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Effect of sirolimus on arteriosclerosis induced by advanced glycation end products via inhibition of the ILK/mTOR pathway in kidney transplantation recipients

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ABSTRACT

To investigate the effect and related mechanism of sirolimus (SRL) in arteriosclerosis(AS) induced by advanced glycation end products (AGEs) in kidney transplantation recipients (KTRs). Human kidney tissues from KTRs before and after treatment with SRL were assessed by hematoxylin-eosin and immunohistochemical staining. Rat vascular smooth muscle cells (VSMCs) were treated with AGEs and/or SRL. The expressions of α-smooth muscle actin (α-SMA), osteopontin (OPN), actinin-associated LIM protein (ALP), proliferating cell nuclear antigen (PCNA), integrin-linked kinase (ILK) and the mTOR signaling pathway proteins were examined using western blot assay. Cytosolic calcium present in VSMCs was also measured by the calcium assay kit and von Kossa staining assay. The expression of α-SMA was remarkably higher while OPN expression was significantly lower in recipients with AS after they were administered SRL. Rat VSMCs treated with AGEs exhibited significantly lower expression of α-SMA and overexpression of OPN, ALP and PCNA than the other groups. In contrast, the expression of α-SMA was significantly higher while the expression of OPN, ALP and PCNA was significantly lower in VSMCs treated with both AGEs and SRL. Moreover, the ILK/mTOR signaling pathway was activated in rat VSMCs treated with AGEs, while treatment with AGEs and SRL led to significant inhibition of the ILK/mTOR signaling pathway.

AGEs play a critical role in the development and progression of AS after kidney transplantation, but SRL can reverse these effects and therefore slow down the development of AS through inhibition of the ILK/mTOR signaling pathway.

1. Introduction

Kidney transplantation is an effective and reliable way to treat end-stage renal disease (ESRD) (Zhu and Everly, 2012; Mikolasevic et al., 2015). However, long-term survival still remains poor because of various long-term complications (Goldberg et al., 2016). Cardiovascular disease (CVD) is one such major complication that accounts for 40–45% of the cases of loss of allograft and death after kidney transplantation, and it also has a strong impact on the quality of life of recipients (Delville et al., 2015). A key event in the pathogenesis of CVD is the development of arteriosclerosis(AS), which is associated with loss of cardiac function and impaired coronary perfusion and also affects cardiovascular outcomes after kidney transplantation (Simic-Ogrizovic et al., 2012; Fishbein and Fishbein, 2015; Tolle et al., 2015).

Advanced glycation end products (AGEs) are various compounds that are formed by non-enzymatic modification of proteins, lipids, and nucleic acids by glucose(Sourris and Forbes, 2009). AGEs are known to induce oxidative stress and inflammatory response and subsequently accelerate the development of arteriosclerosis (Zhu et al., 2014). Data show that serum AGE levels are a predictive marker of post-transplant cardiovascular complications and kidney function (Crowley et al., 2013). Our previous research indicated that KTRs with AS have higher levels of AGEs than healthy volunteers, and that AGEs may play a critical role in the development and progression of AS after kidney transplantation by inducing VSMC-to-osteoblast trans-differentiation via the AGE/RAGE/integrin-ligated kinase (ILK) signaling pathway (Liu et al., 2015).

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Sirolimus (SRL) is a currently available immunosuppressive drug that acts on the mammalian rapamycin receptor. SRL plays a role in inhibition of the immune pathways in organ transplantation patients through direct phosphorylation of the downstream targets p70S6K and selective inhibition of the m-TORC1 signaling pathway via interleukin (IL)-2 (Woillard et al., 2012). As a widely used oral medicine in KTRs, SRL is a promising immunosuppressant that can mitigate renal insufficiency associated with calcineurin inhibitors (CNIs), such as cyclosporine A (Gonzalez-Vilchez et al., 2014; Malvezzi and Rostaing, 2015; Sereno et al., 2015; Yu et al., 2016). It was shown that patients receiving SRL treatment after liver transplantation had a lower risk of coronary artery disease and cerebrovascular accidents than patients who were maintained on CNIs (Weick et al., 2015). Further, CNI withdrawal and conversion to SRL can improve graft function in living donor kidney transplantations (Morales, 2005; Chen et al., 2013). SRL was also shown to provide greater protection against vasculopathy in a rat kidney model of chronic allograft dysfunction than CNIs. However, the role of SRL in AS induced by AGEs after kidney transplantation and the molecular mechanisms involved are still unknown.

In this study, we aimed to investigate the potential effect of SRL in AS induced by AGEs after kidney transplantation and the molecular mechanisms involved.

2. Materials and methods

2.1. Ethics statement

The study protocol was in accordance with the ethical standards of the Declarations of Helsinki and Istanbul. The study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University, and written informed consent was obtained from all the patients.

2.2. Patients and biopsies

We followed up 12 Chinese adult patients who had undergone living donor kidney transplantation between December 2009 and March 2016 at our transplantation center (the First Affiliated Hospital of Nanjing Medical University). Carotid wall thickening (intima-media thickness > 0.9 mm) or the presence of plaque were considered to be indicative of AS. The patients who were included were first diagnosed with AS by kidney biopsy. They were under SRL treatment for over a year, after which kidney biopsy was performed again. Moreover, 25 cases of healthy volunteers from our center of normal physical examination were enrolled as the normal group. All the kidney biopsy samples and blood samples were obtained with the written informed consent of the patients.

2.3. Assessment of serum AGE, creatinine, triglyceride, total cholesterol and fasting blood glucose levels

Serum levels of AGEs were measured using an AGE competitive ELISA kit (Life Science Inc., Wuhan, China) according to the manufacturer's instructions. Serum levels of creatinine (Cr), triglyceride (TG), total cholesterol (TC) and fasting blood glucose (FBG) were measured using an AU5400 automatic chemical analyzer (Olympus, Japan).

2.4. Histological examination and immunohistochemical staining

Paraformaldehyde-fixed kidney biopsy samples were embedded in paraffin and cut into 2-μm sections. The sections were dewaxed in xylene, rehydrated in a series of decreasing ethanol concentrations, and stained with hematoxylin-eosin for morphological analysis. The method for immunohistochemical staining was that described by Liu et al. (2015). Rabbit polyclonal anti-α-smooth muscle actin (α-SMA) antibody (1:200; Abcam, USA) and rabbit polyclonal anti- osteopontin(OPN) antibody (1:200; Abcam, USA) were the primary antibodies used. Colorization was monitored using a diaminobenzidine kit (ZLI-9018; ZSGB-BIO, China) and viewed under a microscope (CX31; Nikon, Japan). Sections were counterstained with hematoxylin, dehydrated, and mounted with resinene. The mean intensity was quantified in five high-powered fields (×400). In each high-powered field, the media thickness of arteries was calculated using the Image-Pro Plus 6.0 software. α-SMA-positive or OPN-positive regions were quantified based on the total arterial area. α-SMA- or OPN-positive staining was expressed as the mean ratio of the α-SMA-positive or OPN-positive area to the total arterial area in the high-powered fields.

2.5. Cell isolation, identification and treatment

The experimental model used was six-week-old male Sprague-Dawley rats (body weight, 180–220 g), which were housed at the Animal Core Facility of Nanjing Medical University and had free access to food and water. Primary VSMCs were isolated from rat thoracic aortas under sterile conditions as described elsewhere29. The cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics at 37 °C in a 5% CO_2 humidified incubator. Indirect immunofluorescence staining was used to identify the VSMCs, and no significant difference was observed between the second and fifth generations of VSMCs with regard to the morphology and number of α-SMA-positive cells. Fifth-generation VSMCs were chosen for further study, and cell growth was arrested by incubation of the cells in serum-free DMEM for 24 h prior to use. There are four groups: control group; AGEs group(cells treated with 200 mg/l AGEs); AGEs+SRL group(cells treated with both 200 mg/l AGEs and10 nmol/l SRL); SRL group(cells treated with 10 nmol/l SRL). Cells were cultured at uniform density in 6-well plates and were treated with AGEs and/or SRL for 24 h or 6 days. Whole-cell lysates were extracted for detection of the expression of α-SMA, OPN, ALP, ILK, and PCNA proteins by western blot analysis.

2.6. CCK8 assay

Fifth-generation VSMCs were seeded in a 96-well tissue culture plate at a density of 3×10^3 cells per well and treated with 200 mg/l AGEs and/or 10 nmol/l SRL for various periods of time. Cell proliferation in each group was assessed using the Cell Counting Kit (KeyGEN BioTECH, Nanjing, China). Absorbance was measured using an Infinite M200 Pro (TECAN, Salzburg, Austria) absorbance microplate reader at a wavelength of 450 nm. The initial data analysis was carried out with the Magellan 7 software (TECAN Group Ltd., Mannedorf, Switzerland).

2.7. Small interfering RNA transfection and inhibition

For small interfering RNA (siRNA) inhibition studies, VSMCs were transfected with 3 μg with negative control siRNA (Cell Signaling Technology, USA) or ILK siRNA (Cell Signaling Technology, USA) via Lipofectamine 2000 (Invitrogen, USA) according to the instructions specified by the manufacturer. After 6 h of transfection and 12 h of low serum starvation, the VSMCs were treated with 200 mg/l AGEs and/or 10 nmol/l SRL for 24 h or 6 days. Cells were divided into five groups: negative control(NC) group; control group; AGEs group(cells treated with 200 mg/l AGEs); AGEs+SRL group(cells treated with both 200 mg/l AGEs and10 nmol/l SRL); SRL group(cells treated with 10 nmol/l SRL). Following this, cytosolic calcium was detected. Cells proliferation rate was measured using CCK8 assay and whole-cell lysates were collected for assessing the expression of α-SMA, OPN, ALP and PCNA proteins by western blot analysis.

2.8. Indirect immunofluorescence assay

After treatment with AGEs and/or SRL, cells were fixed in methanol