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Research paper

Interleukin-33 levels are elevated in chronic allograft dysfunction of kidney transplant recipients and promotes epithelial to mesenchymal transition of human kidney (HK-2) cells

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ABSTRACT

This study is aimed to investigate the potential role of interleukin (IL)-33 in transplanted kidney interstitial fibrosis and the associated mechanism. Serum IL-33 levels were detected using an enzyme-linked immunosorbent assay (ELISA) in healthy volunteers, stable kidney transplantation recipients (KTRs) (stable), KTRs with acute rejection (AR), and KTRs with chronic allograft dysfunction (CAD) (CAD). Immunohistochemical (IHC) staining, Western blotting, and quantitative real-time PCR (qRT-PCR) were used to detect the expression of IL-33 in human kidney tissues obtained from control and CAD patients. In addition, human kidney (HK)-2 cells were treated with human IL-33 at different doses or intervals, and the markers of epithelial to mesenchymal transition (EMT) were assessed by the presence of proteins and mRNA extracted from these cells using Western blotting and qRT-PCR. Cell motility and migration were evaluated with a cell motility and migration assay. The mechanism involved in EMT induced by IL-33 was investigated by Western blot. Finally, fibronectin, E-cadherin, and α-SMA expression, as well as the level of activity in the MAPK signaling pathway in the kidney tissues from the control and CAD group were also detected using a Western blot and an IHC staining assay. The intensity of fibrosis was substantially higher in the CAD group. IL-33 was significantly upregulated in the CAD patients compared to the control group. In vitro, IL-33 could induce EMT in a dose-dependent and time-dependent manner and promoted both the cellular motility and migration capabilities of HK-2 cells. Moreover, the p38 MAPK signaling pathway might be involved in the pathogenesis of EMT induced by IL-33, which was consistent with the in vivo results of the kidney specimens from the control and CAD patients. IL-33 was upregulated in CAD patients and could promote EMT of HK-2 cells.

1. Introduction

Recently, kidney transplantation has become one of the major life-saving treatments for patients with end-stage renal disease (Nemati et al., 2014). With the improvement of immunosuppressive drugs and surgical techniques, the short-term survival of kidney transplantation recipients (KTRs) has substantially increased. However, the long-term survival of both the allograft and transplant recipients remains poor, mainly due to the long-term complications and the toxicity of immunosuppressive drugs (Zhu and Everly, 2012; Nankivell and Kuypers, 2011). Chronic allograft dysfunction (CAD) is one of the major long-term complications leading to allograft loss and is characterized by progressive kidney dysfunction, hypertension, and proteinuria accompanied by the pathological changes of kidney fibrosis (Arias et al., 2011; Nankivell et al., 2001). As defined by the Banff criteria, CAD remains a non-specific pathologic entity encompassing histological changes of kidney interstitial fibrosis and tubular atrophy (IF/TA), glomerulosclerosis, splitting of glomerular capillary basement membranes, and vascular intimal hyperplasia (Maluf et al., 2014). However, the mechanisms underlying kidney interstitial fibrosis in CAD remain unknown. Recently, cytokines, chemokines, growth factors, toxins, and stress molecules have all been found to accelerate the process of kidney

Abbreviations: CAD, chronic allograft dysfunction; EMT, epithelial to mesenchymal transition; IL-33, Interleukin-33; KTRs, kidney transplantation recipients; MAPK, mitogen-activated protein kinase

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interstitial fibrosis, while the suppression of matrix metalloproteinases can lead to matrix accumulation and glomerulosclerosis (Wing et al., 2013; Lan, 2011; Farris and Colvin, 2012; Duffield, 2014).

As a novel member of the interleukin (IL)-1 cytokine superfamily, IL-33 can interact with its receptors, including suppression of tumorigenicity 2 (ST2) and IL-1 receptor accessory protein (IL-1 RACP), to activate intracellular molecules in the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways (Smithgall et al., 2008; Tominaga, 1989; Schmitz et al., 2005). In addition, IL-33 was proved to induce the production of T helper type (Th) 2-associated cytokines, including IL-4, IL-5, and IL-13, which have been demonstrated to play an essential role in the immune response and promotion of fibrosis (Yang et al., 2016).

In humans, the expression of IL-33 can be detected in several organs and tissues, including the kidneys (Yang et al., 2016). Moreover, IL-33 has recently been reported to contribute to various kidney diseases. In a cisplatin-induced mouse acute kidney injury (AKI) model, IL-33 was shown to promote AKI through $CD4^+$ T cell-mediated production of CXCL1, which can exacerbate renal damage (Akcay et al., 2011). IL-33 was also found to enhance host tolerance to *Candida albicans* kidney infections through the induction of IL-13 production by $CD4^+$ T cells, which may benefit patients with systemic candidiasis (Tran et al., 2015). Additionally, the release of IL-33 from necrotic cells might induce autophagy, which can further balance the effects of increased apoptosis secondary to contrast-induced nephropathy in diabetic kidney disease (Demirtas et al., 2016). A study by Shui-Lian et al. (Yu et al., 2013) suggested that IL-33 is involved in kidney fibrosis associated with systemic lupus erythematosus (SLE). Recently, Chen et al. (Chen et al., 2016) demonstrated that the IL-33/ST2 signaling pathway was upregulated in a mouse renal injury model induced by unilateral urinary obstruction (UUO). Thus, the upregulation of the IL-33/ST2 signaling pathway may promote tubular cell injury and interstitial fibrosis. In addition, other studies also suggest that IL-33 is involved in diseases associated with kidney transplantation. A study performed by Thierry et al. (Thierry et al., 2014) demonstrated that the level of IL-33 was significantly increased as early as 30 min post-reperfusion, supporting the potential role of IL-33 as an immune mediator in human transplanted kidneys during kidney ischemia-reperfusion injury (IRI), which is closely associated with CAD. However, the expression of IL-33 in CAD patients and the role of IL-33 in kidney interstitial fibrosis remains unknown.

In the present study, we hypothesized that IL-33 could stimulate the process of interstitial fibrosis in the renal allograft, which would further accelerate the development of CAD in the transplanted kidney. Our findings indicate that IL-33 might be involved in the pathogenesis of interstitial fibrosis in CAD patients and may play a key role in CAD via the p38 MAPK signaling pathway.

2. Materials and methods

2.1. Ethics statement

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

2.2. Patients and samples

We followed up 75 Chinese adults who underwent living or deceased donor kidney transplantation from July 2009 to December 2015 at our kidney transplantation center (First Affiliated Hospital of Nanjing Medical University). Recipients with a serum creatinine level consistently < 141.46 μmol/L (1.6 mg/dl) for at least 12 months following kidney transplantation were assigned to the stable group, whereas patients with consistently elevated serum creatinine above 141.46 μmol/L (1.6 mg/dl) for at least three months and biopsy-proven chronic allograft injury after kidney transplantation were assigned to the CAD group. KTRs with an acute deterioration in allograft function and confirmed biopsy-proven acute rejection following kidney transplantation were assigned to the AR group. In addition, 25 cases of healthy volunteers (HV) from our center that received a normal physical examination were enrolled as the normal group. Volunteers were excluded if they were under treatment for any acute illness or infection. Moreover, 25 cases of kidney samples collected from radical nephrectomy operations were obtained as the controls. Each sample was further than 5 cm from the tumor tissue. Kidney biopsies from the CAD group were obtained. Blood samples were collected from all of the groups after obtaining informed consent. The biochemical parameters of the serum samples were determined using an AU5400 automatic chemical analyzer (Olympus, Japan). Demographic data pertaining to the main clinical characteristics of these groups were collected. All of the blood samples, kidney biopsies, and tissue samples were obtained with written informed consent.

2.3. ELISA

The levels of IL-33 in the serum samples collected from each group described above were detected using an IL-33 Human ELISA kit (eBioscience, San Diego, CA, USA) following the manufacturer's instructions. The serum IL-33 concentrations were estimated from a standard curve and expressed as the mean ± standard deviation (SD).

2.4. Histological analysis and immunohistochemical staining assay

Paraformaldehyde-fixed renal biopsies and kidney samples were stained with hematoxylin and eosin (HE) and Masson's trichrome staining for the morphological analysis. The tissue sections were carefully examined by an experienced observer who was blinded to the sample classification. For each specimen, the mean fibrosis intensity was taken from five fields of view (Farris and Colvin, 2012). The method used for immunohistochemical staining was performed as described elsewhere (Zhu and Carver, 2012). Briefly, 2 μm sections were deparaffinized in xylene and rehydrated in a graded series of alcohol. Non-specific epitopes were blocked by 5% normal goat serum for 30 min at room temperature and were then incubated overnight at 4 °C with primary antibodies. The primary antibodies used consisted of: rabbit polyclonal anti-IL-33 antibody (1:200, Abcam, USA), rabbit polyclonal anti-fibronectin antibody (1:200, Abcam, USA), rabbit polyclonal anti-E-cadherin antibody (1:100, Abcam, USA), and rabbit polyclonal anti-α-SMA antibody (1:200, Abcam, USA). The sections were incubated with biotinylated goat anti-mouse/rabbit IgG (5.0 μg/mL, Abcam, USA) for 1 h at room temperature.

2.5. Western blotting assay

Protein was extracted from renal biopsies, kidney samples, and HK-2 cells. A Western blot was performed as described elsewhere (Liu et al., 2015). The primary antibodies were as follows: rabbit polyclonal anti-IL-33 antibody (1:1000; Abcam, USA), rabbit polyclonal anti- α-SMA (1:1000, Abcam, USA), mouse polyclonal anti-E-cadherin (1:1000, Abcam, USA), mouse polyclonal anti-fibronectin (1:1000, Abcam, USA), rabbit polyclonal anti- phosphorylated-Erk1/2 (1:1000, Cell Signaling Technology, Inc., USA), rabbit polyclonal anti-Erk1/2 (1:1000, Cell Signaling Technology, Inc., USA), rabbit polyclonal anti-phosphorylated p38 MAPK (p38) (1:1000, Cell Signaling Technology, Inc., USA), rabbit polyclonal anti-p38 MAPK(1:1000, Cell Signaling Technology, Inc., USA), rabbit polyclonal anti-phosphorylated-c-Jun38 MAPK (1:1000, Cell Signaling Technology, Inc., USA), rabbit polyclonal anti-c-Jun MAPK (1:1000, Cell Signaling Technology, Inc., USA) and mouse anti-GAPDH (1:5000, ZSGB-BIO, China). The secondary