· Original article ·

# VEGF expression and cell apoptosis in NOD mouse retina

*Cai-Rui Li, Shu-Guang Sun*

Department of Ophthalmology, Hospital of Dali University, Dali 671000, Yunnan Province, China

**Correspondence to**: Cai-Rui Li. Department of Ophthalmology, Hospital of Dali University, Dali 671000, Yunnan Province, China. lcrbrett@hotmail.com

Received:2010-05-31 Accepted:2010-07-19

## Abstract

• AIM: To investigate retinal vascular endothelial growth factor (VEGF) level and retinal cells apoptosis in the early stage of diabetic NOD mouse retina.

• METHODS: Animals were divided into the control group (non-diabetes mice) (2,4,6,8,12 weeks group, $n=30$) and diabetes group (2,4,6,8,12 weeks group, $n=30$). ELISA (Enzyme-Linked Immunosorbent Assay) was performed to detect VEGF level in both serum and retina. Transmission electron microscope method was used to examine retinal cell apoptosis.

• RESULTS: Compared with the control group, VEGF levels in serum and retina were increased significantly in the NOD group (12 weeks: 4.9±0.4μg/g versus 0.19±0.1μg/g in serum sample, $P<0.01$; 165.0±9.0μg/g versus 18.0±4.0μg/g in retinal sample, $P<0.01$). There exists a positive correlation between serum VEGF and retinal VEGF levels in the early diabetic NOD mice ($\gamma=0.9902$, $P=0.001$). The number of the cells apoptosis in the ganglion cells and endothelium can also been found increased significantly in the NOD group($P<0.01$).

• CONCLUSION: The high VEGF expression may be contributed to increase retinal cells apoptosis. Many factors associated with retinal VEGF expression might involve in the early diabetes stage.

• KEYWORDS: retina; apoptosis; vascular endothelium growth factor; NOD mice

DOI:10.3969/j.issn.1672-5123.2010.08.002

Li CR, Sun SG. VEGF expression and cell apoptosis in NOD mouse retina. *Int J Ophthalmol (Guoji Yanke Zazhi)* 2010;10(8):1459-1462

## INTRODUCTION

Diabetic retinopathy is the most common diabetic eye disease and a leading cause of blindness. Diabetic retinopathy is characterized increased microvascular permeability, cell damage, abnormal blood flow autoregulation, disordered angiogenesis and increased adhesive properties of the endothelium. All these factors can cause capillary occlusion, microvascular degeneration and abnormal neovascularization, then eventually leading to irreversible blindness. Hyperglycemia is an independent risk factor for the development of cardiovascular disease[1]. However, the mechanisms of hyperglycemia and hyperglycemia's effect on tissue damage and clinical complications remain unclear. Vascular endothelial growth factor (VEGF) might play a particularly important role in diabetic retinopathy[2]. The expression changes and effect of VEGF on apoptosis in retinal cells induced by hyperglycemia are still elusive.

The nonobese diabetic (NOD) mouse is an useful and important model of autoimmune type 1 diabetes. The incidence of spontaneous diabetes in the NOD mouse varied from 60% to 80% in females and 20% to 30% in males. Diabetes onset typically occurs at 12 to 14 weeks of age in female mice and slightly later in male mice[3]. This study aims to study the expression of VEGF on apoptosis in retinal cells in diabetic NOD mice.

## MATERIALS AND METHODS

**Animals** NOD mice (Jackson Laboratory, Bar Harbor, ME) were bred in the animal house of Central South University. Mice were housed by pairs in plastic cages under a pathogen-free environment, continuously access to food and water on a 12-hour light/dark cycle. All animal experiments were in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were considered to be diabetic when non-fasting blood glucose levels were >16.67mmol/L (One-Touch Lifescan meter; Lifescan, Inc., Milpitas, CA) using whole blood collected from the tail vein. Only females were used in the studies because disease progression in males is slower and less consistent[2]. Then these diabetic NOD mice were divided into 5 groups according to duration of diabetes (2, 4, 6, 8 and 12 weeks group after onset of diabetes, $n=30$). The control groups were made up of sibling without diabetes. Body mass and blood glucose concentration were monitored every week. Diabetic mice were not given routine insulin injections. At 2, 4, 6, 8 and 12 weeks after onset of diabetes, the mice were anesthetized with sodium pentobarbital (100mg/kg, IP) and decapitated. The diabetic NOD mice had significantly elevated concentrations of blood glucose compared with those in control group. At death, the NOD mice also weighed significantly less than the control animals (Table 1).

**Methods**

**VEGF expression** The eyes of both groups were rapidly removed and retinas were dissected, immediately frozen in liquid nitrogen and stored at -70℃. ELISA method was used to determine VEGF protein concentration according to the manufacturer's protocol (Quantikine Mouse VEGF ELISA kit; R&D Systems, Minneapolis, MN). The measurement was performed on three samples for each experimental condition. Four retinas of each sample were placed into 200μL of buffer

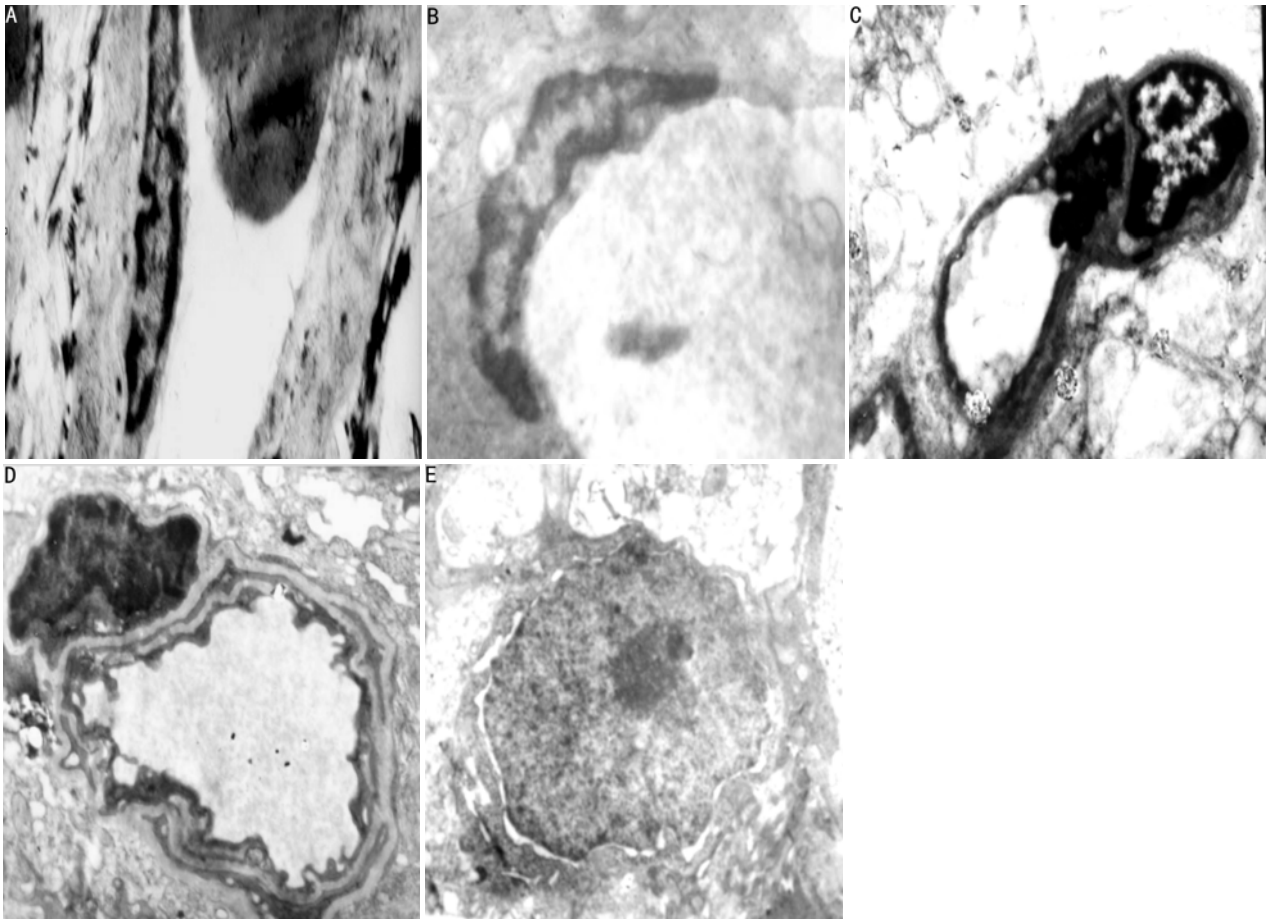


**Figure 1** A: Endothelial cell apoptosis; B: Pericyte apoptosis; C: Perivascular edema, obstruction of microvascular; D: Capillary basement membrane thickening and microvascular wall change; E: Ganglion cells apoptosis.

A and sonicated for 30 seconds at 50Hz. The lysate was centrifuged at 22 000g for 15 minutes at 4℃. VEGF concentration was determined spectrophotometrically (Microplate Reader 680 XR; Bio-Rad) at 450nm. In each experiment, all samples and standards were measured twice. Data were collected as picograms VEGF per milligrams of total protein and averaged in the same graph. Blood was centrifuged at 2 000g for 20 minutes and then serum was stored at -20℃. Same protocol was applied to both serum VEGF and VEGF in retina.

**Ultrastructural changes** The eyeballs from diabetic mice (diabetic 4 weeks and 12 weeks group) were prefixed by immersion in 22g/L glutaraldehyde in 0.1mol/L (pH 7.4) Sorensen's phosphate buffer for 4 hours, and then postfixed in 10g/L osmium tetroxide in 0.1mol/L (pH 7.2) sodium phosphate buffer. The samples were dehydrated in graded ethyl alcohol series and embedded in Araldite CY212. Ultrathin sections were contrasted with uranyl acetate and lead citrate for examination by transmission electron microscope (LEO 906E, Oberkochen, Germany).

**Statistical Analysis** Two-way analysis of variance, *t*-test and correlation analysis via the SPSS statistical program were used to analyze the data. $P<0.05$ was considered statistically significant.

## RESULTS

**VEGF expression** VEGF protein level in the diabetic NOD mice was increased compared with control mice. Retinal VEGF expression in diabetic mice of 2, 4, 6, 8 and 12 weeks group were(μg/g): 23.0±5.0, 33.0±7.0, 64.0±6.0, 92.0±7.0 and 165.0±9.0. There were no significant differences of retinal VEGF expression between 2, 4, 6, 8 and 12 weeks in non-diabetic groups($P<0.01$. Serum VEGF expression in diabetic mice of 2, 4, 6, 8 and 12 weeks group were (μg/g): 0.53±0.10, 0.76±0.10, 1.24±0.20, 2.37±0.20, 4.96±0.40, and no significant differences in non-diabetic mice ($P<0.01$, Table 1). There was linear correlation between retinal VEGF and serum VEGF in diabetic NOD mice($r=0.9902$, $P=0.001$).

**Ultrastructural changes** Retinal capillary basement membrane, endothelial cells and ganglion cells were normal in control group. Increasing membrane thickeness(Figure 1A), microvascular occlusion and perivascular edema can be observed in retinal capillary basement (Figure 1B, 1C); chromatin margination with nuclear deformation in the endothelial cells (Figure 1A) and pericyte (Figure 1B); an irregular aggregation of nuclear chromatin with vacuolar degeneration in the ganglion cells can also be observed in 4 weeks NOD group. Retinal pathological change became more obvious in 12 weeks NOD mice, which includes apparent thickening of capillary basement membrane (Figure 1D); nuclear membrane shrinkage and nuclear chromatin condensation in the endothelial cells (Figure 1D); cell shrinkage, pyknosis of nuclear, distribution of nuclear chromatin and loss of mitochondrial cristae in the ganglion cells (Figure 1E).

**Table 1 Body mass, blood glucose and VEGF levers in diabetes mice** ($\bar{x} \pm s$, $n = 30$)

| Group(wk) | Mass(g) | Glucose(mg/dL) | Retinal VEGF(μg/g) | Serum VEGF(μg/g) |
|---|---|---|---|---|
| Control 2 | 23.0±3.0 | 152.0±9.0 | 16.0±4.0 | 0.18±0.10 |
| 4 | 28.0±3.0 | 138.0±21.0 | 17.0±5.0 | 0.19±0.10 |
| 6 | 31.0±4.0 | 146.0±16.0 | 16.0±3.0 | 0.19±0.10 |
| 8 | 32.0±1.0 | 144.0±5.0 | 18.0±3.0 | 0.19±0.20 |
| 12 | 36.0±2.0 | 140.0±8.0 | 18.0±4.0 | 0.18±0.10 |
| Diabetes 2 | 23.8±1.2 | 479.0±33.0 | 23.0±5.0 | 0.53±0.10 |
| 4 | 24.0±2.3 | 477.0±23.0 | 33.0±7.0 | 0.76±0.10 |
| 6 | 22.0±3.2 | 488.0±27.0 | 64.0±6.0 | 1.24±0.20 |
| 8 | 20.0±4.3 | 558.0±44.0 | 92.0±7.0 | 2.37±0.20 |
| 12[b] | 18.0±3.5 | 576.0±27.0 | 165.0±9.0 | 4.96±0.40 |

[b]$P < 0.01$ *vs* respective 12 weeks value in control group (unpaired *t*-test).

## DISCUSSION

Increased glucose levels and fluctuation in blood glucose are two leading causes of retinal vascular injury in diabetic patients[4]. However, effect of VEGF on the pathogenesis of DR has not clear. Therefore, the study of the effect of VEGF on apoptosis in NOD retinal cells can help us to understand diabetic retinopathy. Increased glucose level can be observed in diabetic NOD mice at age of 12 weeks. After 4 weeks of onset of diabetes, retinal capillary basement membrane became thickening. Perivascular edema, pericytes, endothelial cells and ganglion cells apoptosis can also be found in NOD group. The pathological process became more obvious in the 12 weeks of diabetes. Capillary basement membrane became thicker; under the transmission electron microscope, more apoptosis of endothelial cells and ganglion cells can be observed. The vascular endothelial cell is a dynamic cell layer where they communicate chemical signals with other cells in the vessel wall. These signals contribute to monolayer integrity and vascular function[5]. The balance is seriously disrupted in the diabetic retinal microvasculature because of accelerated apoptosis of pericytes and endothelial cells resulting in progressive vasodegeneration[6]. Elevated glucose levels in diabets can interrupt normal cell substrate communication and vascular function, then induce the endothelial cell loss[7]. Damage of the endothelium is accompanied by breakdown of the inner blood retinal barrier. the process can lead the local release of cytokines and chemokines such as VEGF, IGF-1, SDF-1 by the adjacent endothelium[8,9]. Further more, increased VEGF expression can be found in the retina and serum of 4 weeks and 12 weeks NOD.

The potential relationship between endothelial cell apoptosis and VEGF expression is still ambiguous. Hyperglycemia induces cells hypoxia and vascular inflammation in retina. Then these retinopathy will stimulate retinal cells apoptosis and vascular changes. In the other side, these apoptotic bodies can make uninjured endothelial monolayers to upregulate VEGF expression. VEGF is responsible for the growth of new blood vessels via the stimulation of endothelial cell proliferation. VEGF also stimulates endothelial cell proliferation, migration, and survival. When retinal pigment epithelial cells begin to apoptosis because of lack of nutrition (ischemia), VEGF takes over to create neovascularization and acts as a restorative function in other parts of the body. VEGF can be massively upregulated by hypoxia. Its levels are increased in the retina and vitreous of patients and in animal models of ischemic retinopathy. A recent report had showed that 14 weeks diabetic mice from streptozotocin injection exhibited neuronal cell death in the retinal ganglion cell layer[10]. In this study, we demonstrate that the retinas of the NOD mice showed retinal vascular pathlogical changes, apoptosis of both retinal vascular cells and retinal neuronal cells. These hyperglycemia-induced retinopathy were attenuated with time, which indicated that hyperglycemia plays an important role in the pathogenesis of this disease. These changes become worse with the increasing VEGF expression in the retina and blood. Retinopathy in NOD mice, such as retinal vascular cells and neuronal degeneration were all deteriorated by hyperglycemia and VEGF, which indicated that VEGF and hyperglycemia plays an important role in the pathology of this disease. There are many factors related with retinal VEGF expression in the early diabetes, such as hyperglycemia, increased retinal cells apoptosis, inflammation and ischemia. All these risk factors have an effect on the diabetic retinopathy.

## REFERENCES

1 Dabir P, Marinic TE, Krukovets I, Stenina OI. Aryl hydrocarbon receptor is activated by glucose and regulates the thrombospondin-1 gene promoter in endothelial cells. *Circ Res* 2008;102(12):1558-1565

2 Caldwell RB, Bartoli M, Behzadian MA, , El-Remessy AE, Al-Shabrawey M, Platt DH, Liou GI, Caldwell RW. Vascular endothelial growth factor and diabetic retinopathy: role of oxidative stress. *Curr Drug Targets* 2005;6(4):511-524

3 Lu Y, Tang M, Wasserfall C, Campbell-Thompson M, Gardemann T, Crawford J, Atkinson M, Song S. Alpha1-antitrypsin gene therapy modulates cellular immunity and efficiently prevents type 1 diabetes in nonobese diabetic mice. *Hum Gene Ther* 2006;17(6):625-634

4 Santos KG, Tschiedel B, Schneider JR, KEP Souto, I Roisenberg. Prevalence of retinopathy in Caucasian type 2 diabetic patients from the

South of Brazil and relationship with clinical and metabolic factors. *Braz J Med Biol Res* 2005;38(2):221-225

5 Heissig B, Hattori K, Friedrich M, Rafii S, Werb Z. Angiogenesis: vascular remodeling of the extracellular matrix involves metalloproteinases. *Curr Opin Hematol* 2003;10(2):136-141

6 Feng Y, vom Hagen F, Lin J, Hammes HP. Incipient diabetic retinopathy: insights from an experimental model. *Ophthalmologica* 2007;221(4):269-274

7 Yamagishi S, Imaizumi T. Diabetic vascular complications: pathophysiology, biochemical basis and potential therapeutic strategy. *Curr Pharm Des* 2005;11(18):2279-2299

8 Butler JM, Guthrie SM, Koc M, Afzal A, Caballero S, Brooks HL, Mames RN, Segal MS, Grant MB, Scott EW. l. SDF-1 is both necessary and sufficient to promote proliferative retinopathy. *J Clin Invest* 2005;115(1):86-93

9 Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res* 2004;95(4):343-353

10 Martin PM, Roon P, Van Ells TK, Ganapathy V, Smith SB. Death of retinal neurons in streptozotocin- induced diabetic mice. *Invest Ophthalmol Vis Sci* 2004;45(9):3330-3336

## NOD 小鼠视网膜 VEGF 表达和视网膜细胞凋亡

李才锐,孙曙光
(作者单位:671000 中国云南省大理市,云南大理学院附属医院眼科)

作者简介:李才锐,副院长,副教授,博士,研究方向:玻璃体视网膜疾病。
通讯作者:孙曙光,副教授,博士. lcrbrett@163.com

**摘要**

**目的**:研究 NOD 小鼠早期糖尿病视网膜 VEGF 表达和视网膜细胞凋亡情况,以及二者间的关系。

**方法**:NOD 小鼠分为对照组(非糖尿病小鼠)(2,4,6,8,12wk 组,$n=30$)和糖尿病组(2,4,6,8,12wk 组,$n=30$)。每组小鼠在规定时间处死,提取血液标本,摘除眼球,分离视网膜备用。ELISA 法检测视网膜 VEGF 和血液 VEGF。透射电子显微镜检测小鼠视网膜细胞凋亡情况。

**结果**:糖尿病组血液和视网膜 VEGF 表达与对照组相比明显增高(12wk,血液标本:4.9±0.4μg/g *vs*0.19±0.1μg/g,$P<0.01$;视网膜,165.0±9.0μg/g *vs*18.0±4.0 μg/g,$P<0.01$)。NOD 小鼠早期糖尿病视网膜 VEGF 表达和血液 VEGF 表达呈正相关($\gamma=0.9902$,$P=0.001$)。糖尿病组视网膜神经节细胞和血管内皮细胞凋亡明显增加 $P<0.01$。

**结论**:视网膜 VEGF 表达增加可能与视网膜凋亡增多有关。早期糖尿病 NOD 小鼠视网膜 VEGF 表达增加是多因素的。

**关键词**:视网膜;凋亡;血管内皮细胞;NOD 小鼠