·Basic Research·

Inhibition of corneal neovascularization by vascular endothelia growth inhibitor gene

Hong Wang, Bing Wang

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Department of Ophthalmology, the Provincial Hospital Affiliated to Shandong University, Jinan 250021, Shandong Province, China
Correspondence to: Hong Wang. Department of Ophthalmology, the Provincial Hospital Affiliated to Shandong University, Jinan 250021, Shandong Province, China. ophex2001@yahoo.com.cn
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Abstract

• AIM: To evaluate the effect of Effectene™ lipofectine mediated plasmids encoding human $pcDNA_4$-vascular endothelia growth inhibitor ($pcDNA_4$-VEGI) gene on corneal neovascularization (CNV).

• METHODS: Forty New Zealand albino rabbits were sutured by 5-0 silk on the superior cornea to induce CNV and divided into 4 random teams, ten per each team: team A: transfected by $pcDNA_4$-VEGI gene mediated by Effectene™ lipofectine transfection; team B: by plasmid $pcDNA_4$; team C: by Effectene™, and team D: by normal saline. Length and area of CNV were observed under slit lamp every day after transfection. Immunohistochemistry was performed to detect the expression of VEGI protein in corneas at day 3, 7, 14 and 21.

• RESULTS: 1) Average occurrence of CNV was 6.3 days in team A, 3.1 days in team B, 3.2 days in team C, and 3.2 days in team D. Difference was significant between A and other teams ($P<0.01$); 2) Length and average area of CNV in each period in team A was significantly different from those in team B, C and D ($P<0.01$); 3) VEGI expressions were observed in epithelium, stroma, endothelium and the cliff of CNV in team A at 3 days after transfection by immunohistochemical staining. None VEGI positive cells were found in the control teams (team B, C and D) all the time.

• CONCLUSION: Effectene™ lipofectine transfection technique can effectively transfect $pcDNA_4$-VEGI gene into rabbit cornea and the length and CNV areas can be inhibited by VEGI gene.

• KEYWORDS: corneal neovascularization; vascular endothelia growth inhibitor; gene therapy
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INTRODUCTION

There is no vascular in normal and health corneas. Corneal neovascularization (CNV) is a serious pathologic condition and can cause visual loss. It can also lead the anterior segment's immune privileged (ASIP), which plays a central role in the disequilibrium of ocular cytokine network and decreases reproductive system or tissue repair[1]. Statistics showed that in most progressing countries, CNV was still the most common causes of blindness and disable. Conventional treatments such as corticosteroid, laser, surgical intervention could provide only symptomatic treatment of the disease without addressing the underlying cause [2]. To inhibit the angiogenic stimuli may be able to provide a more effective treatment.

Recently, a new member of the human tumor necrosis factor (TNF) family named as vascular endothelial cell growth inhibitor (VEGI) was reported as a novel cytokine which can inhibit the proliferation of endothelial cells, angiogenesis and tumor growth [3]. VEGI gene has been identified from the human umbilical vein endothelial cell cDNA library, mapped to human chromosome 9q32, and the size of VEGI mRNA was approximately 6.5kb, the cDNA for VEGI encoded a protein of 174 amino acid residues with the characteristics of a type II transmembranous protein. VEGI transcript was found to be expressed in placenta, lung, kidney, skeletal muscle, pancreas, spleen, prostate, small intestine, and colon. Little VEGI signal was detected in heart, brain, liver, thymus, testis, ovary, and peripheral blood lymphocytes. Unlike other members of the TNF family, VEGI is specifically expressed in endothelial cells.

In the first study, VEGI was looked as an anti-tumor factor, and then was observed the activity was mediated through the control of tumor angiogenesis. Although its role in angiogenesis remains unclear, VEGI appears to be a potent inhibitor of endothelial cell growth as well as an inducer of endothelial cell apoptosis[4,5]. VEGI has been shown to cause growth arrest and apoptosis in HUVEC (human umbilical-vein endothelial cells), adult bovine aortic

endothelial cells and bovine pulmonary artery endothelial cells. VEGI also inhibits the proliferation of breast carcinoma (MCF-7), epithelial (HeLa) and myeloid (U-937 and ML-1a) tumor cells. Although VEGI gene in the studies have activated so strikingly, they were protocaryon expressed and only provided temporarily.

In this study, we used our re-constructed eukaryotic expressional pcDNA$_4$-VEGI, with which we could encode the gene into mammalian corneal cells and provide a potential solution for the long-term delivery of anti-angiogenic agents in the cornea and inhibit corneal angiogenesis.

MATERIALS AND METHODS

Materials VEGI gene connected with eukaryotic expressional vector pcDNA$_4$; Effectene (Qiagen, Valencia, CA, U.S.A.); VEGI mouse-anti-human monoclonal antibody (Zymed Laboratories, South San Francisco, CA, U.S.A); Goat-anti-mouse IgG and TMB were purchased from Tianwei (Beijing, China).

Methods

Animal models [6] Forty New Zealand albino rabbits, weighing 1.5 to 2kg, anesthetized with ketamine (30mg/kg, intramuscularly [*i.m.*]) and xylazine (6mg/kg, *i.m.*), were sutured by 2 4-0 silks midstromal depth approximately 1mm from the limbus on the right cornea to induce neovascularization. Ofloxacin eye drop to prevent infection. To determine the length and area of CNV, slit-lamp photographs in a standardized magnification were taken on day 1, 3, 7, 14 and 21 after surgery. Photographs were digitized, and the length of CNV (in millimeters) determined, the area of the CNV (in square millimeters) were determined using the rule $A=C/12\times3.1416[r^2-(r-L)^2]$. All values were presented as means and standard errors.

Gene transfection After surgery, 40 rabbits were divided into 4 random teams at once, 10 per each team: team A: transfected by pcDNA4-VEGI gene mediated by Effectene™ lipofectine transfection (pcDNA$_4$-VEGI DNA 20.0μL, Effectene™ Reagent 230.0μL, Enhancer 40.0μL, the total was 290.0μL), team B: by Plasmid pcDNA$_4$, (pcDNA$_4$ 20.0μL, Effectene™ Reagent 230.0μL, Enhancer 40.0μL, the total was 290.0μL); team C: by Effectene™, (Effectene™ lipofectine transfection 290.0μL); and team D: by normal saline (290.0μL).The liquids were injected under the conjunctiva near the silk sutured place. Length and area of CNV were measured under slit-lamp every day after transfection.I Immunohistochemistry was used to detected the expression of VEGI protein in cornea on day 1, 3, 7, 14 and 21.

Histology and immunohistochemistry On day 1, 3, 7, 14 and 21 after gene transfection, 2 rabbits were random chosen from each team and killed by an overdose of pentobarbital sodium. Corneas were took off immediately after killed, and fixed in 10% neutral buffered formalin for histology and immunohistochemistry test. Tissues were dehydrated, embedded in paraffin and serially sectioned at 4μm, dewaxed, and rehydrated using routine procedures immunolocalization. The sections were blocked for endogenous peroxidase (1% H_2O_2 in PBS for 10 minutes), treated with acid, and labeled with a biotin-conjugated mouse anti-VEGI antibody (1.25μg/mL, Zymed Laboratories, South San Francisco, CA, USA). Stained sections were imaged on an Olympus AX-70 light microscope equipped with computer-controlled digital camera.

Statistical Analysis Results were expressed as the mean ± SD for at least three independent experiments. Statistical differences between means were determined using one-way ANOVA followed by Bonferroni's post hoc test or two-tailed Student's *t*-test when appropriate with the software SPSS 10.0 For Windows. $P<0.05$ was considered statistically significant.

RESULTS

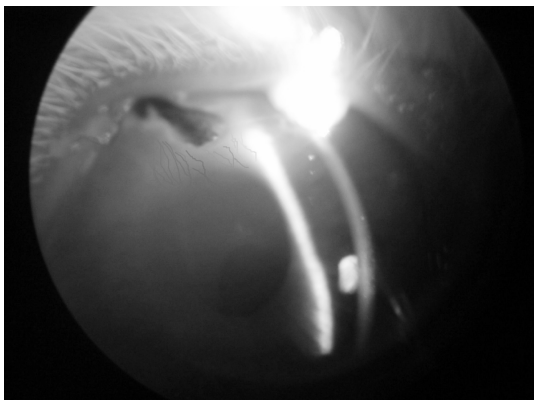
Animal Experiment Average occurrence of CNV in the pcDNA4-VEGI gene transfected team (team A) was 6.3 days, in plasmid pcDNA$_4$ control team (team B) was 3.1 days, in Effectene™ lipofectine control team (team C) was 3.2 days, in normal saline control team (team D) was 3.2 days. Difference between A and B, C, D was significant ($F=39.838$, $P=0.000$), while difference in B, C and D was meaningless ($F=0.064$, $P=0.938$).

CNV were not observed in any corneas of 4 teams on day 1. Some CNV were observed in teams B, C and D but not team A on day 3 after surgery. On day 7 CNV could be seen in each cornea and the length and areas of CNV, the corneal edema and opacity were different form team A to team B, C and D. The CNV were less and were found to grow only around the sutured silks in team A (Figure 1). In teams B, C and D, the CNV were longer, thicker and the corneas were more edemous. The length of the longest CNV was 2.9mm, the clock point was 0.4 to 1.3. On day 14 post-operation, all the CNV were longer but in team A they became sparser and the cornea became more transparent. The length of the longest CNV in team A was 4.0mm while that was 6.4mm in teams B, C and D, the clock point was 3.2; On the 21st day after surgery, part of the CNV were extinct, there were much less CNV in team A than that in teams B, C and D. The length of the longest CNV in teams B, C and D was 8.8mm and the clock point was 1.5. Statistic showed that length and average area of CNV in each period in team A was

Table 1 Comparison of average length of the longest vessel and average area of CNV (mean±SD)

Groups	*n*	Average length of the longest vessel (mm)			Average area of CNV (mm^2)		
		7d	14d	21d	7d	14d	21d
A	10	0.2±0.1	3.4±0.4	6.2±0.9	1.1±0.7	28.5±2.0	31.9±0.9
B	10	2.0±0.5	5.8±0.3	8.0±0.6	26.9±2.2	52.7±0.6	46.1±1.1
C	10	2.3±0.3	5.7±0.3	7.7±0.5	29.0±2.9	52.7±0.8	46.5±1.4
D	10	2.3±0.4	5.7±0.5	8.0±0.5	30.0±3.9	52.6±1.4	45.7±1.5
q_1[a]		17.3860	20.9435	8.8922	30.2375	57.8195	35.5431
P_1[a]		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
q_2[b]		19.4876	19.7947	7.4831	32.6071	57.8434	36.6532
P_2[b]		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
q_3[c]		19.5831	20.4133	8.9408	33.8732	57.5898	34.7243
P_3[c]		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
q_4[d]		2.1016	1.1488	1.4091	2.3696	0.0239	1.1119
P_4[d]		>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
q_5[e]		2.1971	0.5302	0.0486	3.6356	0.2297	0.8169
P_5[e]		>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
q_6[f]		0.0955	0.6186	1.4577	1.2660	0.2536	1.9288
P_6[f]		>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

Note: [a] was: q_1, P_1 of the compare between group A and B; [b] was: q_2, P_2 of the compare between group A and C; [c] was: q_3, P_3 of the compare between group A and D; [d] was: q_4, P_4 of the compare between group B and C; [e] was: q_5, P_5 of the compare between group B and D; [f] was: q_6, P_6 of the compare between group C and D



Figuer 1 Seven days after VEGI gene transfection, little and sparse CNV and less edema in the rabbit cornea under the slip-microscope

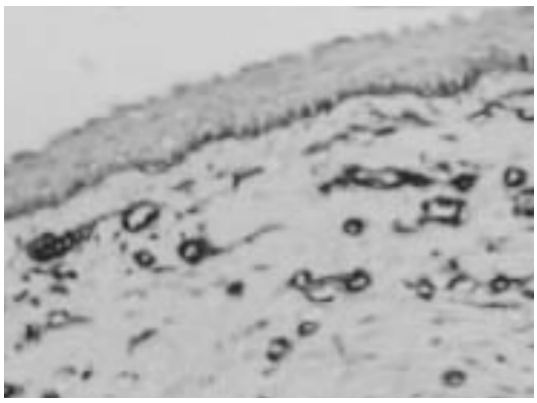


Figure 2 Seven days after VEGI gene transfection, lots of yellow-brown stained VEGI positive cells in the the tubal wall of the CNV, the inner- and inter-kytoplasm in cellular columnoepithelialis of basal membrane under the microscope (×200)

significantly different from those in teams B, C, and D, while differences among teams B, C and D were meaningless (Table 1).

Immunohistochemistry Results Immunohistochemistry study revealed the following: in team A, on the 1st day after transfection, the 5 layers of the cornea were well-distributed stained yellow-brown; on the 7th day after transfection, there were large amounts of stained yellow-brown cells in the matrix, the collagenous fibers, the tubal wall of the CNV, the inner- and inter-kytoplasm in cellular columnoepithelialis of basal membrane (Figure 2). In the contrast teams B, C and D, there were none VEGI positive cells all the time.

DISCUSSION

Application of the Transgenic Technology As the development of molecular biology[7,8], it has been proved that the transgenic technology was a very effective method to change the bionomics of the cells. With the technology of genetic recombination, establish expressional genetic vector and transfect the exogenous gene into the recipient cells and express the protein, implement the proportional contribution. Positive ion liposome is a kind of phospholipids molecule with positive charge, which could transfect the exogenous gene into the recipient cells by parceling DNA under the cellular phagocytosis or fusion [9]. It has the qualities of safe, hypo-toxin, non-antigenicity, convenience using and much cheaper. In our study, we used the improved liposome-

Effectene™ (Qiagen, Germany), its transfecting rate was approximate 30-40% , could successfully mediate the recombinant exogenous gene pcDNA$_4$-VEGI into the animal tissues.

Inhibition of CNV of pcDNA$_4$-VEGI Gene Transfection Mediated by Liposome Since 1997 when bolted from cDNA lib, VEGI has been paid more and more attention[10]. Many studies have proved that VEGI was a type of transmembranous protein specifically expressed by endothelial cells, and could strongly inhibit proliferation of vascular endothelial cells by combining the receptor on the cell surface [11,12]. However, little was known about the eukaryotic expressional VEGI gene or how it worked in genetic level.

In our pre-research, we have changed the expressional vector from prokaryotic PBV$_{220}$ plasmid to eukaryotic vector pcDNA$_4$, RT-PCR, enzyme cutting and computer automatic sequence analysis have identified the correct of the gene. In this study, we transfected the eukaryotic gene by the form of Effectene™ lipofectine-pcDNA$_4$-VEGI unit into the animal tissues to examine while it could express bioactive fusion protein and inhibit CNV. The results showed that on every time of experiment, the secreted VEGI protein could be seen in immunohistochemistry test and the CNV was obviously inhibited in VEGI transfection team compared with the control teams.

In summary, mediated by liposome, eukaryotic pcDNA$_4$-VEGI is able to express bioactive fusion protein in the cornea. It can reduce the CNV. The mid-and long-term outcome and problems that may arise from the gene therapy itself need further observation and study in our ongoing experiments.

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