Original Article
Effect of HIF-1α, VEGF and MVD expression on angiogenesis in diabetic nephropathy

Xiaomin Zhang[1,3], Lianhuan Ma[2], Hua Dong[3], Zhimei Lv[1], Yunqi Liu[3], Jinxiang Gao[3], Rong Wang[1]

[1]Department of Nephrology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, Shandong, P.R. China; [2]Department of Diagnostics, Clinical Medical College, Binzhou Medical University, Yantai 264003, Shandong, P.R. China; [3]Department of Nephrology, Binzhou Medical University Hospital, Binzhou 256603, Shandong, P.R. China

Abstract: Hypoxia-inducible factor1-α (HIF-1α) and vascular endothelial growth factor (VEGF) are associated with the occurrence and development of diabetic nephropathy (DN) and the process of angiogenesis. This research adopts the streptozotocin (STZ) to create animal models with DN, detect the levels of HIF-1α and VEGF and analyze the impact of HIF-1α expression on VEGF and microvessel density (MVD) in rats with DN. STZ was adopted to create the rat models with DN and retinal venous blood and renal tissues of rats were selected. Enzyme linked immunosorbent assay (ELISA) and immunohistochemical (IHC) method were used to detect the expressions of HIF-1α and VEGF in serum and tissues, observe the MVD and analyze the relations between HIF-1α and VEGF, MVD. Levels of HIF-1α and VEGF of rat serum in experimental group were higher than that in control group (P<0.05). The positive rate of HIF-1α in experimental group had reached to 80%, VEGF reached to 70% and MVD reached to (33.9±15.2)/200 visual fields, which were higher than that in control group (P<0.05). HIF-1α stayed positive correlation with VEGF and MVD (P<0.05). HIF-1α, VEGF and MVD in DN were increased and the HIF-1α stayed positive correlation with VEGF and MVD.

Keywords: Hypoxia-inducible factor1-α (HIF-1α), diabetic nephropathy (DN), vascular endothelial growth factor (VEGF), microvessel density (MVD)

Introduction

Diabetes, at present, is a clinical common endocrinal and metabolic disease. One of the common complications of diabetes is diabetic nephropathy (DN), the manifestation of which mainly includes sclerosis of glomerulus and renal small vessels and lesion in important micrangium [1-3]. An experiment points out that the pathogenesis of DN is closely related with the significant cytokines, such as TGF-β (transforming growth factor-β) within body, etc., which participate in corresponding signal transduction pathways and regulate the generating process of DN [4]. As the important transcription factor within body, HIF-1α widely exists in multiple tumor cells and can play a role of transcription in a timely manner, regulate the gene expression, promote the neovascularization of organs and tissues and facilitate the histocyte

to take more energy once tissue stays at a hypoxic microenvironment. HIF-1α can also adjust the VEGF expression [5, 6]. The growth, invasion and metastasis of malignant tumors need a continuous supply of blood and oxygen. VEGF is one of the important cytokines within body that shows relation with the angiogenesis of malignant tumors, with highly direct and strong action. In the fundamental research, it focuses on VEGF, considered as the target, to be the treatment direction [7]. The Microvessel density (MVD) can reflect the tumor angiogenesis and be used to detect activity of angiogenesis. Till now, several studies have investigated the role of HIF-1α, VEGF and MVD in various solid tumors, such as colorectal cancer [9], gastric cancer [10]. Expression of HIF-1α and VEGF were positively observed in patients with colorectal cancer and significantly associated with tumor stage, lymph nodes, liver metasta-

ses and overall survival [9]. In addition, HIF-1α was positively correlated with VEGF [9]. Apart from colorectal cancer, gastric cancer patients also displayed positive expression of HIF-1α, which was significantly associated with expressions of VEGF and MVD [10].

Considering the role of abnormal angiogenesis in DN [11], plus closely relationship among HIF-1α, VEGF and MVD, we hypothesized that HIF-1α, VEGF and MVD might be involved in the pathogenesis of DN. To test this hypothesis, we established DN model rats to investigate the expressions of HIF-1α, VEGF and MVD and analyze the impact of HIF-1α on VEGF and MVD in DN.

Materials and methods

Experimental animals

The experimental animals, 80 Kunming mice fed under SPF condition, were selected. Their weight was kept between 18 and 20 g, their age was at about four weeks and male and female each accounted for 50% (Laboratory Animal Centre of Shandong University).

Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Provincial Hospital Affiliated to Shandong University.

Experiment reagents

Streptozotocin (STZ) was bought from Sigma Company of America. HIF-1α and VEGF ELISA kits, HIF-1α and VEGF confining liquid+primary antibodies and rabbit antimouse second antibody kits were bought from Changzhou Peaks Chemical, CO., LTD. of China, Shanghai Feige centrifugal machine was bought from Shanghai AnTing Electronic Instruments Plant and inverted microscope was bought from Olympus Company of Japan.

Experimental methods

Creation of DN rat models: 80 rats were divided randomly into two groups (40 for each group) with weight between 18 and 20 g and age at about four weeks. Rats in experimental group were fed with high lipid feed while rats in control group were given with equivalent regular feed and normal saline at a same time once daily. It was definite that insulin resistance

existed in rats at the end of the tenth week and the intraperitoneal injection concentration was 35 mg/kg of STZ. Keep feeding with the regular feed, collect venous blood of rat tail to detect the random blood glucose, which showed >15.7 mmol/L, collect the rat urine to detect 24 h urine protein at the end of the twelfth week and finally collect serum to detect blood glucose. 24 h urine protein and blood glucose of DN rats were increased. Intervention with STZ was higher than that before, which showed that the DN animal model was successfully created. Healthy rats were selected as the control group.

Specimen collection: Fix the selected Kunming mice at the edge of experiment platform, firmly grasp neck skin of mouse with left hand to fix its head, insert a capillary into its eye ground, and drop the blood into the pre-prepared EP tube continuously along the capillary. After stopping bleeding, seal the tube as soon as possible and preserve under low temperature.

Lavage the kidney of selected rat with 0.9% sodium chloride solution in a environment of 4°C, excise both kidneys of rat during the laparotomy and divide them into pieces with the same sizes, about 0.5 cm × 0.5 cm. Save these pieces at -80°C in refrigerator.

Detection of levels of HIF-1α and VEGF in rat blood with ELISA method

Select venous blood from eye ground and centrifuge it to take supernatant. Standard substance was diluted and prepared by concentration demultiplication method according to the specification. Extract 120 μl of standard substance in the kit, add it into an EP tube, add another 120 μl of diluent in the kit, respectively prepare the concentration of standard liquid according to the requirement of each detection factor specification, set repeated wells and add specimen into them one by one. After finishing, seal the plate and place in a condition of 37°C to incubate. Use the Phosphate Buffer Solution (PBS) to wash each well and add fluid A and B for developing. Finally, measure the light absorption value at the wave length of 450 nm.

Detection of expressions of HIF-1α and VEGF in rat kidney tissue with IHC method

First, fix specimens, and then embed the specimen tissue with formalin and following with pre-prepared paraffin. Laboratorial microtome was

Table 1. Detection of content of HIF-1α and VEGF in patients' blood with ELISA method

Group	Case number	HIF-1α (ng/ml)	VEGF (ng/ml)
Experimental group	40	1.25±0.14*	1.57±0.12*

Note: *Compared with control group, the difference was statistically significant, P<0.05.

Table 2. Detection of HIF-1α protein expression in rat kidney tissuewith IHC

Group	Case number	HIF-1α expression strength			
		-	+-++	+++	Positive rate (%)
Experimental group	40	8	10	22	80*
Control group	40	36	4	0	10

Note: *Compared with control group, the difference was statistically significant, P<0.05.
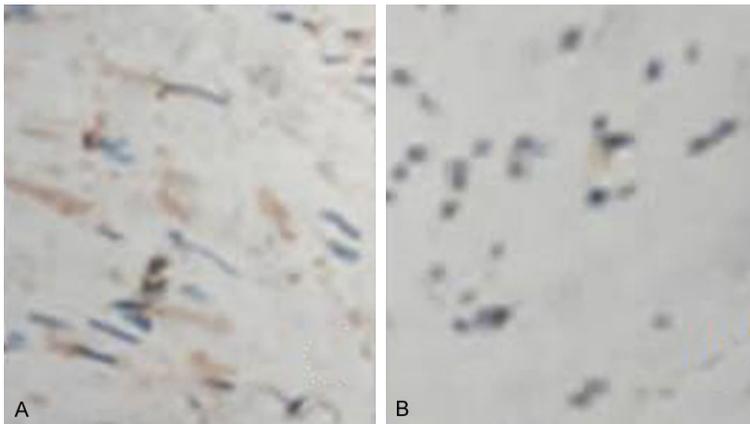
Figure 1. Expression of HIF-1α protein in rat kidney tissue. A: Experimental group HIF-1α (+++). B: Control group HIF-1α (-).

used to cut out the paraffin straps in a thickness of 3 um. Tile every paraffin strap on glass slide, put them into a thermostat with temperature of 60°C and stay overnight. Add xylene for 10 min and add ethyl alcohol with gradient of 95%, 85% and 75% for 5 min. After boiling the specimens with high temperature in a pressure cooker, wash them with running water to cool them to room temperature, drop hydrogen peroxide and add confining liquid into. Drop the diluted primary antibody and then wash after 1 h. Drop the diluted second antibody, add DAB for developing after ten minutes, stain with hematoxylin and seal plate by differentiation.

There was brown yellow particle deposition on cell membranes, cytoplasm or karyon. The color intensity was higher than that unspecific staining, which was defined as positive [12]. Observe the sectionbydouble-blindmethod.Negative (-): colorless; weakly positive (+): faint yellow; positive (++): yellow; strong positive (+++): brown yellow.

Data processing

SPSS 17.0 statistics software was used to do data processing. χ^2 test was used for getting enumeration data and T-test was adopted for measurement comparison, indicated with ($\overline{x}$±s). P<0.05 meant the differences showed with statistical significance.

Results

Detection of levels of HIF-1α and VEGF in rat serum with ELISA method

Take venous blood from eye ground to detect levels of HIF-1α and VEGF in rat serum. The results showed that HIF-1α in experimental group was (1.25±0.14) ng/ml, VEGF was (1.57±0.12) ng/ml, which was significantly higher than that in control group (P<0.05). See **Table 1**.

Detection of HIF-1α proteinexpression in rat kidney tissue with IHC

Detect the expression of HIF-1α protein in rat kidney tissue and find that HIF-1α positive rate in experimental group had reached to 80%, which was significantly higher than that in control group (P<0.05). See **Table 2** and **Figure 1**.

Detection of expressions of VEGF and MVD in rat kidney tissue with IHC

Detect the VEGF expression of patients' bone tissue and find that VEGF positive rate in experimental group had reached to 70% and MVD reached to (33.9±15.2)/200 visual fields. Which was significantly higher than that in control group (P<0.05). See **Table 3** and **Figure 2**.

Table 3. Detection of expressions of VEGF and MVD in rat kidney tissue with IHC

Group	Case number	VEGF expression strength			Positive rate (%)	MVD (/200 visual fields)
		-	+-++	+++		
Experimental group	40	12	18	10	70*	33.9±15.2*
Control group	40	36	4	0	10	20.1±9.8

Note: *Compared with control group, the difference was statistically significant, P<0.05.
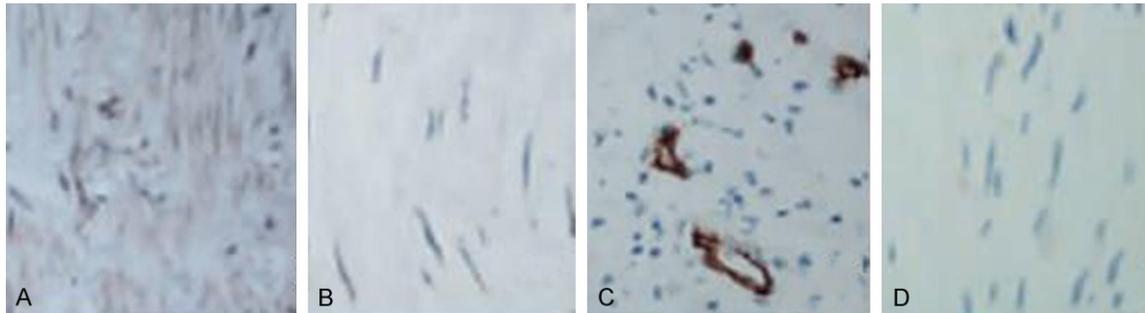
Figure 2. Expressions of VEGF and MVD in rat kidney tissue (×200). A: Experimental group VEGF (+++). B: Control group VEGF (+). C: Experimental group MVD (+). D: Control group MVD (-).

Relation between HIF-1α and VEGF expressions in rat kidney tissue of experimental group

Analyze the relation between HIF-1α and VEGF and find that HIF-1α showed significant positive correlation with VEGF (P<0.01). Specific results were shown in **Table 4**.

Relation between HIF-1α and MVD expressions in rat kidney tissue of experimental group

Analyze the relation between HIF-1α and MVD and find that HIF-1α showed significant positive correlation with MVD (P<0.01). Results were shown in **Table 5**.

Discussion

There are numerous complications of diabetes, one of which is DN (Diabetic Nephropathy), one of the most common complications. In the different classifications, type I diabetes accounts for 15%-25% and type II accounts for 30%-40% [2]. Researches show that one of the important reasons of DN occurrence is that local renal tissue is in lack in oxygen. Once the renal tubular cells stay at hypoxic microenvironment, they will be easy to suffer from inflammation, tissues fibrosis, even cell apoptosis and sometimes epithelial-mesenchymal transition, which

will leads to rapid aggravation of renal interstitial fibrosis and renal failure further [13]. When the body is at hypoxic microenvironment, HIF-1 exists in multiple histocytes and participates into the neovascularization and the decomposition and transport of saccharides [10, 14]. VEGF is a kind of angiogenesis factor. When body histocytes is lacking in oxygen, the VEGF can play an action combined with HIF-1. The action of VEGF is the most powerful and wide factor. The most important element for inducing its expression activity is the ischemia and hypoxia microenvironment [15].

This paper has created STZ DN rats to detect and find that levels of serum HIF-1α and VEGF in DN rats are increased. Clinical experiment has studied patients with diabetes and research findings showed that VEGF shows significant high expression in patients with diabetic foot, but shows weak expression in normal group [16]. Select the kidney tissue of rats to detect and find that HIF-1α protein, VEGF protein and MVD of rats tissue in experimental group are increased, which has showed that the HIF-1α, VEGF and MVD existing in kidney tissue of rats with DN has been increased. The neovascularization capacity can be estimated by accumulating the microvessel count. VEGF is a kind of important cytokine stimulating vascularization. The increasing of VEGF expression

Table 4. Relation between HIF-1α and VEGF expressions

Group	Case number	HIF-1α		χ²	P
		-	+		
VEGF (-)	12	8	4		
VEGF (+)	28	8	20	0.859	0.002

Table 5. Relation between HIF-1α and MVD expressions

Group	Case number	MVD		χ²	P
		Low	High		
HIF-1α (-)	8	6	2		
HIF-1α (+)	32	6	26	0.882	0.001

level promotes the formation of new blood vessels, which contributes to the increasing of MVD. VEGF is the important growth factor of body and secreted by multiple cells with powerful action. Mainly depending on the interactions with tyrosine kinase receptors, the VEGF can exert biological function. There are experiments pointed out that VEGF is the core cytokine during the process of neovascularization. It shows very powerful function in promoting the normal mitosis of VEGF cells and enhancing the vascular permeability [17]. Now, the immunohistochemical method (IHC) is adopted to mark the micrangium typically, country the microvessel quantity in unit area, i.e. MVD, and reasonabely determine the neovascularization capacity [18]. Celletti has conducted fundamental experiment study on experimental rats with apo-E/apo-B100 and rabbits with hypercholesteremia and interfered with VEGF to find that VEGF could effectively promote the atherosclerotic plaque formation. Not only that, the research also found that VEGF could not only promote angiogenesis, but also induce the monocyte chemotaxis and exert facilitation on the process of arterial restenosis [19]. There are other basic experimental researches have created DN rats to detect and found that, when rats growing in hypoxic environment, levels of HIF-1α and VEGF were subsequently heightened [20].

Correlation analysis reveals that HIF-1α shows positive correlation with VEGF and MVD. Researches indicates that HIF-1, combing with VEGF-5', can increase its transcriptional activi-

ty and participate into the occurrence and development of DN [21]. When the body stays at an ischemic condition, HIF-1α is the important cytokine to start the body signal pathways. Meanwhile, expressions of cytokines like VEGF will be increased, which affect the survival and permeability of blood. Inflammatory reaction cells take chance to enter, accelerating the atherosclerosis and increasing the risk of atheromatous plaque formation. The high expressions of HIF-1α and VEGF show us two aspects: One, interaction between HIF-1α and VEGF can promote the normal metabolism of cells and enhance the cell adaptablity of body when body stays at hypoxia environment. High expression will stimulate more abnormal expressions of body cytokines to lead to corresponding progression and deterioration of diseases [22]. Two, VEGF increasing can promote the rising of new vessels. However, the new vascular structure, different from normal vessels, has poor function and low oxygen carrying capacity, which makes tumor stay at hypoxia survival state. That is to say, when the body cells stay in hypoxia microenvironment, the HIF-1α level will be increased obviously. MVD, therefore, will be unceasingly increased due to the increasing of abnormal new vessels promoted by VEGF. Once the normal body vascular microenvironment is changed, a series of response elements and signal pathways may work tohether, i.e. HIF-1α has positive correlation with MVD.

Conclusion

The DN HIF-1α, VEGF and MVD are significantly elevated and the HIF-1α shows positive correlation with VEGF and MVD. In recent years, molecular diagnostic technique has developed and innovated unceasingly. Researcher has paid more attention to the molecule level and gene level to deeply investigate the pathogenetic mechanism. More and more cytokines, genes and expression and conduction ways of corresponding signal pathway are gradually discovered and have been used for diagnosis and treatment of diseases. In the following fundamental and clinical researches, researchers can focus on considering from the points of HIF-1α and VEGF, MVD to do experiment and take full advantage of the relationship s among those three, which may improve and update

better ideas for treating DN, enhance therapeutic effect on DN. However, the specific molecular mechanism, signal pathway need further research and exploration.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Rong Wang, Department of Nephrology, Shandong Provincial Hospital Affiliated to Shandong University, No.324 Jing 5nd Wei 7nd Road, Jinan 250021, Shandong, P.R. China. Tel: +860531-68776294; Fax: +86-0531-68776294; E-mail: rongwangun@sina.com

References

[1] Farag YM and Al Wakeel JS. Diabetic nephropathy in the arab gulf countries. Nephron Clin Pract 2011; 119: c317-322; discussion c322-313.

[2] Kim DK, Nam BY, Li JJ, Park JT, Lee SH, Kim DH, Kim JY, Kang HY, Han SH, Yoo TH, Han DS and Kang SW. Translationally controlled tumour protein is associated with podocyte hypertrophy in a mouse model of type 1 diabetes. Diabetologia 2012; 55: 1205-1217.

[3] Ferguson KK, Loch-Caruso R and Meeker JD. Exploration of oxidative stress and inflammatory markers in relation to urinary phthalate metabolites: NHANES 1999-2006. Environ Sci Technol 2012; 46: 477-485.

[4] Kung WJ, Lin CC, Liu SH and Chaung HC. Association of interleukin-10 polymorphisms with cytokines in type 2 diabetic nephropathy. Diabetes Technol Ther 2010; 12: 809-813.

[5] Chen L, Shi Y, Yuan J, Han Y, Qin R, Wu Q, Jia B, Wei B, Wei L, Dai G and Jiao S. HIF-1 alpha overexpression correlates with poor overall survival and disease-free survival in gastric cancer patients post-gastrectomy. PLoS One 2014; 9: e90678.

[6] Feng Y, Wang W, Hu J, Ma J, Zhang Y and Zhang J. Expression of VEGF-C and VEGF-D as significant markers for assessment of lymphangiogenesis and lymph node metastasis in non-small cell lung cancer. Anat Rec (Hoboken) 2010; 293: 802-812.

[7] Chen G, Liu XY, Wang Z and Liu FY. Vascular endothelial growth factor C: the predicator of early recurrence in patients with N2 non-small-cell lung cancer. Eur J Cardiothorac Surg 2010; 37: 546-551.

[8] Weis SM and Cheresh DA. Tumor angiogenesis: molecular pathways and therapeutic targets. Nat Med 2011; 17: 1359-1370.

[9] Cao D, Hou M, Guan YS, Jiang M, Yang Y and Gou HF. Expression of HIF-1alpha and VEGF in colorectal cancer: association with clinical outcomes and prognostic implications. BMC Cancer 2009; 9: 432.

[10] Isobe T, Aoyagi K, Koufuji K, Shirouzu K, Kawahara A, Taira T and Kage M. Clinicopathological significance of hypoxia-inducible factor-1 alpha (HIF-1alpha) expression in gastric cancer. Int J Clin Oncol 2013; 18: 293-304.

[11] Nakagawa T, Kosugi T, Haneda M, Rivard CJ and Long DA. Abnormal angiogenesis in diabetic nephropathy. Diabetes 2009; 58: 1471-1478.

[12] Granados Lopez AJ and Lopez JA. Multistep model of cervical cancer: participation of miR-NAs and coding genes. Int J Mol Sci 2014; 15: 15700-15733.

[13] Belaiba RS, Bonello S, Zahringer C, Schmidt S, Hess J, Kietzmann T and Gorlach A. Hypoxia up-regulates hypoxia-inducible factor-1alpha transcription by involving phosphatidylinositol 3-kinase and nuclear factor kappaB in pulmonary artery smooth muscle cells. Mol Biol Cell 2007; 18: 4691-4697.

[14] Sharma S, Kapahi R, Sambyal V, Guleria K, Manjari M, Sudan M, Uppal MS and Singh NR. No association of hypoxia inducible factor-1alpha gene polymorphisms with breast cancer in North-West Indians. Asian Pac J Cancer Prev 2014; 15: 9973-9978.

[15] Micko AS, Wohrer A, Wolfsberger S and Knosp E. Invasion of the cavernous sinus space in pituitary adenomas: endoscopic verification and its correlation with an MRI-based classification. J Neurosurg 2015; 122: 803-811.

[16] Yuan Y. Expressions of hypoxia-inducible factor-1α and vascular endothelial growth factor in diabetic foot patients. J Med Postgra 2011; 5: 490-494.

[17] Decoster L, de Marinis F, Syrigos K, Hirsh V and Nackaerts K. Bisphosphonates: prevention of bone metastases in lung cancer. Recent Results Cancer Res 2012; 192: 93-108.

[18] Li J and Sun Y. Expression and significance of microvascular density and vascular endothelial growth factor in lung cancer and their intracranial metastasis. Acad J PLA Postgrad Med Sch 2001; 24: 232-234.

[19] Celletti FL, Waugh JM, Amabile PG, Brendolan A, Hilfiker PR and Dake MD. Vascular endothe-

lial growth factor enhances atherosclerotic plaque progression. Nat Med 2001; 7: 425-429.

[20] Katavetin P, Miyata T, Inagi R, Tanaka T, Sassa R, Ingelfinger JR, Fujita T and Nangaku M. High glucose blunts vascular endothelial growth factor response to hypoxia via the oxidative stress-regulated hypoxia-inducible factor/hypoxia-responsible element pathway. J Am Soc Nephrol 2006; 17: 1405-1413.

[21] Mace KA, Yu DH, Paydar KZ, Boudreau N and Young DM. Sustained expression of Hif-1alpha in the diabetic environment promotes angiogenesis and cutaneous wound repair. Wound Repair Regen 2007; 15: 636-645.

[22] Liu L, Marti GP, Wei X, Zhang X, Zhang H, Liu YV, Nastai M, Semenza GL and Harmon JW. Age-dependent impairment of HIF-1alpha expression in diabetic mice: correction with electroporation-facilitated gene therapy increases wound healing, angiogenesis, and circulating angiogenic cells. J Cell Physiol 2008; 217: 319-327.