Original Article

Autophagy is involved in the protective effect of rapamycin on renal allograft injury in rats

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Abstract: Aims: Rapamycin has potential beneficial effects on interstitial fibrosis and inflammatory cell infiltration in various models of kidney injury. However, its role in renal allograft injury has not been well-defined. This study aimed to investigate whether rapamycin exerts protective effect on tubular atrophy and interstitial fibrosis in a rat model of kidney transplantation. Methods: Recipients were divided into three groups after kidney transplantation: isograft, allograft, and allograft treated with 0.8 mg/kg/d rapamycin. Grafts were harvested at 4 and 8 weeks post-transplantation, and renal function and histopathology were examined. Grafts were also evaluated by electron microscopy. The autophagy-associated molecules LC3-II, Beclin-1, and p62 were measured using western blot analyses, immunohistochemistry, and immunofluorescence staining. Results: Serum creatinine levels were increased significantly at 8 weeks after kidney transplantation. This increase was diminished in the rapamycin group compared with the allograft group (P < 0.05). Histopathology revealed less kidney damage and interstitial collagen deposition at 4 and 8 weeks after transplantation in the rapamycin group compared to the allograft group (P < 0.05). Expression levels of the autophagy markers LC3-II and Beclin-1 were higher, and p62 levels were lower, around tubules in the rapamycin group at 4 and 8 weeks compared to the allograft group (P < 0.05). Furthermore, immunofluorescence staining and electron microscopic analysis showed that rapamycin increased the LC3-II level and autophagic vacuoles, at 8 weeks compared to the allograft group (P < 0.05). Conclusions: Rapamycin may effectively reduce the progression of renal interstitial fibrosis and tubular atrophy in rats following kidney transplantation. These effects occur, at least in part, by increasing autophagy around the renal tubules.

Keywords: Rapamycin, interstitial fibrosis/tubular atrophy, kidney transplantation, autophagy

Introduction

Successful short-term allograft survival has been achieved by using immunosuppression drugs, especially cyclosporine A. In contrast, drug therapy has not improved long-term allograft survival significantly. Thus the focus of transplant clinicians has shifted toward improving the long-term outcomes. Interstitial fibrosis/tubular atrophy (IF/TA), once known as chronic allograft nephopathy, remains an important cause (40% to 60%) of renal allograft loss and is characterized by tubular atrophy, interstitial fibrosis, and glomerulopathy [1]. The development of IF/TA is progressive and irreversible, eventually giving rise to chronic renal dysfunction. However, Seron et al. demonstrated that renal allografts may develop chronic lesions despite stable and even optimal graft function [1].

Rapamycin, a mammalian target of rapamycin antagonist, originates from the metabolic products of Streptomyces hygroscopicus [2]. Rapamycin was later discovered to be an effective immunosuppressant and was introduced as an adjunct to calcineurin inhibitors to suppress acute rejection in kidney transplant recipients. Rapamycin is a macrolactone that does not inhibit calcineurin [3]. Thus, this novel immunosuppressive agent is expected to lack the nephrotoxicity of calcineurin inhibitors, such as fibrogenesis. Recently, reports about the antifibrotic effects of rapamycin emerged. Wang, et al. found that rapamycin attenuates renal interstitial fibrosis by reducing the number of fibroblasts and myofibroblasts in the renal interstitium in a model of obstructive nephropathy [4]. In several rat models of kidney injury and renal transplant patients, rapamycin dramatically decreased renal tubular damage, glo-
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merular hypertrophy, and mesangial fibrosis [5, 6]. Therefore, rapamycin might be beneficial in the treatment of IF/TA.

This study was designed to investigate the ability of rapamycin to diminish the progression of IF/TA in a rat model of kidney transplantation to explore the potential mechanism of this effect. The structural and functional changes of grafts, as well as autophagic activity, were evaluated.

Methods

Animals

Inbred male Fischer (F344, RT1\(^1\)) and Lewis (LEW RT1\(^1\)) rats weighing 250-300 g were purchased from Beijing Vital River Laboratory (Beijing, China). Rats had access to standard rat chow and water ad libitum. The animal study protocols were approved by the institutional Animal Care and Use Committee of Wenzhou Medical University, Wenzhou, China.

Rat renal transplant model

The left kidney from a Fischer or Lewis rat was transplanted into a Lewis rat orthotopically according to the modified technology described by Kamada [7], thereby generating Fischer-Lewis allografts and Lewis-Lewis isografts, respectively. Briefly, under anesthesia, the left kidney was perfused with chilled heparin sodium chloride solution (0-4°C). The recipient underwent a left native nephrectomy, an end-to-side anastomosis was performed, and the donor ureter was anastomosed to the dome of the bladder. After the transplant procedures was completed, all recipients were given cyclosporine 5 mg/kg/d for 10 days and then divided randomly into three groups: group 1, Lewis-to-Lewis, untreated control (isogeneic transplanted control group, n = 8); group 2, Fischer-to-Lewis, untreated control (allogeneic transplanted control group, n = 8); group 3, Fischer-to-Lewis allograft, rapamycin (Pfizer, New York, NY, USA) given intragastrically, 0.8 mg/kg/d (allogeneic transplanted (rapamycin) group, n = 8). The renal grafts and serum samples were harvested at 4 and 8 weeks post-transplantation. Only kidneys without apparent complications of grafting, such as hydronephrosis or pyelonephritis, were evaluated. Representative portions of the kidneys were snap frozen in liquid nitrogen and stored at -80°C for western blot analyses. Arterial blood was collected quickly into calcium-containing tubes. Other samples were fixed in 4% formalin or 3% phosphate buffered glutaraldehyde for histological and electron microscopic evaluations, respectively.

Renal function and histological evaluation

Serum samples were obtained to measure the level of serum creatinine (SCr) using an AU5800 automatic biochemistry analyzer (Beckman Counter., Brea, CA, USA). Kidney specimens were fixed in 4% formalin, embedded in paraffin, cut into 4 μm sections, and stained with hematoxylin-eosin or Masson’s trichrome. Interstitial fibrosis, tubular atrophy, glomerulopathy, vasculopathy, and other specific changes suggesting chronic rejection were graded from 0 for no to 3 for severe according to the Banff 2013 working classification of chronic allograft pathology [8].

To further analyze the degree of interstitial collagen deposition, Masson’s trichrome stained sections were graded (0, no staining; 1, < 25% staining; 2, 25 to 50% staining; 3, 50 to 75% staining; 4, 75 to 100% staining of the section) [9, 10]. Morphometric analyses of the kidney grafts were performed using a DM4000 B LED microscope system (Leica Microsystems, Wetzlar, Germany) and a DFC 42°C 5 M digital microscope camera (Leica Microsystems).

Immunohistochemistry

4 μm thick sections were deparaffinized in xylene and rehydrated through a sequential ethanol. Sections were then incubated in 3% H\(_2\)O\(_2\) for 10 min followed by primary antibodies for Beclin-1 (1:200), LC3B (1:200), or SQSTM1/p62 (1:300) (all from Abcam, Shanghai, China) overnight at 4°C. Subsequently, immunohistochemical staining was performed using solutions containing an avidin-biotin peroxidase complex and the Liquid DAB Substrate Kit (ZSGB-BIO, Beijing, China). All samples were semi-quantitatively or quantitatively assessed by two independent investigators in a blinded manner.

Western blot analyses

The expression levels of Beclin-1, LC3B, and SQSTM1/p62 in transplanted kidneys were
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measured using western blot analysis. Tissues were homogenized in lysis buffer and the protein concentrations determined by a Micro BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Proteins (20 μg) per specimen were separated by a SDS-PAGE, and blotted onto nitrocellulose membranes. Filters were incubated overnight at 4°C with antibodies (all from Abcam) specific for Beclin-1 (1:2000), LC3B (1:1000) or SQSTM1/p62 (1:1000). Anti-β-actin antibodies (Bioworld technology, Inc., Minnesota, USA) were used to detect actin as the loading control.

Immunofluorescence

Sections were dewaxed with xylene, rehydrated through a sequential ethanol gradient, and heated in a microwave oven with 0.1% sodium citrate buffer (pH 6.0) to retrieve antigenic determinants. Slides, blocked with goat serum for 20 min, were incubated with a primary antibody specific for LC3B (1:400, Abcam) at 4°C overnight, and then treated with a FITC-conjugated goat anti-rabbit IgG secondary antibody (1:400, Abcam). Samples were semi-quantitatively or quantitatively assessed by two independent investigators in a blinded manner.

Electron microscopy

Transplanted tissues were fixed at room temperature in 0.1 M sodium phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde and post-fixed in 2% osmium tetroxide for 1 h. These samples were then dehydrated in sequential ethanol and propylene oxide, embedded in epoxy resin, and cut into ultrathin sections. Tissue sections were counterstained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (Hitachi, Tokyo, Japan).

Statistical analysis

The results are expressed as means ± standard deviation (SD). One-way analysis of variance or a two-tailed t-test was used to determine differences between groups. A P-value < 0.05 was considered statistically significant. All analyses were performed using SPSS 19.0 software (IBM, Chicago, IL, USA).

Results

Establishment of kidney transplantation in rats

A rat model of kidney transplantation was established successfully at 4 and 8 weeks, including modeling of IF/TA at 8 weeks post-transplantation, rather than 4 weeks. Two transplant recipients in the rapamycin group died 4 and 8 days after transplantation because of incomplete intestinal obstruction and infection. In addition, one rat in the allograft group and one in the isograft group died on days 4 and 6, respectively, due to incomplete intestinal obstruction.
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Effect of rapamycin on renal function and histology

The evaluation of SCr levels and renal ultrastructure was performed in all rat groups. No significant differences of SCr levels were observed among the three groups at 4 weeks post-transplantation ($P > 0.05$). However, the Banff scores were lower in the rapamycin group and isograft groups than in the allograft group ($P < 0.05$). At 8 weeks, SCr levels and Banff scores were significantly higher in the allograft group compared with those in the rapamycin and isograft groups ($P < 0.05$). Except for the isograft group, the SCr levels and Banff scores increased from 4 to 8 weeks (Figures 1A and 2A).

Although no obvious signs of acute rejection were seen in any of the three groups, we did observe time-dependent histopathological changes. At 4 weeks, the allograft and rapamycin groups, but not the isograft group, showed slight pathological changes, such as the infiltration of a wide variety of lymphocytes or mononuclear cells, and the appearance of casts and tubular dilation. In addition, mild IF/TA was observed in several rats. At 8 weeks post-transplantation, the SCr levels and Banff scores increased significantly in all groups (Figures 1A and 2A).
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plantation, typical IF/TA lesions were noted in the allograft and rapamycin groups, including moderate interstitial fibrosis, tubular atrophy, glomerulosclerosis, and arteriosclerosis. The allograft group exhibited more severe histopathological lesions than the rapamycin group (Figure 1B).

To assess the degree of interstitial fibrosis, Masson’s trichromestain was applied to evaluate the deposition of total collagen in the transplanted kidneys. Collagen was present mainly among the renal interstitium and increased significantly as time progressed except in the isograft group. The deposition of interstitial collagen was reduced significantly by rapamycin, especially at 8 weeks post-transplantation (Figure 2B).

These results demonstrated that rapamycin at this dose exerted protective effects on kidney function and structure and could to some extent alleviate renal interstitial fibrosis.

Western blotting revealed that, compared with the allograft and isograft groups, the expression of LC3-II and Beclin-1 was higher in the rapamycin group at both 4 and 8 weeks post-transplantation ($P < 0.05$) (Figures 3 and 4). This suggested that the autophagic activity was higher in the rapamycin group than in the allograft and isograft groups. Interestingly, autophagy occurred mainly around renal tubules. Although the majority of renal tubules were damaged or even vanished by 8 weeks, the retaining tubules exhibited a high degree of autophagic activity.

Evaluation of the autophagic response with immunofluorescence and electron microscopy

No individual assay is sufficient to examine autophagy. Thus, to confirm the autophagic response, we also assessed tissues, using immunofluorescence and electron microscopy. Immunofluorescence analyses confirmed the

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**Figure 3.** Immunohistochemistry analyses for LC3-II and Beclin-1 expression. A. Semi-quantitative assessment of LC3-II expression from the isograft, allograft, and rapamycin groups 4 and 8 weeks post-transplantation. Original magnification × 200. B. Semi-quantitative assessment of Beclin-1 expression from the isograft, allograft, and rapamycin groups 4 and 8 weeks post-transplantation. Original magnification × 200.

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**Autophagy in transplanted kidneys**

Autophagy plays a pivotal role in the homeostasis and viability of renal cells, and might be a mechanism for renal cells to cope with stress. Thus to better understand the occurrence of autophagy following kidney transplantation, the autophagy markers LC3-II and Beclin-1, and the autophagic flux marker SQSTM1/p62, were measured. Immunohistochemical staining demonstrated that LC3-II and Beclin-1 levels were increased significantly and p62 was decreased in the rapamycin group compared with the isograft and allograft groups both at 4 and 8 weeks post-transplantation ($P < 0.05$) (Figures 3 and 4). This suggested that the autophagic activity was higher in the rapamycin group than in the allograft and isograft groups.
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increased expression of LC3-II in the rapamycin group compared to the allograft and isograft groups at 8 weeks ($P < 0.05$) (Figure 6A). Renal tissue examined by electron microscopy, especially renal tubular epithelial cells (RTECs), showed that rapamycin group had numerous autophagic vacuoles including autophagosomes and autolysosomes compared with the isograft and allograft groups 8 weeks post-transplantation (Figure 6B).

**Discussion**

In the present study, we evaluated the effect of rapamycin on the progression of IF/TA. In rats that underwent kidney transplantation, rapamycin exerted beneficial effects as shown by reduced SCr levels and Banff grading scores. Although kidney injuries caused by IF/TA could not be eliminated, graft function was always better and the parenchymal damages less serious at 4 weeks compared to 8 weeks post-transplantation in all three groups. Importantly, rats treated with rapamycin exhibited superior kidney function, a lower degree of interstitial fibrosis and tubular atrophy (the prominent histological feature of IF/TA), and decreased infiltration of inflammatory cells compared with the allograft group both at 4 and 8 weeks. Consistent with previous reports, our results suggest that rapamycin may be beneficial in the treatment of IF/TA.

The current study investigated the potential role of autophagy in the progression of IF/TA. Enhanced autophagy was observed in the rapamycin group as evidenced by the correlation between the increased expression of autophagic regulators, including LC3-II and Beclin-1, and the decreased level of p62, and autophagic activity. However, autophagy markers did not significantly change in a time-dependent manner either in the rapamycin or allograft groups. There are two major reasons for this
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lack of a time-dependent effect. First, there was a progressive deterioration of renal parenchyma, including tubules and glomeruli, that may down-regulate or eliminate autophagic activity. Second, a steady-state of autophagy may become established over time.

Immunofluorescence and electron microscopy provided reliable evidence that autophagy activity was higher in the rapamycin group than in the allograft and isograft groups. Because rapamycin has renal protective effect, we conclude that this drug may delay the progression of IF/TA by activating autophagy. Furthermore, we observed that autophagy occurred mainly around renal tubules in transplanted kidneys, rather than in the glomerulus. This indicated that rapamycin may defend RTECs against noxious stimuli and damage via up-regulated autophagy thereby protecting grafts from structural and functional deterioration.

In the context of kidney transplantation, grafts will encounter continuously both acute and chronic stresses, including ischemia and reperfusion injuries, immunological assaults and infections [11]. These stresses will lead to acute and chronic rejection, tissue remodeling, function deterioration and ultimately result in allograft [12, 13]. The renal tubular epithelium plays a critical role in the development of acute and chronic structural deterioration of the kidney, particularly through the generation of inflammatory and profibrogenic cytokines [14, 15]. Our results also suggested that the remaining tubules, with the help of autophagy, are pivotal to grafts survival. Indeed, autophagy is a protective mechanism for cell survival [16]. Research using in vitro and murine models of cisplatin and ischemia reperfusion-induced RTEC injury demonstrated that inhibiting autophagy, through pharmacologic means, genetic knockdown, or RNA interference-induced decreases of key autophagy genes, can exacerbate damages in an AKI model [17-19]. Yamahara et al. reported that autophagy in renal tubular cells was stimulated by proteinuria, and was important for preventing proteinuria-induced apoptosis [20]. Conversely, excess calorie uptake, for example in high-fat diet, restrains autophagy in renal tubular cells, resulting in higher susceptibility of tubular injury from proteinuria.

Glomerular visceral epithelial cells, also known as podocytes, are specialized epithelial cells that cover the outer layer of the glomerular basement membrane. These cells form the final barrier to protein loss and thus podocyte injury is typically associated with proteinuria. Data from Hartleben et al. demonstrated that proteinuria was dramatically worsened by treatment with doxorubicin in mice with podocyte-specific knockout of autophagy protein 5 [21]. This suggested that autophagy was essential to defend the kidney from glomerular diseases. Other research has shown the protective role of autophagy on polycystic kidney disease and diabetic nephropathy [22].

Some studies have indicated the importance of autophagy in glomerular podocytes [21, 23, 24]. A lack of autophagy can contribute to slowing the progressive loss of podocytes and glomerulosclerosis in aged mice [21]. Nevertheless, in our transplanted model, glomeruli, including glomerular podocytes, showed lower autophagy activity relative to renal tubules. This indicated that renal tubules, rather than glomeruli, through the induction of autophagy, may play a pivotal role in IF/TA.
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the protective effect of autophagy on RTECs, we also observed less fibrogenesis in the interstitium when autophagy was induced. Based on this observation, the increased level of autophagy by rapamycin may have anti-fibrogenic activity, although the precise mechanism remains to be investigated.

The pathogenesis of IF/TA is still largely unclear although a considerable number of stresses, both immune and non-immune, may contribute to its progression. The principal biological activators of autophagy are starvation and inflammatory mediators, which are also key stressors leading to IF/TA [16]. Thus, autophagy may play a role in the progression of IF/TA. Autophagy is an evolutionally conserved catabolic process, and is the major protein degradation system. Autophagy is pivotal to the maintenance of intercellular homeostasis by eliminating bulky cellular constituents [25, 26]. This interesting role of “self-eating” means that autophagy can break down harmful components and is thus regarded as a self-protective mechanism.

Autophagy is involved in a broad spectrum of diseases, particularly oncology- and neurology-related disorders. Nevertheless, research focusing on the relationship between autophagy and kidney diseases has been limited. To date, the majority of published papers in kidney diseases have investigated potential mechanism of acute kidney injury (AKI). Results from rat models of AKI indicate that autophagy protects the renal parenchyma from various stresses induced by renal ischemia-reperfusion, nephrotoxicity caused by cisplatin and sepsis, and kidney transplantation [17, 27, 28]. However, there is comparatively little research investigating the role of autophagy on kidney injury in chronic kidney diseases such as IF/TA.

In summary, our data demonstrated that rapamycin induced autophagy in allografts, and that this activity had a significant negative correlation with characteristic pathologic features of IF/TA. Therefore, the autophagy signaling pathway may be involved in the pathogenesis of IF/TA. Based on our data and other studies, the possible molecular mechanism of rapamycin’s protective effect in renal transplantation might involve activation of the autophagy signaling pathway and reduced infiltration of inflammatory cells leading to a protection of renal tubules from atrophy and degeneration, and decreased collagen deposition in the renal interstitium. However, further researches, including studied with autophagy inhibitors and different doses or concentrations of rapamycin, will be required to clearly identify the role of autophagy-mediated processes in the pathogenesis of IF/TA.

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Disclosure of conflict of interest
None.

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References
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[12] El-Zoghby ZM, Stegall MD, Lager DJ, Kremers W, Chien CT, Shyue SK and Lai MK. Bcl-xL aug...


[27] Chien CT, Shyue SK and Lai MK. Bcl-xL augmentation potentially reduces ischemia/reperfusion induced proximal and distal tubular apoptosis and autophagy. Transplantation 2007; 84: 1183-1190.