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IGF-1 regulates Ang II and VEGF signaling pathways in retinal neovascularization

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Abstract. – OBJECTIVE: To investigate the effect of insulin-like growth factor (IGF)-1 on regulating the occurrence of retinal neovascularization and the expression mechanism of Angiotensin II (Ang II) and vascular endothelial factor (VEGF).

MATERIALS AND METHODS: A total of 40 C57BL/6J healthy mice of either gender were selected, and randomly divided into 4 groups: control group, model group, IGF-1 overexpression carrier intervention group and IGF-1 siRNA intervention group (10 in each group). Mice in control group were fed in the air without any further processing. The mice in other 3 groups were used to build high oxygen-induced retinal neovascularization model. The vitreous cavity of mice in the model group was injected with the equal amount of normal saline, while the mice in the IGF-1 overexpression carrier intervention group were injected with 1 μl IGF-1 liposomes mixture. At the same time, IGF-1 siRNA intervention group mice were continuously injected with 1 μl IGF-1 siRNA liposomes mixture for 3d. Stretched preparation of retina was used to observe the neovascularization form; HE staining tissue section were prepared to observe the amount of vascular endothelial nuclei out of the retina boundary; IGF-1, Ang II and VEGF expression levels of retinal tissue were detected by RT-PCR and Western blot.

RESULTS: A large area of non-perfusion and tortuous expansion form of the blood vessel was found in the optic nerve surroundings of retinal serial sections in model group, while neovascularization plexus and fluorescence leakage were observed between perfusion and non-perfusion area. Non-perfusion and neovascularization have experienced a decrease in IGF-1 overexpression group but an increase in IGF-1 siRNA group. The number of neovascularization endothelial nuclei out of the internal limiting membrane in model group was remarkably higher than that in control group. This number in IGF-1 over-expression group decreases while in IGF-1 siRNA group it increases, with a significant statistical difference ($p < 0.05$). Histone IGF-1 expression level in the model group was lower than that in the control group, but Ang II and VEGF expression level were higher. IGF-1 in over-expression group was higher but with a lower expression level of Ang II and VEGF; however, the results in siRNA group were opposite. The difference was statistically significant ($p < 0.05$).

CONCLUSIONS: The occurrence of retinal neovascularization is related to the reduction of IGF-1 expression and the increase of Ang II and VEGF.

Key Words:
IGF-1, Retinal neovascularization, Ang II, VEGF.

Introduction

Proliferative diabetic retinopathy, retinopathy of prematurity (ROP) and retinal vein occlusion are retinal neovascular diseases which are severe blinding eye disease worldwide and account for 30-60% of the total disease incidence[1]. Retinal laser photocoagulation, cryotherapy, and vitrectomy are a common clinical treatment for blinding eye disease, but no significant improvement or prevention of vision progress have been observed[2]. A topical application of vascular endothelial growth factor (VEGF) antagonists to some extent has a good effect on the treatment, but it cannot completely inhibit the angiogenesis and usually has a high recurrence rate[3]. The formation of retinal neovascularization is a fine adjustment process with the participation of a variety of cytokines. Ischemia and anoxia are important inducing factors which can cause an increase of hypoxia-inducible factor (HIF) and insulin-like

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growth factor expression level[4,5]. Also, multiple cell signaling pathways like tumor growth factor (TGF)-β and nuclear factor (NF-κB)[6] can regulate the secretion and expression of angiotensin (Ang) II, VEGF and matrix metalloproteinase[7,8] to eventually promote the formation of neovascularization. Our research mainly focuses on providing a reference for finding the effective disease intervention target by establishing mice model and analyzing the mechanism of IGF-1 on regulating the formation of neovascularization, Ang II, and VEGF expression.

Materials and Methods

Animals

40 C57BL/6J healthy mice of either gender with an age of 7 d have been selected; these mice were purchased from Beijing Vitalriver Laboratory Animal Co., Ltd., (Beijing, China). The study was approved by the Animal Ethics Committee of Wuhan University Animal Center. Model establishment: mice and their breastfeeding mothers were put together in the airtight oxygen cabin with a pressure of about 100kPa, oxygen flow rate of 1.5 L/min and oxygen concentration of (75±2)%; the exhaust vent was connected to CY-100 digital oxygen meter, indoor ventilation and temperature (22±2)°C were maintained well; fluorescent lamp was used for lighting, both light and dark time were 12 h. The breastfeeding mothers were replaced every two days; after 5 days, the mice could be taken out of the oxygen cabin and continuously being fed in the air.

Research Method and Observational Index

All 40 mice were randomly and evenly divided into 4 groups: control group, model group, IGF-1 Overexpression carrier Intervention group and IGF-1 siRNA intervention group. The mice in control group were fed in the air without any further processing. The mice in other 3 groups were used to build high oxygen-induced retinal neovascularization model. The vitreous cavity of mice in the model group was injected with the equal amount of normal saline, while the mice in the IGF-1 overexpression carrier intervention group were injected with 1 μl IGF-1 liposomes mixture. IGF-1 siRNA intervention group mice were continuously injected with 1 μl IGF-1 siRNA liposomes mixture for 3 d and, thereafter, sacrificed. Stretched preparation of retina was used to observe the neovascularization form; HE staining tissue section was prepared to observe the amount of vascular endothelial nuclei out of the retina boundary, and the IGF-1, Ang II and VEGF expression level of retinal tissue were detected by RT-PCR and Western blot. Based on the mRNA sequence of IGF-1 gene of mice in GeneBank, IGF-1 overexpression and siRNA base sequence have been synthesized by Nanjing Kgi Biological Technology Development Co., Ltd. The overexpression sequence is (F): 5'-CCAAGACUCUAGACAACUA-3'; (R): 5'-GGUUCUGAGAUCUGUUGAU-3'; the siRNA sequence is (F): 5'-GCAACAUGCUCAAGCCAAA-3'; (R): 5'-CGUUGUACGAGUUCGGUUU-3'. Reorganized with the carrier after digestion, Lipofectamine™2000 was transfected into mice vascular endothelial cells by liposome transfection, as described in the manual.

Retinal Serial Sections

Cavity injection of anesthesia for mice was made with 0.01 ml 1% pentobarbital sodium; 1 ml dextran fluorescein isothiocyanate FITC-Dextran solution (50 mg: 1 ml phosphate-buffered saline, PBS) was drawn by avoiding light syringe; hearts were continuously perfused till the tip of ear, nose and mouth, extremities and tail were stained to green rapidly. The eyeball was taken out and fixed in 4% paraformaldehyde for 10 min, a snip was opened into the retina radially from the center of the nipple, and anti-fluorescence quenching tablets were dropped on the surface of the retinal serial sections, which were covered with a coverslip and observed under the fluorescence microscope.

HE Staining

After fixation, dehydration and embedding, eyeballs were cut into 5 μm-thick sections. 8 sections without optic nerve were randomly selected, stained with HE and observed under an inverted optical microscope. 80 sections were selected in each group and counted, and the average was taken.

RT-PCR

The normal TRIzol agent was used to extract the total RNA in cells, UV spectrophotometer was applied for the concentration and purity test, and cDNA was synthesized using reverse transcription kits. Primer sequences were synthesized by Shanghai Sangon Ltd., based on the sequences in GeneBank: IGF-1: (F) 5'-CAGGCAGGTATGCTAGGAGC-3', (R) 5'-TCAAGGTATTTCCCAGTGCC-3'; Ang

II: (F) 5'-CCTTGTGGCTACTCTTCCTC-3', (R) 5'-ACTTGGTCACGGGTTACTCT-3'; VEGF: (F) 5'-CCTGGCTTTACTGCTGTACCT-3', (R) 5'-GCTGGTAGACGTCCATGAACT-3'; GAPDH (F): 5'-CGCGAGAAGATGACCCAGAT-3', R: 5'-GCACTGTGTTGGCGTACAGG-3'. The reaction system is as follows: 2 μL cDNA, 3 μL forward and 3 μL reverse primers, 0.5 μL Taq polymerase, 1 μL dNTPs, 3 μL $MgCl_2$, 5 μL 10×Buffer and 2.5 μL ddH_2O_2. The reaction conditions are as follows: 95°C for 5 min, 95°C for 30 s, 58°C for 30 s and 75°C for 50 s, a total of 45 cycles, and terminated at 76°C for 5 min. 2% agarose gel electrophoresis was used to detect the PCR product, followed by UV imaging via gel imaging analysis system and gray value analysis using a digital camera; the results were presented as $2^{-\Delta\Delta Ct}$.

Western Blot

RIPA lysate was added into the tissue homogenate. Total proteins were extracted from the cells, and the bicinchoninic acid (BCA) method was used to detect the concentration and purity of proteins. 10 μg total protein were taken and separated via 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred from separation area onto polyvinylidene difluoride (PVDF) membrane; then, rabbit anti-mouse IGF-1, Ang II and VEGF monoclonal antibodies (1:2000, Sigma-Aldrich, St. Louis, MO, USA) were added overnight. Thereafter, sheep anti-rabbit polyclonal secondary antibody (1:500, Sigma-Aldrich, St. Louis, MO, USA) was added for incubation at ambient temperature for 4 h. Phosphate-buffered saline (PBS) was used to wash the mixture, and ECL was used for color development. The results were scanned and saved, followed by semi-quantitative analysis using Lab Works4.5 gel imaging software (Invitrogen, Carlsbad, CA, USA). The results were expressed as integral optical density (IOD) with β-actin as an internal reference gene.

Statistical Analysis

Quantitative data are expressed as mean ± standard deviation after normality test. One-way ANOVA was used for the intergroup comparison. Least significant difference (LSD) *t*-test was applied for the pairwise comparison; $p < 0.05$ suggested that the difference was statistically significant. Statistical Product and Service Solutions (SPSS) 20.0 software package (IBM SPSS, Armonk, NY, USA) was used for analysis.

Results

Retinal Serial Section Analysis

The fluorescence microscope showed the clear and uniform arrangement of retinal vessels, radial distribution of superficial vessels, net structure of deep vessels without neovascularization plexus, as well as thin superficial vessels and thick deep vessels in control group. A large non-perfusion area could be found around optic nerve head, vessels were tortuous and expanded, and neovascularization plexus and fluorescence leakage could also be observed between perfusion and non-perfusion areas in model group. Non-perfusion area and neovascularization were decreased in IGF-1 overexpression group but increased in IGF-1 siRNA group.

HE Staining Analysis

The number of neovascular endothelial nuclei passing through the internal limiting membrane in model group was remarkably larger than that in control group. The number of IGF-1 overexpression group was decreased but increased in IGF-1 siRNA group, and the difference was statistically significant ($p < 0.05$) (Figure 1).

RT-PCR Analysis

The IGF-1 mRNA expression level in model group was evidently lower than that in control group, but the Ang II and VEGF expression levels were increased; the IGF-1 mRNA expression level in overexpression group was increased, but the mRNA expression levels of Ang II and VEGF were decreased; the results in siRNA group were the opposite. The differences were statistically significant ($p < 0.05$) (Table I).

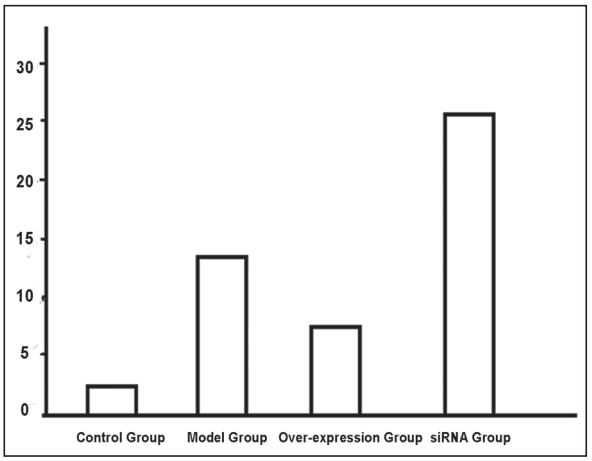

Figure 1. Number of neovascularization endothelial nuclei out of the internal limiting membrane (200×).

Table I. RT-PCR analysis.

Group	Control group	Model group	Over-expression group	siRNA group
IGF-1	0.2652 ± 0.0659	0.1235 ± 0.0532	0.5216 ± 0.2145	0.0659 ± 0.0057
Ang II	0.1427 ± 0.0458	0.3569 ± 0.1024	0.0639 ± 0.0056	0.4247 ± 0.2136
VEGF	0.1758 ± 0.0356	0.5214 ± 0.1635	0.0527 ± 0.0049	0.5869 ± 0.2658

Western Blot Analysis

The IGF-1 protein expression level in model group was significantly lower than that in control group, but Ang II and VEGF expression levels were increased. The IGF-1 protein expression level in overexpression group was increased, but the protein expression levels of Ang II and VEGF were decreased; the results in siRNA group were the opposite. The differences were statistically significant ($p < 0.05$) (Table II).

Discussion

IGF-1 can play a role as insulin-like substance metabolism in the body, which also has important regulatory functions for cell proliferation and differentiation[9]. Kong et al[10] found that the decrease of serum IGF-1 level is related to the occurrence of ROP. Methimazole can delay the normal retinal vascular development of neonatal mice and induce the neovascularization, which is probably related to the decrease in serum IGF-1 level[11]. The VEGF expression level in retinal tissues of mice with IGF-1 knockout is significantly decreased. Exogenous application of VEGF can only partially reverse the angiogenesis, indicating that IGF-1 plays an important role in regulating the retinal neovascularization[12]. Growth retardation is an important factor of the immature retinal neovascularization. Neovascularization usually has an incomplete endothelial cell structure, few gap junctions, immature functions, and high vascular permeability. Plasma, blood cells, and other visible components tend to congregate in the intercellular space and cause microcirculation disturbance of neovascularization with extracellular matrix (ECM), further inducing the expressions of a variety of inflammatory cytokines and cell adhesion molecules[13]. In this research, the structure and function of neovascularization in retinal serial sections were immature so IGF-1 might exert functions through vascular maturation[14]. Many studies[15,16] have shown that the interaction between Ang II and VEGF jointly promote the retinal neovascularization. Renin-angiotensin system (RAS) exists in the retina of human and many other organisms. Ang II is a main effector molecule, regulating the vascular activity and acting as a growth factor with type-1 and type-2 receptors. Besides, Ang II can promote the migration and aggregation of vascular endothelial cells, fibroblasts, and monocyte-macrophages, and regulate the cell growth, differentiation, apoptosis, and extracellular matrix deposition[17]. Ang II has an essential effect in the treatment of hypertension, myocardial reperfusion injury, atherosclerosis, and cancer[18]. VEGF has been identified as the most effective cytokine to promote the angiogenesis[19]. This research found that the retina neovascularization was related to the decrease of IGF-1 expression level and the increase of Ang II and VEGF expression levels. IGF-1 could negatively regulate the expressions of Ang II and VEGF. However, some studies[20] have also found that IGF-1 can positively promote the effect of VEGF in angiogenesis. The effect of VEGF on promoting angiogenesis is weak in case of inadequate secretion of IGF-1. IGF-1 acceptors are widely distributed in capillary endothelial cell of retinal nerve sensory

Table II. Western blot analysis.

Group	Control group	Model group	Over-expression group	siRNA group
IIGF-1	0.24 ± 0.11	0.13 ± 0.05	0.56 ± 0.21	0.08 ± 0.02
Ang II	0.12 ± 0.06	0.37 ± 0.13	0.05 ± 0.01	0.44 ± 0.22
VEGF	0.15 ± 0.07	0.55 ± 0.19	0.06 ± 0.01	0.56 ± 0.28

layer, pigment epithelium layer, choroidea, and retina. IGF-1 is a polypeptide generated after the intraocular vessels being activated[21]. The decrease of IGF-1 expression, which is earlier than neovascularization, can be observed in the acid- and hyperoxia-induced retinal neovascularization model. The increase of VEGF expression level is consistent with the peak area of neovascularization[22].

Conclusions

We showed an important theoretical basis for the application of IGF-1 in the treatment of retinal neovascular lesions. Further investigations are needed to verify its application and dosage and whether it is also effective in systemic application, so as to provide important intervention targets for the clinical treatment.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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