·Basic Research·

Effects of high-concentration insulin on expression of vascular endothelial growth factor in cultured Müller cells *in vitro*

E Song [1], Wei Yang [1], Zhi-Hua Cui [1], Jia-Xiang Wu [2]

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[1]Department of Ophthalmology, the First Affiliated Hospital of Jilin University, Changchun 130021, Jilin Province, China

[2]Department of Pathology, School of Basic Medicine, Jilin University, Changchun 130021, Jilin Province, China

Correspondence to: E Song. Department of Ophthalmology, the First Affiliated Hospital of Jilin University, Changchun 130021, Jilin Province, China. songe23@21cn.com

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Abstract

• AIM: To study the effect of high-concentration insulin on the expression of vascular endothelial growth factor (VEGF) in cultured rabbit retinal Müller cells *in vitro*.

• METHODS: Müller cells were cultured with insulin of different concentrations (4×10^3, 8×10^3, 12×10^3 U/L). Immunocytochemistry, *in situ* hybridization and ELISA were conducted to assay the expression of VEGF in cultured Müller cells *in vitro* at different insulin concentrations qualitatively and quantitatively.

• RESULTS: VEGF expression was enhanced obviously by high concentration of insulin.

• CONCLUSION: Insulin plays an important role in neovascularization of diabetic retinopathy (DR) by stimulating the transcription of the VEGF gene in Müller cells so as to enhance the expression of VEGF protein.

• KEYWORDS: insulin; vascular endothelial growth factor; Müller cells; diabetic retinopathy

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INTRODUCTION

Diabetic retinopathy (DR) is one of severe microangiopathic complications of diabetes in which neovascularization is the main pathological change. Recent studies prove that many growth factors take part in the formation and development of DR, among which vascular endothelial growth factor (VEGF) plays a crucial role [1]. Meantime, high dosage insulin has been recognized as an independent perilous factor for DR [2]. In this paper, the effects of insulin at different concentrations on VEGF expression in Müller cells *in vitro* were observed so as to find out how insulin enhances the development of the DR.

MATERIALS AND METHODS

Materials

Reserch animals Five adult New Zealand rabbits weighing from 2.5 to 3.0 kilograms, of either sex, were provided by the Experimental Animal Center of Jilin University.

Main reagents Main reagents include Dulbecco's modified Eagle's medium (DMEM), neocalf serum of Gibco Company, rabbit antihuman glial fibrillary acidic protein (GFAP) monoclonal primary antibody, VEGF monoclonal primary antibody, horseradish peroxidase labeled avidin (SP reagent), biotin labeled goat anti-rabbit IgG, VEGF ELISA reagent(Boshide Bioengineering Co. Ltd., Wuhan) and DAB color reagent (Maixin Bioengineering Co. Ltd., Fuzhou). The sequence of VEGF is synthesized by Shanghai Biological Detection Kit, which is: 5'-GAGCCTTGGTGGACATCTT-3', 5'-ATGATCTGCATGGTGACATT-3.

Methods

Primary culture of Müller cells Pentobarbital sodium 30g/L (1mL/kg) was injected into the periauricle vein. The eyeball was enucleated and dissected along the ora serrata. Lens and vitreous body were removed while the posterior half of the ocular wall was kept and immersed into the DMEM solution. The pigment epithelium was removed from the retina and the latter was cut into pieces(each about $1mm^2$). The tissue was left in an incubator with 50mL/L CO_2 at 37℃ for 7 days when the rim of the tissue blocks became stuck to the wall, shining and cells began to grow out. Then tissue blocks were aspirated for centrifugation at

800r/min for 5 minutes. The sedimentation was gently pipetted to be cultured for two weeks until cells grew from the margin of the tissue block and 80% of them were merged. These cells were digested and subcultured.

Cell identification The subcultured cells were taken out when they were nearly merged, rinsed with D-Hank's solution, and then fixed in 40g/L polyoxymethylene for 20 minutes. The endogenous peroxidase was blocked for 20 minutes with 30mL/L H_2O_2, and the non-specific background pigmentation was also blocked with normal goat serum for 20 minutes. GFAP (dilution 1:100) was added in the experimental group, while PBS was added in the control group (0.01mol/L, pH 7.2). Then they were kept at 4℃ overnight. The next day, biotin labeled goat-anti-rabbit IgG was added, which was followed by horseradish peroxidase labeled avidin (SP reagent, Bosdon company). DBA was used to color the cells and cells with brown yellow filamentous network in the cytoplasm were considered GFAP positive.

Grouping The second-generation cells were used for the experiment. Cover-slips were placed onto the 24-pore board and cells were dropped evenly into the pores. Seven days later when 80% cells merged, the nutrient solution containing serum was replaced with the serum-free nutrient solution. After 24 hours' culture, the serum-free solution was discarded, and cells were then cultured in insulin at different concentrations (0, 4, 8, 12kU/L) for 24 hours.

VEGF immunohistochemistry VEGF antibody acted as the primary antibody and the other steps were the same as those in cell identification.

VEGF *in situ* hybridization All appliances used for *in situ* hybridization should be sterilized by conventional antisepsis and liquid sterilized by autoclavng. The main procedures were as follows. The cover-slip was fixed in polyoxymethylene with 1/1 000 DEPC. The endogenous peroxidase was blocked with 30mL/L H_2O_2. The cells were blocked with newly prepared 5mL/L H_2O_2 and digested with citrate compounded pepsin. Probe (5'-GAGCCTTGGTGGAC ATCTT-3', 5'- ATGATCTGCATGGTGACATT-3) was added after 2-4 hours' prehybridization and incubated at 37℃ overnight. The solution was washed with 2×SSC, 0.5× SSC and 0.2×SSC respectively. The non-specific background pigmentation was blocked, and then the rabbit anti-digoxin was dropped . Biotin labeled goat-anti-rabbit IgG, SABC and Biotin labeled peroxidase were added subsequently. DBA was used to color the cells and the nuclei were restained. The specimens were dehydrated with graded ethanol and clarified with xylene. Finally the cover-slips were sealed by neutral gum.

ELISA The reagent kit was purchased from Bosdon Company. We followed the instructions and the result was determined by enzyme labeling. ① Preparation of the standard samples: after VEGF and 1mL dilution were mixed evenly by shaking, 250μL of the solution were transferred into appen 1. Into appen 1 was added 750μL dilution and they were mixed evenly. Take out 200μL appen 1, add appen solution 800μL to it and mix them evenly-this procedure was repeated 4 times to obtain 5 samples with different concentrations. ② DMEM 100μL was added into a well as negative control and 100μL of the sample was added into the other wells. ③ Preparation of the monoclonal antibody: monoclonal antibody and 3.5mL dilution were mixed. Add 25μL of the mixed solution into each well. Leave it for 3 hours with the cover-slip on. ④ Preparation of the rinsing solution: the condensed rinsing solution was diluted with ion-free water in proportion of 1:20. ⑤ Preparation of the enzyme: condensed enzyme was diluted with 6mL dilution. To each well, 50μL enzyme was added. Leave it for 45 minutes. ⑥ Discard the supernatant, add the wash solution 250μL and elute 5 times. ⑦ Preparation of the coloring reagent: reagent A and B were mixed in proportion of 1:1. To each well, 200μL coloring reagent was added. Leave it at room temperature for 20 minutes. Then the absorbance of 492nm was measured.

Result evaluation ① Image analysis of immunocytohistology and *in situ* hybridization: seven visual fields on each section were selected randomly to be analyzed. The amplitude of positive cells was confirmed in each visual field and the gray levels were determined. The results were recorded as $\bar{x}\pm s$. ② VEGF ELISA result determination: we made a double logarithm curve based on the determined values of the standard substance and their corresponding concentrations. The results were recorded as $\bar{x}\pm s$. ③ Statistical processing: all the results were analyzed by *t*-test(Table 1).

RESULTS

Culture and Identification of Müller Cells In the 7-day primary culture, the tissue blocks began to stick to the wall and several pseudopodia grew from the rims of the blocks, which gradually crept out of the tissue blocks, thus giving birth to isolated cells. On the 14th day, more than 80% cells merged. In HE staining, cells were of ellipse shape, rich in cytoplasm; the nuclei which were ellipse in shape and light

Table 1 VEGF expressions in cultured Müller cells at different insulin concentrations ($\bar{x} \pm s$)

Method	Control	4×10^3U/L	8×10^3U/L	12×10^3U/L	*F* value
Immunocytohistorlogy	172.3 ± 4.9	132.5 ± 5.2[a]	179.1 ± 5.1[a]	179.8 ± 5.3[a]	3.298
In situ hybridization	124.7 ± 4.9	132.5 ± 5.2[a]	133.1 ± 4.9[a]	133.8 ± 5.0[a]	5.054
ELISA	20.3 ± 3.4	28.5 ± 3.0[a]	29.1 ± 2.6[a]	30.1 ± 2.6[a]	16.942

[a]$P<0.05$ *vs* normal control

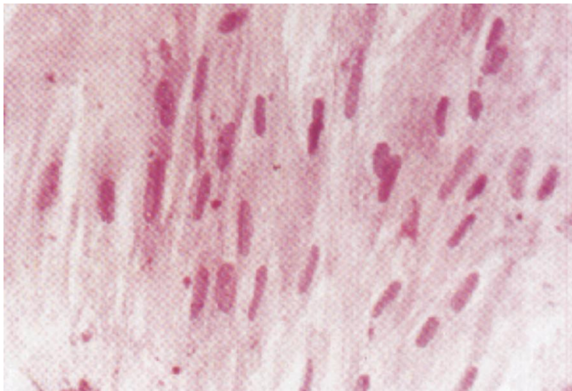

Figure 1 Müller cells in HE staining

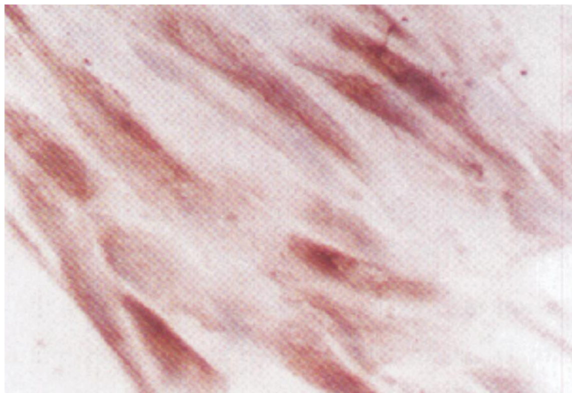

Figure 2 Müller cells in GFAP immunocytohistology

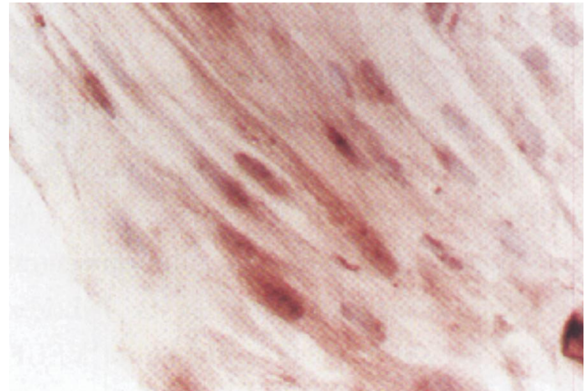

Figure 3 Expression of VEGF protein in cultured Müller cells

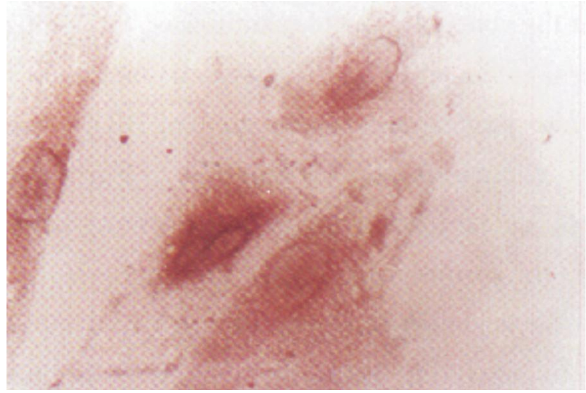

Figure 4 Expression of VEGF mRNA in cultured Müller cells

in staining were located in the center of the plasm, and had 2 or more nucleoles (Figure 1). Using GFAP immunocyto-histology, yellow-brown filamentous reticular structure was detected in cytoplasm of more than 80% cells (Figure 2). Subcultured cells (P_1 and P_2) showed configuration similar to that of the primary cultured cells. After one week, the cells began to merge and more than 90% cells were positive by using GFAP immunocytohistology. In further subcultured cells, the normal cell shape was lost. We adopted P_2 cells as the experimental cells.

Effects of Insulin on the Expression of VEGF in Müller Cells The higher the concentration of insulin, the stronger the expression of VEGF protein and mRNA in cultured Müller cells; consequently, the difference between the experiment groups and the control group became more and more significant with the increase of insulin concentration ($P<0.05$, Figure 3,4). However, no significant difference was detected in VEGF expression among the experimental groups of different insulin concentrations (4, 8 ,12kU/L).

DISCUSSION

VEGF is a specific regulating factor of vascular growth. Whether in normal physiological conditions (such as embryo development and wound repairing) or in pathological conditions (such as inflammation, proliferated diabetic retinopathy, tumor growth, central retinal vein occlusion and premature retinopathy), VEGF is closely related to development and proliferation of blood vessels [3]. In DR, VEGF can promote splitting and proliferation of vascular

epithelial cells through binding to high-affinity antibody and leading to retinal neovascularization [4]. Because VEGF plays an important role in the formation and development of DR, many researches are being made about its functional mechanisms and regulation.

Expression of VEGF induced by hypoxia is the main cause of DR, in which VEGF is excreted mainly by Müller cells [5]. In our previous researches, expression of VEGF, mainly in epithelium cells and glial cells, enhanced remarkably when the DR animal model was established for 5 months [6]. High dosage insulin can induce hyperinsulinism and has proved an independent perilous factor for DR[2]. In this experiment, we observed the change of VEGF expression in cultured Müller cells *in vitro* at different insulin concentrations. Results of immunocytochemistry, *in situ* hybridization and ELISA showed that the expression of VEGF and mRNA was stronger at higher insulin concentrations compared with the control group (P<0.05) and the higher the concentration of insulin was, the stronger the expression tended to be. So it is demonstrated that insulin may promote the development of DR by stimulating the expression of VEGF protein. Further studies need to be performed to investigate how insulin increases the expression of VEGF protein.

It has been shown that insulin can depolarize membrane by activating the K^+ channel of retina pericytes, make pericytes contract, decrease the blood volume, lead to retinal ischemia and hypoxia, enhance the capillary permeability, and eventually cause DR [7]. Some researches have proved that insulin can lead to retinal neovascularization by stimulating DNA synthesis in vascular epithelial cells, which accelerates proliferation of these cells and promotes generation of vascular sprout [8]. Our experiment showed that *in situ* hybridization, the mRNA VEGF expression was stronger in Müller cells cultured with insulin than in the control group, and the higher the concentration was, the stronger the expression seemed to be. This result shows that high concentration of insulin *in vitro* can induce transcription of VEGF gene and enhance VEGF protein expression in Müller cells to induce the occurrence and development of DR, which offers another approach to understanding the role of insulin in DR development.

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