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Expression of vascular endothelial growth factor in the early stage of rats' kidney with Chronic Allograft Nephropathy

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Abstract:

Objective *The purpose of this study was to investigate the expression of vascular endothelial growth factor (VEGF) in early stage of rats' kidney with chronic allograft nephropathy (CAN).*

Method *The left kidneys of Fischer (F344) rats were orthotopically transplanted into bilaterally nephrectomized Lewis(LEW) recipients. Meanwhile, the F344 rats and LEW rats with removed right kidney are acted as control groups. Animals were sacrificed at the 12 weeks after transplantation for kidney function,*

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histologic, immunohistochemistry analysis. VEGF expression was determined by immunohistochemistry, Western-blot and RT-PCR.

Result *Our data show that the expression of VEGF was up-regulated in the rats' kidney of CAN and increased more significantly in the tubulointerstitial. The expression of VEGF was significantly correlated with 24h urine protein excretion, the number of tubulointerstitial infiltrated mononuclear cells, the number of smooth muscle cells in vascular wall.*

Conclusion *VEGF might play a key role in the mechanism of pathological changes in the early stage of CAN, the up-regulated VEGF is correlated with mononuclear cells infiltration and vascular smooth muscle cells migration in the early stage of CAN.*

Key words: Rat; Kidney transplantation; Chronic Allograft Nephropathy (CAN); vascular endothelial growth factor (VEGF)

INTRODUCTION

Chronic allograft nephropathy (CAN) is the leading cause of late renal allograft failure. It impedes graft long-term survival (Zarjou et al. 2012, Ganji and Harririan 2012, Fletcher, Nankivell, and Alexander 2009, Wali and Weir 2008, Najafian and Kasiske 2008, Koskinen, Lemstrom, and Hayry 1996). Typical morphologic changes of CAN include glomerulosclerosis, mesangial matrix increase, vasculopathy, interstitial inflammation and fibrosis (Shrestha and Haylor 2014, Han et al. 2011, Zou et al. 2005). It is well known that the infiltration of inflammatory cells, the migration and proliferation of vascular smooth muscle cells are two key pathological changes of the early stage of CAN(Zhou et al. 2015, Shrestha and Haylor 2014, Han et al. 2011, Fletcher, Nankivell, and Alexander 2009). However, the mechanism remains elusive. Vascular endothelial growth factor (VEGF) is a heparin binding growth factor, largely secreted by epithelial cells, myocytes, and macrophages (Ferrara 2009, Ozdemir et al.

2005, Shweiki et al. 1992, Kitamoto, Tokunaga, and Tomita 1997). It produces endothelial specific mitogen, exerts much of its action in a paracrine fashion via binding to the specific receptors of endothelial cells. It has been demonstrated that VEGF plays an important role in endothelial cell growth, angiogenesis, vascular permeability, chemoattraction for inflammatory cells, proliferating of extracellular matrix. VEGF expression is up-regulated in pathological conditions, such as infiltration of inflammatory cells, chronic tubular injury, hypoxic states, wound healing, etc.(Guo et al. 2016, Weis and Cheresh 2005, Grone, Simon, and Grone 1995). However, its function is not fully elucidated. These observations suggest that VEGF may contribute to the pathogenesis of chronic allograft rejection. It is very likely that VEGF is also an important factor of the early stage of CAN. Thus, we investigated the role of VEGF expresses in early stage of CAN in classic CAN rat models.

MATERIAL AND METHODS

Animals

Naive inbred male rats with a body weight of 150~220g and aged 8~10 weeks were obtained from Experimental animal center of China, Peking. Fischer (F344) rats acted as donors and Lewis (LEW) rats as graft recipients. They were kept in animal facility with temperature of 24℃, humidity of 50% and 24h day and night cycle, free access to water and standard rat food. All animal experiments were conducted in accordance with the institutional policies and the recommendations for care and use of laboratory animals. The experimental protocol was approved by the Institutional Ethics Committee of Animal Research.

Drugs

Cyclosporine A(CsA) injection (Novartis Pharma AG, Basel, Switzerland) was dissolved in the cremophor as an excipient and administered subcutaneously with the dose of 1.5mg/kg.

Experimental design and kidney transplantation

Ten F344 kidneys were grafted orthotopically to ten LEW recipients, respectively. Twelve uninephrectomized F344 and twelve LEW rats were acted as controls. Kidney transplantation was performed under general anesthesia. Each rat was anesthetized intraperitoneally with 2% ketamine (100mg/kg body weight; Huadong-Pharma, Shanghai, China). The left donor kidney was isolated, flushed in situ with saline, removed, preserved in saline on ice. The donor kidney was transplanted orthotopically into a recipient rat, in which left renal vessels had been dissected free, clamped, and the native kidney removed. Donor and recipient renal vessels and ureter were anastomosed end-to-end used 11-0 prolene sutures. Vascular clamps were released immediately to recover blood reperfusion after the vascular anastomoses were completed. Mean ischemia time was 25 minutes (ranges 22-31min). To suppress acute rejection, the recipients were administered with CsA (1.5mg/kg/day) immediately after surgery for 10 days. The native right kidney in the recipient was removed on the 10^{th} day after transplantation, at which time the transplanted kidney was checked for surgical or urological complications. Recipient rats were excluded with any overt signs of unsuccessful operation.

Functional measurements

Rats were put into metabolic cages at 12 weeks after renal transplantation. 24 hour urine samples were collected from each recipient rat to evaluate proteinuria quantitation.

Meanwhile, serum and urine creatinine levels were detected and creatinine clearance was calculated.

Harvesting

Twelve recipients and twelve controls (6 F344 and 6 LEW) rats were sacrificed humanely at 12 weeks and the transplanted kidneys were removed. Only the kidneys with no apparent grafting complications such as pyelonephritis or hydronephrosis were evaluated for histopathology. Representative portion of the kidney were snap-frozen in liquid nitrogen and stored at -80°C for later RNA and protein analysis or fixed in formalin (4%) for histological and immunohistological evaluation.

Histopathology

Formalin-fixed and paraffin embedded tissue sections were cut at 4um thickness and stained with hematoxylin eosin to assess pathological changes of grafts. Tissue sections were coded and examined in a blinded manner by light microscopy. At least 100 glomeruli were counted in each section. Renal structural damage was scored semi-quantitatively on a scale from 0 to 3+ for interstitial cellular infiltration, tubulopathy, glomerulopathy and arterial intimal fibroplasia using the Banff criteria(Campistol et al. 2009) and the sum of scores (0-12+) was calculated for each sample.

Immunohistochemistry

The expression of VEGF in the sections of kidney was identified by a standard immunohistochemical method. In a word, Formalin-fixed and paraffin-embedded sections were removed paraffin and rehydrated through a graded alcohol series. Antigen retrieval in tissue sections was performed by boiling the slides in citrate buffer for 2 minutes with high pressure boiler. After blocking with normal goat serum, the tissue sections were incubated with a 1:100 dilution of a mouse

monoclonal VEGF antibody (Novus Biologicals. USA), The secondary antibodies (anti-mouse) were anti-mouse IgG antibody conjugated with biotin from ZAGB-BIO (Beijing, China).The procedure is according to PV-9002 Polink-2 plus®Polymer HRP Detection System. The intensity of tissue staining for VEGF was evaluated in a blinded manner and the following scale was used: "0" as negative, "1" as pallide-flavens, "2"as buffy, "3" as brown. With regard to the area stain, there are 5 degrees expressed as 0–4 according to staining areas, "0" for non-staining, "1" for areas $<$ 25 %, "2" for 25–50 %, "3" for 50–75 %,and "4"for more than 75 % area stained.

Western-blotting

Samples were grinded and lysed by radio immunoprecipitation assay (RIPA) buffer. Protein concentrations were determined with a Pierce BCA Protein Assay Kit (Pierce Biotechnology).The total protein was diluted in sample dye and denatured by in boiling water for 10 min. The proteins along with the marker were separated by 10% polyacrylamide gel with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto transferred onto polyvinylidene fluoride (PVDF) membranes after gel electrophoresis, The membranes were blocked in 1xTBST (Tris-Buffered Saline Tween-20) containing 0.5%W/V BSA(Albumin from bovine serum) for 0.5h at room temperature, membranes were incubated with primary antibodies against VEGF (dilution 1:2,000, Novus Biologicals. USA) at 4°C overnight. Then the membranes were washed with 1x TBST, followed by incubation with 1:2,000 diluted secondary antibodies (Abcam, Cambridge, MA, USA) conjugated with horseradish peroxidase (HRP) for 1 h. Then membranes were washed again as above and incubated with westernBright ECL (advansta, USA) for 1 min. The proteins were quantified with Quantity One Analysis Software (Bio-Rad, Hercules, CA, USA).

RNA isolation AND RT-PCR

Representative portions of the kidney samples were stored in -80℃. 60-100mg frozen tissue samples were used to isolated total RNA by TRIzol reagent (Invitrogen, USA), then total RNA was transcribed to the complementary DNA(cDNA) by reverse transcription utilizing PrimeScript RT reagent Kit (TaKaRa Bio Inc. Japan),according to the manufacturer's instruction. VEGF and β-actin were amplified in the Stratagene Mx3000p Real-time PCR systems (Agilent Technologies, Germanny) β-actin was acted as endogenous control to normalize gene expression. SYBR Premix Ex Taq (TaKaRa Bio Inc. Japan) served for relative quantification of cDNA. The primers used to quantify the expression of targeted genes (Biosune, Shanghai, China) as follows: beta-actin-forward: 5'-atggagggaatacagccc-3' and reverse: 5'- ttctttgcagcrccttcgtt-3'. The length of the amplified product was 149bp.VEGF-forward: 5'-aatgctttctccgctctgaa-3'and reverse: 5'-ctcaccaaagccagcacata-3'. The length of the amplified product was 126bp. Target gene expression levels were normalized to those of the control gene and were calculated using the method of $2^{-\Delta\Delta CT}$.

Statistical analysis

All data are expressed as mean ± SD. Statistical analysis was performed by SPSS software, using One-Way ANOVA test and two variable non-parameter correlation analyses were used to compare the data of experimental and control group. p-values <0.05 was considered as statistically significant.

RESULTS

Functional changes

24-hour urine protein excretion in the transplant group was significantly higher than that in two control groups at 12weeks. Serum creatinine levels in transplant group were also

significantly higher as compared to LEW controls at 12 weeks, but not statistical difference between transplant animals and F344 controls (Table 1).

Histological changes

At 12 weeks, mesangial expansion, glomerulosclerosis and adhesions to Bowman's capsule, tubulopathy and interstitial mononuclear cells infiltration, intimal proliferation in allografts were found to be significantly increased in allografts as compared with LEW/F344 controls. The sum of Banff scores was significantly higher in allografts than that in F344 controls or LEW controls. There was no significant difference of the percentage of interstitial fibrosis between allografts and F344 controls or LEW controls (Table 1, Fig1).

VEGF mRNA and Protein Expression

VEGF mRNA expression increased significantly higher in allografts as compared with F344/LEW controls (Fig.2 A). Similarly, the expression of VEGF protein in allografts was also significantly increased as compared to two controls. (Fig.2 B).

Immunohistochemistry

The prominent expression of VEGF was observed in tubules and interstitium of allografts, and next in the glomerular and the arterial wall. The levels of VEGF expression in allografts were significantly increased as compare with F344/LEW controls, (Fig.3).

Correlation analysis

The expression of VEGF was positively correlated with 24h urinary protein excretion($r=0.95$, $p< 0.01$), degree of mononuclear cells infiltration($r=0.757$, $P<0.05$) and the amount of SMCs in vascular wall($r=0.741$, $P<0.05$).

DISCUSSION

It is shown that the previous studies of chronic allograft nephropathy (CAN) rat models(Zununi Vahed et al. 2016, Xue et al. 2016, Zhou et al. 2015, Shrestha and Haylor 2014, Li and Zhuang 2014, Han et al. 2011, Fletcher, Nankivell, and Alexander 2009, Najafian and Kasiske 2008, Zou et al. 2005, Viklicky, Matl, and Heemann 1999, Koskinen, Lemstrom, and Hayry 1996)mild focal mononuclear cell infiltration was observed at the 1st week after transplantation, whereas at the 12th week, Typical pathological changes of CAN were tubulointerstitial mononuclear cell infiltration and vascular intimal proliferation. Meanwhile, the major appearance was proteinuria and renal function begun to abnormal. But interstitial fibrosis, glomerulosclerosis and arteriosclerosis were also observed at the 16th week and thereafter.

The overall data from these studies indicate that until the 12th week after renal transplant is the early stage of CAN which characterized by reversible interstitial mononuclear cells infiltration, vascular smooth muscle cells (SMC) migration, minor proteinuria, and normal serum creatinine levels. Later, the onset of chronic stage of CAN is characterized by development of persistent and irreversible lesions such as glomeruloarterial sclerosis and renal interstitial fibrosis.

Vascular endothelial growth factor (VEGF) is a highly selective vascular endothelial mitogen. To date, VEGF has been shown to be key molecules implicated in endothelial cell proliferation, angiogenesis, vascular permeability, migration, tumor progression and cardiovascular disease (Weis and Cheresh 2005, Ozdemir et al. 2005, Otto et al. 2002). VEGF can be detected in normal kidney (Kitamoto, Tokunaga, and Tomita 1997). Besides VEGF is expressed in endothelial cells of glomerular and peritubular capillaries. It is also observed in proximal and distal convoluted tubules. VEGF has been

clarified to play an important role for kidney growth and development(Cao et al. 2006, Pilmore et al. 1999, Kitamoto, Tokunaga, and Tomita 1997).

VEGF expression is up-regulated in pathological conditions, such as infiltration of inflammatory cells, chronic tubular injury, hypoxic states, wound healing, etc. (Shweiki et al. 1992, Ozdemir et al. 2005, Weis and Cheresh 2005). B.Handan et al. found that VEGF expression increased on tubular and interstitial in patients with CAN, acute CSA toxicity and acute rejection. There were significant differences as compare with control groups. Moreover, tubular and interstitial VEGF expression was a significant positive correction with interstitial macrophage infiltration (Ozdemir et al. 2005). Similarly, Grone HJ, et al. reported that tubular and interstitial VEGF expressions were significantly increased in patients with CAN (Grone, Simon, and Grone 1995). Many researchers suggested the characterizing pathological changes of early stage of CAN include infiltration of mononuclear cells and SMC replication in the vascular wall and migration from the media into the intima (Koskinen, Lemstrom, and Hayry 1996, Mannon 2012, Tilney et al. 1993).

In this study, we have evaluated significance of the VEGF expression in the early stage (12 weeks) of CAN with the F344 to LEW classic transplant rat model. We found that VEGF expression was up-regulated in kidney allografts, especially within the tubulointerstitium, and relatively lower expression was observed in the glomeruli and arteries. The increased VEGF expression correlated with the typical pathologic changes of the early stage of CAN such as tubulointerstitial mononuclear cells infiltration, SMC migration in vascular wall, and urinary protein excretion. We found that 24-h urinary protein excretion at the 12th week was found to be significantly elevated in rats of CAN. Furthermore, VEGF expression and 24h urinary protein are correlated positively.

Meanwhile the VEGF expression showed a significant positive correlation with the mononuclear cells infiltration and SMCs migration in the vascular wall at the 12th weeks after transplantation;

Based on the above evidence, we also indicate that overexpression of the VEGF in renal tubules and interstitium at the early stage of CAN might be the key event to induce or promote the disease pathogenesis through infiltration of mononuclear cells and migration of vascular wall SMCs. Above these data suggest that VEGF plays an important role in the early development of CAN pathogenesis involving interstitial mononuclear cells infiltration and SMCs proliferation migration in vascular wall.

In conclusion, our data show that expression of the VEGF of graft is significantly increased at the early stage of CAN, and VEGF expression has a significant positive correlation with the characteristic pathologic features of CAN in the early stage such as mononuclear cells infiltration and vasculopathy.

Competing Interests

The authors declare no conflict of interests.

Acknowledgments

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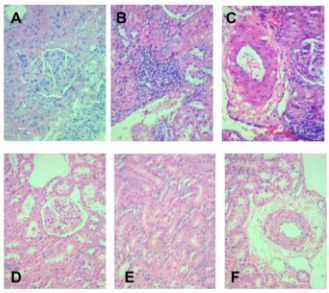
Table 1. Functional measurements and histological evaluation

	N	24h urine protein excretion(mg/d)	Serum creatinine (umol/l)	Banff sums score	quantity of interstitial mononuclear cells	Quantity of SMCs in vascular wall	degree of interstitial fibrosis
Allografts	10	26.4±5.8	92±8.5	4.4±1.32	26.4±5.8	12.0±2.21	0.086±0.026
Control(LEW)	10	19.2±4.3[a]	73.4±6.8[a]	1.5±0.23[a]	5.3±1.6[a]	7.36±2.16[a]	0.067±0.021
Control(F344)	10	15.4±5.2[a]	82.3±5.9	1.4±0.28[a]	6.0±1.7[a]	7.01±1.94[a]	0.068±0.025

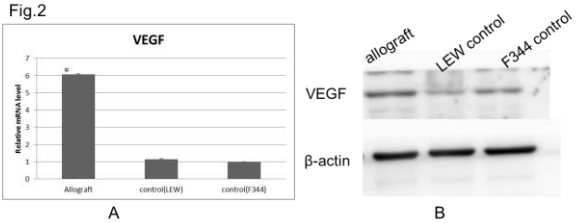
a: $p < 0.05$,compared to allografts at the same time point

Fig **1 renal histology**



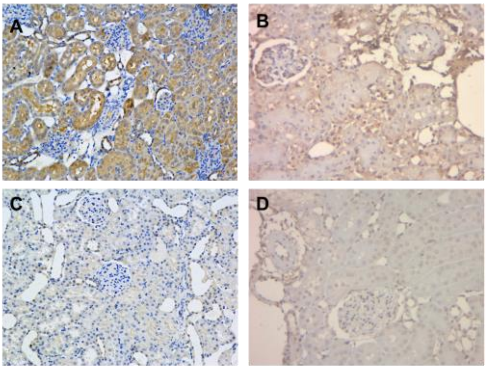
Formalin-fixed and paraffin embedded tissue sections were stained with hematoxylin and eosin to assess pathological changes of grafts(X200). A glomerular cellular infiltration and glomerulosclerosis in allografts;B tubulointerstitial cellular infiltration in allgrafts;C intimal proliferation in allgrafts; D glomeruli in controls; E tubulointerstitial in controls; F vascular wall in controls.

Fig.2



A RT-PCR was used to evaluate VEGF mRNA levels in kidney tissues. Al data are expressed as mean±SD and β-actin mRNA is used as an internal control （n=3）.*p＜ compared with LEW controsl and F344 conrols. B western blot evaluate VEGF protein expression in allografts, LEW controls, F344 controls. β-actin was analysed as the loading control.

Fig.3 immunohistochemical location of VEGF in kidney



Immunohistochemical staining for VEGF in the Rat kidney tissue of allografts and controls. A tubular and interstitial VEGF expression in allograft; B glomerular and arterial expression of VEGF in allograft; C tubular and interstitial expression of VEGF in control; D glomerular and arterial expression of VEGF in control; original magnification x 200.