Decreased expression of sirtuin 3 protein correlates with early stage chronic renal allograft dysfunction in a rat kidney model

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Abstract. Chronic renal allograft dysfunction (CRAD) is the primary factor affecting the long-term survival of patients who have undergone renal transplantation. Oxidative stress and inflammation serve an important role in the pathological damage caused by CRAD in the early post-transplantation phase. Previous studies have demonstrated that sirtuin 3 (sirt3) protects cells from oxidative stress and inflammation. A model of renal orthotopic transplantation was established in the current study and kidney samples were harvested from the rats 12 weeks following surgery. Compared with the control groups, there were significantly increased levels of serum creatinine, blood urea nitrogen and 24 h urinary protein in the allograft group (P<0.05). Pathological examinations indicated mononuclear cell infiltration and intimal proliferation in the allograft group, which had a higher Banff score compared with the control groups. There were increased levels of malondialdehyde, decreased sirt3 protein expression and decreased superoxide dismutase enzyme activity in the allograft group compared with the control groups (P<0.05). In addition, there was a negative correlation between the expression of sirt3 and 24 h urinary protein excretion, serum creatinine levels, tubulointerstitial mononuclear cell infiltration, smooth muscle cell migration in the vascular wall and Banff scores. Thus, sirt3 may serve an important protective role in the early stage of CRAD.

Introduction

Kidney transplantation is a method of treating patients with end stage renal disease. The development of immunosuppressive treatments and improvements in transplant surgery have led to a decrease in acute rejection of transplanted kidneys in the early stage following kidney transplantation, and the 1-year survival rate is now ~90% (1). However, maintaining long-term allograft function remains challenging and the 10-year survival rate is only 68% in living donor kidney allograft recipients (1). It has been demonstrated that chronic renal allograft dysfunction (CRAD) is the primary factor affecting the long-term survival of patients receiving renal allografts. Early pathological changes in CRAD include inflammatory cell infiltration into tubulointerstitial spaces, and smooth muscle cell (SMC) proliferation and migration (2). Immune and non-immune factors contribute to the pathogenesis of CRAD, including renal ischemia-reperfusion (I/R) injury, which inevitably occurs following kidney transplantation and may cause graft dysfunction. This determines the short- and long-term outcomes of transplant recipients (3). Oxidative stress serves a role in the pathophysiology of renal injury in I/R. During reperfusion, cellular metabolism increases rapidly, resulting in the increased generation of reactive oxygen species (ROS), cytokines and chemokines, which promote inflammatory cell infiltration and lead to tissue damage and graft dysfunction (4).

Sirtuin 3 (sirt3) is involved in the regulation of antioxidant pathways. Sirt3 is a member of the sirtuin protein family and is a nicotinamide adenine dinucleotide (NAD\(^+\)) dependent protein deacetylase. It is highly expressed in well-oxygenated tissues, including the brain, muscles, heart, kidneys and adipose tissue (5). Sirt3 is primarily localized in the mitochondrial matrix, and its overexpression increases the oxidative capacity of mitochondria and causes a concurrent decrease in ROS levels (6). Increased expression of sirt3 protein inhibits the nuclear factor-xB (NF-xB) dependent transcriptional activity of inflammatory genes, reduces malondialdehyde (MDA) levels and increases superoxide dismutase (SOD) activity. Subsequently, overexpression of SIRT3 increases the oxidative capacity of mitochondria and produces a parallel decrease in ROS levels (7). This may be the mechanism by which sirt3 exerts antioxidant and anti-inflammatory effects in proximal tubular cells (7). However, the role of sirt3 in CRAD remains unknown. Thus, the current study investigated the expression and role of sirt3 in the early stage of CRAD in a standardized rat model.

Materials and methods

Animals. A total of 60 naive inbred male Fischer (F344) (n=30) and Lewis (Lew) rats (n=30) weighing 150-220 g,
aged 8-12-week-old were purchased from Charles River Laboratories, Inc. (Beijing, China). The rats were kept in a specific pathogen free laboratory at the Animal Experimental Center of the Southern Medical University (Guangzhou, China). The rats were fed a standard rat chow diet, had free access to water, were housed under standard conditions with a controlled temperature (24±2°C), humidity (40-60%) and a 12-h light/dark cycle. The rats were acclimatized for 1 week prior to the experiment. The current study was approved by the Committee on the Ethics of Animal Experiments of the Southern Medical University (permission no. 00119486) and was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (8).

**Study design.** Kidneys from Fischer (F344) rats were orthotopically transplanted into Lewis (LEW) rats. The Fischer-Lewis model has become the most intensively studied and widely accepted animal model of chronic renal allograft rejection because of its similarity to the progress of events following human renal transplantation, thus the Fischer-Lewis model was established in the present study (9). Rats were split into three groups as follows: i) A Lew control group consisting of uninephrectomized male Lew rats (n=10); ii) a F344 control group consisting of uninephrectomized male F344 rats (n=10); and iii) an allograft group consisting of Lew rats receiving transplants (n=10).

**Uninephrectomy surgery.** Surgery was performed under general anesthesia with 3% sodium pentobarbital (30 mg/kg; Hangzhou Zhongmei Huadong Pharmaceutical Co., Ltd., Hangzhou, China) on the F344 rats and the LEW rats. A half-inch incision was made on the left flank portion of abdomen and the kidney was pulled out of the abdomen by holding the perirenal fat at the lower pole with blunt forceps, which separated the kidney from the surrounding fat and supra renal gland. Furthermore, ligation of the renal artery and vein and ureter ~0.5 cm below the level of hilum with a non-absorbable surgical suturing thread was performed and the kidney was snap resected. The uninephrectomized rats received ceftriaxone via i.p. injection for 3 days.

**Kidney transplantation.** A total of 15 male Fischer (F344) weighing 150-220 g, aged 8-12-week-old were used for this procedure. A surgical ASX-2 microscope was purchased from Shanghai Anxin Optical Instrument Manufacture Co., Ltd. (Shanghai, China). Orthotopic kidney transplantation was performed as previously described (9). Surgery was performed under general anesthesia with 3% sodium pentobarbital (30 mg/kg) administered via intraperitoneal (i.p.) injection. The left kidney from the recipient Lew rat was removed and the left renal vessels were clamped. The left donor kidney from a F344 rat was then removed, cooled and positioned orthotopically in the recipient Lew rat. The renal arteries, veins and ureters of the donor and recipient were then anastomosed end-to-end with 10-0 prolene (Ningbo Lingqiao Biological Technology, Ningbo, China). No ureteral stents were used. A low dose of cyclosporine A (1.5 mg/kg/day; Novartis International AG, Basel, Switzerland) was administered for 10 days following transplantation to suppress acute rejection. To prevent infection, recipients received ceftriaxone (Rocephin; 20 mg/kg/day) via i.p. injection for 3 days. The right native kidney of each recipient was removed 10 days following surgery. Rats exhibiting overt signs of unsuccessful surgeries were excluded from the current study. Subsequently there were 10 rats per group following exclusion.

**Tissue harvesting.** All rats were anesthetized with i.p. injection of 60 mg/kg sodium pentobarbital and subjected to cardiac exsanguination 12 weeks following surgery. Kidneys were harvested from rats in all groups. However, only kidneys with no visible grafting complications, including pyelonephritis or hydrenephrosis, were selected for subsequent experimentation. The kidney of each rat was cut into two sections: One section was stored at -80°C in liquid nitrogen for western blot analysis. Rat serum samples (from 2 ml blood) were used for the evaluation of blood urea nitrogen, as well as creatinine with an automatic biochemical analyzer (AU5400; Olympus, Tokyo, Japan). Urine protein excretion was measured via an enzymatic kinetic method using commercial kits purchased from Nanjing Jiancheng Biological Engineering Research Institute (Nanjing, China) and 1 ml 24-h urine samples that were taken prior to sacrifice. The second part of the kidney was fixed in 4% formalin for the histopathological examination and immunohistochemistry.

**Renal histopathology.** Kidney tissues were fixed in 4% paraformaldehyde at room temperature for 24 h and embedded in paraffin. Paraffin sections with a thickness of ~2 µm were stained with hematoxylin and eosin or periodic acid-Schiff (PAS) at room temperature for 30 min to assess the extent of renal pathological changes and sections were observed with a light microscope. There were ≥100 glomeruli counted in each kidney section. Using the Banff 07 Classification (10), interstitial cellular infiltration, tubulopathy/intertstitial fibrosis, glomerulopathy and arterial intimal fibroplasia were scored on a scale from 0 to 3+, and the sum of these scores (range, 0-12+) was calculated for each sample.

**Immunohistochemistry.** Immunohistochemistry was used to detect the expression of sirt3 protein in the renal allograft tissue. Antigens were retrieved using the citric acid buffer (pH 6.0) microwave antigen retrieval method and detected via PV-9001 staining (Golden Bridge Biotechnology Co., Beijing, China). Tissues were boiled for 15 min at a temperature ranging 95-100°C and staining with PV-9001 was performed at room temperature for 60 min. The paraffin sections 4 µm thick were hydrated in an alcohol gradient (75, 85, 95, and 100% at 37°C for 3 min per concentration) and 3% hydrogen peroxide was used to block the endogenous peroxidase. Sections were then incubated with rabbit polyclonal anti-sirt3 antibodies (cat. no. 2627, 1:100; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C. Sections were washed with PBS, incubated with the goat anti-rabbit secondary antibody (cat. no. PV-9001, diluted 1:100; Golden Bridge Biotechnology Co., Ltd., Beijing, China) for 20 min at room temperature and washed a further three times with PBS. Sections were then incubated in a 3,3’-diaminobenzidine chromogen solution (OriGene Technologies, Inc., Rockville, MD, USA) and re-stained with hematoxylin at 37°C for 1 min. The intensity of
positive staining for sirt3 was quantified using Image-Pro Plus software version 7.0.1.658, Media Cybernetics, Inc. (Rockville, MD, USA).

Western blot analysis. Kidney tissues stored in the -80°C freezer were homogenized on ice in a lysis buffer consisting of 0.1 mol/l Tris buffer (pH 7.4), 0.1 mmol/l EDTA, 1 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulfonyl fluoride, a protease inhibitor cocktail (Roche Applied Science, Madison, WI, USA) and a protein phosphatase inhibitor. The protein concentrations of the samples were measured using a BCA Protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Protein samples (50 µg/lane) were loaded on to a 10% SDS gel and SDS PAGE was performed. Subsequently, samples were transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA), which were then blocked with 5% bovine serum albumin (cat. no. B2064; Sigma-Aldrich; Merck KGaA) in TBST for 1 h at 4°C. The membranes were then washed three times with TBST and incubated with anti-sirt3 antibodies (cat. no. 2627, 1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C. Membranes were washed and then incubated with a goat anti-rabbit IgG, horseradish peroxidase conjugate secondary antibody (cat. no. CW0156S, 1:5,000; CWBiotech, Beijing, China) for 1 h at room temperature. The target bands were observed using the SuperSignal West Pico enhanced chemiluminescent substrate (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All analyses were repeated in triplicate. Protein band intensities were quantified using Image J software (version 1.46, National Institutes of Health, Bethesda, MD, USA).

Measurement of SOD activity and MDA levels. The SOD and MDA levels in the renal tissues were assessed using commercial Total Superoxide Dismutase and Malondialdehyde assay kits. (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s protocol. SOD activity was detected using a xanthine oxidase-based technique and MDA content was assayed using the thiobarbituric acid method.

Statistical analysis. Statistical analyses were performed using SPSS 19.0 for Windows (IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard error of the mean (SE). A one-way analysis of variance followed by the LSD test was used to determine the significance of differences in multiple-group comparisons. Spearman’s rank correlation analysis was used to assess the association between different factors in the groups. P<0.05 was determined to indicate statistically significant difference.

Results

Renal functional changes. Orthotopic kidney transplantation in rats had been successfully established (Fig. 1) The levels of serum creatinine, blood urea nitrogen and 24 h urinary protein excretion were significantly increased in the allograft group compared with the F344 and Lew rat controls (P<0.05; Table I).

Histological changes. Kidney sections from the F344 and Lew control groups did not exhibit marked histological changes or SMC migration. However, kidney sections from the allograft group exhibited interstitial mononuclear cell infiltration, intimal proliferation and SMC migration (Fig. 2). The Banff scores of the allograft group were significantly
higher compared with those of the two control groups (P<0.05; Table I). Early pathology change of CRAD was indicated by infiltration of mononuclear cells during tubulointerstitial inflammation and the proliferation and migration of smooth muscular cells (SMCs). The late change was observed by sclerosis of renal blood vessel and tubulointerstitial fibrosis. These findings suggested that after 12 weeks, kidney sections from the allograft group exhibited interstitial mononuclear cell infiltration. The difference in interstitial fibrosis among groups was not significant, indicating that a rat kidney transplantation model at the early stage of CRAD was successfully established.

**SOD and MDA levels in renal tissues.** SOD activity was decreased while the levels of MDA were increased in the renal tissues in the allograft group compared with the F344 and Lew controls (P<0.05; Table II). These results suggest that oxidative stress was present in the kidneys at the early stage of CRAD in the model established in the current study.

**Sirt3 expression.** Immunohistochemical analysis demonstrated that sirt3 was strongly expressed in the cytoplasm of tubular epithelia cells and the renal arterioles of the kidneys from control rats. However, the expression of sirt3 was significantly decreased in the kidneys from the allograft group (P<0.05; Fig. 3). Western blotting also demonstrated that the expression of sirt3 was significantly decreased in the allograft group compared with the F344 and Lew controls (P<0.05; Fig. 4).

**Correlation analysis.** Spearman's rank correlation analysis identified that there were negative correlations between the expression of sirt3 and serum creatinine levels (r=-0.656; P<0.05; Fig. 5A), 24 h urinary protein excretion (r=-0.925; P=0.002; Fig. 5B), tubulointerstitial mononuclear cell infiltration (r=-0.564; P<0.05; Fig. 5C), the number of SMCs in the vascular wall (r=-0.669; Fig. 5E).
P<0.05; Fig. 5D) and the sum of BANFF scores in allografts (r=-0.637, P<0.05; Fig. 5E).

Discussion

The current study determined the relevance of the expression of sirt3 in the pathogenesis of CRAD in a rat model. Sirt3 expression was significantly decreased in rats with CRAD at week 12. The present study demonstrated that the early changes in the pathology of rats with CRAD were tubulointerstitial mononuclear cell infiltration and SMC proliferation and migration. The decreased expression of sirt3 was correlated with the pathological hallmarks of CRAD, including urinary protein excretion, serum creatinine levels, tubulointerstitial mononuclear cell infiltration and SMC migration in the vascular wall. These results suggest that sirt3 may serve a role as an endogenous protective factor in the early stage of CRAD.

CRAD is the primary factor affecting the long-term survival of patients receiving renal allografts (1). Transplanted kidneys are prone to oxidative stress-mediated injury caused by I/R, which generates large amounts of ROS (11). Reperfusion injury and reactive oxygen compounds serve an important role in the pathophysiology of acute allograft rejection and kidney function in the early post-transplantation phase but also affect long-term kidney function (11). Levels of oxidative stress are increased in patients who have undergone transplantation, suggesting that supplementary antioxidants may be beneficial. However, the molecular mechanisms of oxidative stress in CRAD remain unclear and further studies are required.

Oxidative stress promotes inflammation by activating NF-κB, which may then aggravate oxidative stress-mediated injury. Oxidative stress and inflammation cause endothelial injury and arteriosclerosis, which are histopathological characteristics of CRAD (12). MDA is the product of lipid peroxidation inside cells and may be used as a marker to detect the level of oxidative stress occurring in an organism (13). SOD is an enzyme that removes free radicals, leading to a reduction in the production of lipid peroxides, thereby protecting cells and tissue (14). The current study investigated oxidative stress in the early stage of CRAD by establishing...
a rat model of the condition 12 weeks post-transplantation. SOD levels were significantly decreased while the levels of MDA were significantly increased in the renal tissues of rats in the allograft group compared with F344 and Lew control rats (P<0.05). These results are consistent with those of previous studies, which demonstrated that the levels of products from oxidizing proteins, including MDA, carbonylated proteins and 8-hydroxy-2′-deoxyguanosine are increased and glutathione levels are decreased 1 year following transplantation. These changes are also associated with atherosclerosis and high levels of serum creatinine (15,16). It has also been demonstrated that ROS are important pro-fibrogenic cytokines in chronic allograft nephropathy and oxidative stress is increased in the presence of interstitial fibrosis and tubular atrophy, which generally precede chronic allograft failure (17). A previous study conducted on 40 patients who had received kidney transplants demonstrated that increased MDA levels 1 day following kidney transplantation may be an early prognostic indicator of delayed graft function and increased levels on day 7 may be a useful predictor of 1-year graft function (11). Increased levels of MDA reflecting lipid peroxidation have been detected in rat models of chronic allograft tubular atrophy/interstitial fibrosis (18). In the current study, an increase in MDA levels and a decrease in SOD activity were detected in the renal tissues of rats in the allograft group, indicating that transplantation caused oxidative damage in the transplanted kidneys of rats in the early stage of CRAD. This is consistent with the results of the aforementioned previous studies.

To the best of our knowledge, there have been no previous studies focusing on the role served by sirt3 in the early stage of CRAD in rat models. Sirt3 is a human NAD+-dependent protein deacetylase in humans that belongs to the sirtuin family, which contains 7 proteins identified in mammals (sirt1, sirt2, sirt3, sirt4, sirt5, sirt6, sirt7) (19). Sirt3 localizes primarily in the mitochondria, regulating respiratory chain function, the tricarboxylic acid (TCA) cycle and fatty acid β oxidation. It also exhibits antioxidant activity (20). The ability of sirt3 to protect cells from oxidative damage has been demonstrated previously, supporting the idea that sirt3 serves an important role in regulating ROS homeostasis (21). Sirt3 also reduces cellular ROS levels via a mechanism dependent on the activation of superoxide dismutase 2, peroxiredoxins 3 and 5, glutathione peroxidases and the TCA cycle enzyme isocitrate dehydrogenase 2 (22,23). Sirt3 is highly expressed in renal tissues (24,25) and the sirt3 protein has been widely studied in relation to kidney disease. In a murine model of cisplatin-induced acute kidney injury, it was demonstrated that
increased oxidative stress and mitochondrial damage were associated with reduced levels of sirt3. Following treatment with the antioxidant agent acetyl-l-carnitine, which enhanced the expression and activity of sirt3, renal function was improved and the extent of tubular injuries were decreased in mice (5). The overexpression of sirt3 inhibits the NF-κB signaling pathways, decreases the phosphorylation of ERK1/2 and p38, decreases the expression of inflammatory cytokines and reduces ROS levels in an experimental model of free fatty acid-mediated tubulointerstitial inflammation. This may be the molecular mechanism by which sirt3 induces antioxidant and anti-inflammatory effects (25). The results from an in vitro experiment demonstrated that increasing the expression of sirt3 may inhibit the mesangial hypertrophy induced by high glucose levels, primarily by adenosine monophosphate-activated protein kinase-mammalian target of rapamycin signaling pathways (26). The current study demonstrated that the expression of sirt3 was increased in the cytoplasm of tubular epithelia cells and in the renal arterioles of rats in the F344 and Lew control groups, whereas the expression of sirt3 was decreased in the allograft group. The mean optical density values for sirt3 in the allograft group were lower than those of the F344 and Lew control groups (P<0.05). Western blotting also indicated a decrease in the expression of sirt3 protein in the allograft group (P<0.05). There was a negative correlation between the expression of sirt3 and mononuclear cell infiltration and SMC migration in the vascular wall, indicating that decreased expression of sirt3 protein serves a significant role at the early development stage of CRAD pathogenesis. The signaling mechanisms related to oxidative stress in rat models of CRAD and the role of sirt3 in transplanted kidneys remain unclear. Sirt3 has antioxidant and anti-inflammatory effects and the results of the current study suggest that the sirt3 related signaling pathway may serve an important role in mediating the important early effects of CRAD, including mononuclear cell infiltration and vascular SMC proliferation and migration.

The current study has some limitations. The experimental grouping design is based on our previous study as follows: The allograft group consisted of Lew rats orthotopically transplanted with kidneys from F344 rats and the control groups consisted of uninephrectomized male Lew and F334 rats (9,27,28). However, the current study lacked a Lew-Lew syngeneic transplant model should be used as a control in future studies. In addition, the pathological changes and renal function following the up-or down-regulation of sirt3 expression were not investigated due to limits of the experimental conditions. Further studies are required to investigate the pathogenic mechanisms that lead to changes in the expression of sirt3 in renal tissue during chronic allograft dysfunction.

In conclusion, to the best of our knowledge, this is the first study measuring sirt3 expression in a rat kidney transplantation model at the early stage of CRAD. The results indicate that sirt3 may be involved in pathogenic processes at the early stage of CRAD. Thus, increasing the expression of sirt3 at the early stage may be a novel therapeutic approach for attenuating the development of CRAD.

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Competing interests

The authors declare that they have no competing interests.

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