón for his outstanding technical assistance and Dr Enrique Granados for his excellent help and support.

Parts of this work have been presented in abstract form at the Congress of the Societat Catalana de Trasplantament 2007 in Barcelona, Spain and at the congress of the Gesellschaft für Nephrologie 2008 in Tübingen, Germany.

Funding. This study was supported by grants from Wyeth Pharmaceuticals, from 'Redes Temáticas de Investigación cooperativa V-2003-REDC03' and from the 'Fondo de Investigación Sanitaria, FIS 03/0557'.

Conflict of interest statement. None declared.

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Received for publication: 23.7.10; Accepted in revised form: 5.5.11

Nephrol Dial Transplant (2012) 27: 541–547
doi: 10.1093/ndt/gfr308
Advance Access publication 30 June 2011

Using microdialysis for early detection of vascular thrombosis after kidney transplantation in an experimental porcine model

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Abstract

Background. In kidney transplantation (KTx), vascular thrombosis has a major impact on morbidity and graft survival. The ischaemia, caused by thrombosis, can lead to interstitial metabolite changes. The aim of this experimental study was to create conditions in which the graft would be prone to vascular thrombosis following KTx and then to evaluate the role of microdialysis (MD) for its early detection.

Methods. Sixteen randomized pigs in the control group received heparin and immunosuppressive drugs, while the case group received none. Based on histopathological evidence of vascular thrombosis, the case group was subdivided into mildly and severely congested subgroups. Using MD, we evaluated the interstitial concentrations of glucose, lactate to pyruvate ratio, glutamate and glycerol in the transplanted grafts during different phases of KTx.

Results. Following reperfusion, we noted considerable changes. The severely congested subgroup showed a low and decreasing level of glucose. Only in this group did the lactate to pyruvate ratio continue to increase until the end of monitoring. The glycerol level increased continuously in the entire case group and this increase was most significant in the severely congested subgroup. In all of the study groups, glutamate concentration remained in a low steady state until the end of monitoring.

Conclusions. MD can be an appropriate method for early detection of vascular complications after KTx. Decreasing glucose levels, increased lactate to pyruvate ratio and