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Cellular and Molecular Diol

Tubular epithelial cell and podocyte apoptosis with *de novo* sirolimus based immunosuppression in renal allograft recipients with DGF

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Summary. Sirolimus is associated with prolonged delayed graft function (DGF) following renal transplantation and exacerbation of proteinuria. We assessed renal allograft biopsies from DGF patients treated with *de novo* sirolimus (n = 10) for renal tubular cell and podocyte apoptosis and expression of activated caspase-3, Bcl-2, and mTOR and compared them to biopsies from DGF patients not receiving sirolimus (n =15). Both groups received mycophenolate mofetil, prednisone and antibody induction. Apoptosis was assessed using terminal deoxynucleodidyl transferase mediated dUTP nick end labeling (TUNEL) staining. Caspase-3, Bcl-2, and mTOR expression were assessed by immunohistochemistry. Sirolimus treated patients had 334±69 TUNEL positive cells per 5 high power fields compared to 5.5±2.9 TUNEL positive cells in control patients (p<0.001). The number of TUNEL positive cells correlated with tubular architectural disruption. Expression of activated caspase-3, Bcl-2, or activated mTOR did not differ between groups. 60% of biopsies from sirolimus treated patients compared to 7% of biopsies from controls showed diffuse podocyte apoptosis (p = 0.007). There was no podocyte expression of activated mTOR, activated caspase-3, or Bcl-2 in either group. These data suggest that DGF patients treated with sirolimus have increased renal tubular cell apoptosis and podocyte apoptosis.

Key words: Sirolimus, Apoptosis, Podocyte, Delayed graft function, Kidney

Introduction

There have been a number of reports suggesting that immunosuppression using sirolimus prolongs the recovery from delayed graft function (DGF) immediately following deceased donor renal transplantation (McTaggart et al., 2003; Simon et al., 2004; Smith et al., 2003; Stallone et al., 2004; Boratynska et al., 2005). Smith et al. (2003) reported that kidney recipients whose initial immunosuppression included sirolimus in addition to tacrolimus were significantly more likely to have DGF, defined as the need for dialysis beyond 24 hours after transplantation, compared to a group of patients treated with cyclosporine and mycophenolate. They also noted, as did McTaggart et al. (2003) that recovery from DGF was prolonged in patients receiving sirolimus in the early post-transplantation period. Simon et al. (2004) analyzing the USRDS data base, subsequently reported that de novo sirolimus was an independent risk factor for the development of DGF. Others have reported a delay in recovery from DGF but not an increased incidence of DGF (Stallone et al., 2004; Boratynska et al., 2005). Despite prolonging recovery from DGF, Stallone et al. (2004) found that recipients with DGF treated with sirolimus and cyclosporine had no difference in allograft function one year after transplantation compared to those treated with cyclosporine and mycophenolate mofetil.

Sirolimus has well described anti-proliferative effects. The only known molecular target of sirolimus is the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase that regulates cell growth, proliferation, survival, protein synthesis, and gene transcription. mTor has emerged as a key enzyme regulating cellular responses to a variety of cell survival signals (Hay and Sonenberg, 2004; Sarbassov et al.,

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2005). Sirolimus binds to FKBP-12 and this complex inhibits the kinase activity of mTOR perhaps through effects on binding of raptor, a regulatory protein associated with activation of mTOR (Oshiro et al., 2004; Yonezawa et al., 2004; Sarbassov et al., 2006). Inhibition of mTOR mediated pathways regulating the responses to cell survival signals is known to result in increased apoptosis in a variety of experimental systems. It is possible that one mechanism of prolonged DGF following renal transplantation is increased apoptosis of renal tubular cells, slowing recovery from injury induced by ischemia and reperfusion.

The objective of this study was see if there is histologic evidence of increased apoptosis in renal transplant recipients with DGF treated with sirolimus based immunosuppression compared to those not receiving sirolimus. In this retrospective, case controlled analysis, renal allograft biopsies from these two patient groups were compared by staining for markers of apoptosis and by immunohistochemical assessment of pathways associated with regulation of apoptosis.

Materials and methods

Patients and study design

This is a retrospective, case controlled study of renal allograft biopsies from two groups of patients with DGF following deceased donor renal transplantation. Patients treated with a *de novo* sirolimus immunosuppressive protocol were compared to a historical control group treated with a calcineurin based protocol. The aim of this study was to assess these biopsies for histologic evidence of apoptosis using TUNEL staining as well as immunohistochemical assessment of selected pathways involved in the regulation of apoptosis and cell cycle progression. All patients in both groups had oligoanuric renal failure and were dialysis dependent. Since all patients had oligoanuric dialysis dependent renal failure, we feel, based on clinical indicators, that the degree of pre-transplant renal injury is comparable between the two groups although cold ischemia time is not available. The biopsies were obtained 7 to 10 days after transplantation as part of the usual clinical care of these recipients. Patients remained dialysis dependent at the time of biopsy. Archived renal biopsy material was used for this retrospective study. 10 patients with DGF transplanted using a de novo sirolimus based immunosuppressive regimen were studied. A control group of 15 recipients with DGF, matched for age, gender, HLA match, PRA, and donor demographics (Table 1), were selected from a group of patients transplanted using a calcineurin based protocol.

Immunosuppression

All Patients received induction with 1.5 mg/kg/d rabbit anti-thymocyte globulin (Thymoglobulin, Genzyme Corporation, Cambridge, MA, USA) for 7 to

10 days, or two 20 mg doses of basiliximab (Simulect, Novartis AG, Basel, Switzerland) on days 1 and 4. 500 mg doses of methylprednisolone were given on days 1-3 followed by tapering to 10 mg/d. Mycophenolate mofetil (Cellcept, Hoffman La-Roche, Nutley, NJ, USA) was started on day 1 at 1000mg twice a day and continued at this dose unless a dose reduction was needed for leucopenia or gastrointestinal side effects. Sirolimus (Rapamune, Wyeth, Madison, NJ, USA) was started on day 2 with a 15 mg loading dose followed by 10 mg/d for 2 days with subsequent adjustments to achieve a 24 hr trough of 10-15 ng/ml in the study group. For patients in the control group, tacrolimus (Prograf, Astellas Pharma Inc, Tokyo Japan) was started following the renal allograft biopsy at day 7 to 10 with a 12 hr trough goal of 8-12 ng/ml. Antimicrobial prophylaxis included trimethorim-sulfamethoxazole, clotrimazole troches, and oral ganciclovir.

Histology, Immunohistochemistry, and TUNEL assay

Renal allograft biopsies obtained during DGF were preserved in formalin and embedded in paraffin. Subsequently, for the purposes of this study, immunohistochemical stains and deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assays were done on slides cut from the stored blocks of paraffin embedded tissue. Hematoxylin and eosin (H&E) stains were done using conventional techniques.

Immunohistochemical stains for phospho-mTOR used a monoclonal antibody recognizing mTOR phosphorylated at Ser2448, (Cell Signaling Technology, Beverly, MA catalog #2976). Immunohistochemical staining for Bcl-2, an inhibitor of apoptosis, used a monoclonal antibody raised against amino acids 1-205 of Bcl-2 of human origin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, catalog #sc-7382), and staining for active caspase 3, an pro-apoptosis protease, used a polyclonal antibody detecting the cleaved p17 fragment of human caspase 3 (Chemicon International, Temecula, CA catalog #AB3623). Staining was also done for the podocyte marker WT-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, catalog #sc-846). All antibodies used for immunohistochemistry had been tested to work in paraffin-embedded human tissue.

DNA fragmentation was detected in apoptotic cells by TUNEL assay using immunoperoxidase staining techniques (ApopTag Plus Peroxidase In Situ Apoptosis Dectection Kit, Chemicon International, Temecula, CA).

The number of cells with positive staining in both the immunohistochemical stains and TUNEL assay were counted and expressed per 5 high power fields. The degree of apoptotic changes were further assessed for severity using a grading scheme in which grade 1 was defined as TUNEL positive tubular cells remaining in situ, grade 2 as TUNEL positive tubular cells with sloughing into the tubular lumen, and grade 3 as TUNEL positive tubular cells with disruption of tubular architecture (Fig. 1)

Statistical Analysis

Cell counts are expressed as the mean \pm SEM. Statistical analysis was performed using Spearman correlation coefficients, chi square, Fisher's exact test and Student's t tests as appropriate. This study was approved by the institutional review board of the University of Maryland School of Medicine.

Results

Demographics

Recipient and donor demographics are shown in Table 1. All patients in both groups underwent deceased donor renal transplants. Both recipient groups were similar in age and were predominantly male. Most recipients had diabetes or hypertension as their cause of ESRD. HLA mismatches and prior antibody sensitization were comparable in both groups. Sirolimus Table 1. Demographics.

	Sirolimus N=10	Control N=15	p Value
% DDT	100	100	n/a
Recipient age, years	45±5	54±3	NS
% male	60	80	NS
Cause of ESRD, % diabetes, hypertension	90	80	NS
HLA mismatch	3.7±0.6	4.3±0.3	NS
PRA % > 80	0	7	NS
Recipient race, % African American	100	47	0.008
Donor age, years	33±5	43±5	NS
Donor sex, % male	60	53	NS
Donor race, % Caucasian	80	73	NS



treated recipients were more likely to be African American. Donor demographics were also similar between the groups.

TUNEL and H&E Staining

The results of TUNEL staining in the initial day 7 to 10 protocol biopsies are shown in Table 2. TUNEL positive tubular epithelial cells, consistent with tubular epithelial cell apoptosis, were significantly increased in the sirolimus treated recipients compared to patients not receiving sirolimus, 333 ± 69 vs 6 ± 3 cells per 5 high power fields respectively (p<0.001). Comparing only African American recipients also resulted in significant differences between sirolimus treated patients and control patients (333±69 vs 10±5 cells per 5 high power fields respectively, p<0.001). Glomerular podocyte TUNEL positive staining was also significantly different between groups. 60% of sirolimus treated recipients showed diffuse TUNEL positive staining of glomerular podocytes (Fig. 2a). This compares to only 7% of tacrolimus recipients who showed significant TUNEL staining of glomerular podocytes (Fig. 2b). The identity of these cells was confirmed by positive staining with

Table 2. Biopsy results.

the podocyte marker WT-1. Glomerular TUNEL staining and WT-1 staining in adjacent sections from a sirolimus treated patient are shown in Figure 3.

By conventional light microscopy, using the grading system developed for this analysis, the sirolimus treated recipients had an increase in the degree of disruption of renal tubular architecture compared to control patients, 2.6 ± 0.2 vs 1.5 ± 0.2 respectively (Table 2). These changes were also significant when only African American recipients were compared (2.6 ± 0.2 vs 1.4 ± 0.2 , p<0.001). The number of TUNEL positive cells correlated with tubular architectural disruption (R = .96). The differences in tubular architectural disruption were also apparent in H&E stains. Representative H&E stains from both tacrolimus and sirolimus treated patients are shown in Figure 4.

Immunohistochemistry

Results of renal tubular cell immunohistochemical staining for activated mTOR, activated caspase 3, and activated Bcl 2 are shown in Table 2. Sirolimus treated recipients and control patients showed similar levels expression of these three markers. Despite evidence of

	Sirolimus N=10	Control N=15	p Value
TUNEL positive tubular cells / 5 hpf	334±69	6±3	< 0.001
Tubular architectural disruption	2.6±0.2	1.5±0.2	< 0.001
% diffuse TUNEL positive podocytes	60	7	P = 0.007
mTOR positive tubular cells / 5 hpf	122±47	151±42	NS
Caspase 3 positive tubular cells / 5 hpf	304±82	326±80	NS
Bcl 2 positive tubular	1541±197	1280±105	NS



Fig. 2. Diffuse podocyte TUNEL positive staining in a representative sirolimus treated patient (a). Lack of significant TUNEL staining in a representative tacrolimus patient is (b). Original objective magnification x 40.



Fig. 3. Adjacent cuts in a glomerulus from a sirolimus treated patient showing TUNEL staining (a) and WT-1 staining (b). Original objective magnification x 40.



Fig. 4. H&E stains in representative sections showing glomerular and tubular structure from sirolimus treated patients (a, b) and tacrolimus treated recipients (c, d). Original objective magnification x 40.

podocyte apoptosis in sirolimus treated recipients by TUNEL staining, there was no evidence of glomerular expression of activated mTOR, activated caspase 3, or activated Bcl 2 by immunohistochemistry (Fig. 5). Similarily, the glomeruli from control tacrolimus patients showed no expression of these markers.

Discussion

Inhibition of mTOR by sirolimus results in cell cycle arrest and can increase apoptosis in proliferating cell populations within the kidney (Lieberthal et al., 2001; Lui et al., 2006). It has been suggested that the delay in recovery of renal function after renal transplantation in patients treated with sirolimus based immunosuppression may be related to increased apoptosis of renal tubular epithelial cells. In experimental models of renal injury, ischemia and reperfusion has been shown to result in increased apoptosis of renal tubular epithelial cells (Daemen et al., 1999, 2002; Supavekin et al.,

2003). Similar findings have been observed in human renal allografts. Burns et al. (1998), comparing pre and post reperfusion biopsies of human renal allografts, found evidence of DNA fragmentation characteristic of apoptosis post-reperfusion. Castaneda et al. (2003) found increased apoptosis, primarily in tubular cells, in human renal allografts following reperfusion. mTOR could control apoptosis through effects on mediators of apoptosis acting through the Bcl-2 or caspase pathways. In animal models of ischemia/reperfusion injury, increased expression of Bcl-2 induced through gene transfer inhibits apoptosis (Cooke et al., 2005; Kienle et al., 2005). Similarly, failure of Bcl-2 up-regulation in proximal tubular epithelial cells in human kidney allografts has been associated with apoptosis and delayed graft function (Schwarz et al., 2002). Inhibition of the pro-apoptotic enzyme caspase 3, by small interfering RNA, decreases ischemia-reperfusion injury in animal models (Contreras et al., 2004).

In our study we found that transplant recipients with



DGF who received initial immunosuppression with sirolimus had a significantly increased amount of renal tubular cell apoptosis as evidenced by increased DNA fragmentation measured by TUNEL assay. This was compared to a group of patients with DGF who did not receive sirolimus. This increased renal tubular cell of apoptosis was correlated with histologic evidence of disruption of renal tubular architecture. Of note, the 'cast nephropathy' observed by others in sirolimus treated patients with DGF was not observed in our patients. These findings support the idea that renal tubular cell apoptosis in this clinical setting may be in part responsible for the reported increased incidence of DGF as well as the prolonged recovery from DGF.

The cellular pathways by which mTOR inhibition contributes to increased apoptosis in this situation are not clear. The regulation of cell survival signals by mTOR involves multiple interrelated pathways. We examined the tubular cell expression of activated caspase 3, an enzyme which promotes apoptosis and found that expression of the active form of caspase 3 was the same in both sirolimus treated and tacrolimus treated recipients with DGF. Regulation of apoptosis through the mTOR pathway is only partially understood and interaction with other regulatory pathways affecting caspase activity requires additional study. Similaily we looked at the expression of Bcl 2, an enzyme which promotes cell survival and decreases apoptosis and found no decrease in its expression induced by sirolimus. These findings suggest that the renal tubular cell apoptosis associated with sirolimus is not mediated by changes in expression of caspase 3 or Bcl 2. Enzyme activity was not, however, measured in this morphologic study.

Sirolimus has also been reported to exacerbate proteinuria in renal transplant recipients following conversion from calcineurin inhibitors to sirolimus based immunosuppression (Butani, 2004; Letavernier et al., 2005; Ruiz et al., 2006; Franco et al., 2007). This has been proposed to result from effects on proximal tubular cell uptake of filtered proteins (Coombes et al., 2005; Straathof-Galema et al., 2006). It has also been suggested that the hemodynamic effects of the withdrawal of calcineurins may have a role in the effect. Little is known about the effects of sirolimus on glomerular structure or function although the Akt-mTOR pathway has been suggested to affect mesangial cell proliferation and apoptosis. Izzedine et al. (2005) reported increased expression of vascular endothelial growth factor in the glomeruli of a patient who developed proteinuria and collapsing focal segmental glomerulosclerosis following conversion from cyclosporine to sirolimus eight years following transplant. Letavernier et al. (2007) suggested that high levels of sirolimus may induce *de novo* focal segmental glomerulosclerosis in renal transplant recipients and they noted changes in markers of podocyte differentiation. In experimental models, proteinuria has been associated with apoptosis of renal proximal tubular cells (Thomas et al., 1999; Tejera et al., 2004).

We observed in this study a substantial amount of podocyte apoptosis in the glomeruli of patients treated with sirolimus with 60 percent of sirolimus treated recipients having diffuse podocyte apoptosis. This was strikingly different from the findings in biopsies of patients not treated with sirolimus. These findings are of interest in light of the reports of exacerbation of proteinuria in patients converted to sirolimus. Our results suggest that in addition to effects on proximal tubular cell protein uptake there may also be a component of sirolimus-induced podocyte dysfunction or loss, as evidenced by the diffuse increase in podocyte apoptosis that we observed. We were not, however, able to measure urinary protein excretion in our study since these patients were oligoanuric. The functional results of this effect on podocytes are not clear from our current study and will require additional long term clinical evaluation.

Using immunohistochemistry, we were unable to demonstrate podocyte expression of activated mTOR. This suggests that the effect of sirolimus on podocytes may be independent of the inhibition of mTOR and may be evidence of sirolimus toxicity to podocytes. It is possible that ischemic tubules may have altered nonspecific antibody binding that could affect our results. Our findings require confirmation and additional study.

This study is exploratory in nature. The findings are limited by the retrospective design and small sample size. The finding that renal transplant recipients with DGF who are treated with sirolimus have increased renal tubular cell apoptosis does, however, suggest a mechanism by which sirolimus may delay the recovery from DGF. The finding of podocyte apoptosis also suggests a possible mechanism by which sirolimus may affect glomerular structure and function and give insight into the association of sirolimus with increased proteinuria in renal transplant recipients.

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