·Basic Research·

Inhibition of corneal neovascularization by vascular endothelia growth inhibitor gene

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Abstract

• AIM: To evaluate the effect of Effectene™ lipofectine mediated plasmids encoding human pcDNA4-vascular endothelia growth inhibitor (pcDNA4-VEGI) gene on corneal neovascularization (CNV).

• METHODS: Forty New Zealand albino rabbits were sutured by 5-0 silk on the superior cornea to establish the animal model and divided into 4 random group, ten per each group: group A: transfected by pcDNA4-VEGI gene mediated by Effectene™ lipofectine transfection, group B: by Plasmid pcDNA4, group C: by Effectene™, and group D: by normal saline. Length and area of CNV were measured under slit lamp every day after transfection, immunohistochemistry was used to detected the expression of VEGI protein in cornea at 3, 7, 14 and 21 days.

• RESULTS: Average occurrence of CNV in the pcDNA4-VEGI gene transfected group (group A) was 6.3 days, in plasmid pcDNA4 control group (group B) was 3.1 days, in Effectene™ lipofectine control group (group C) was 3.2 days, in normal saline control group (group D) was 3.2 days. Differences between groups A and B, C, D were statistically significant ($P<0.01$), while differences in groups B, C and D were meaningless ($P>0.05$). Lenth and average area of CNV in each period in group A was meaningful different from that in groups B, C, and D ($P<0.01$), while differences in group B, C and D were meaningless ($P>0.05$). Immunohistochemistry result: VEGI positive cells could be seen in epithelium, stroma, endothelium and the cliff of CNV in group A at 3 days after transfection. VEGI cells changed with the decrease of CNV. None positive cells were in the control groups (groups B, C and D) all the time.

• CONCLUSION: Effectene™ lipofectine transfection technique can be effectively used in transfecting pcDNA4-VEGI gene into rabbit cornea and the lenth and areas of CNV 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 neovascularisation (CNV) is a serious pathologic condition and an important cause of 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 tumour 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) tumour cells. Although VEGI gene in the studies have activated so strikingly, yet 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, USA); VEGI mouse-anti-human monoclonal antibody (Zymed Laboratories, South San Francisco, CA, USA); Goat-anti-mouse IgG and TMB were purchased from Tianwei (Beijng, China).

Methods

Animal models [6] Forty New Zealand albino rabbits, weighing 1.5-2.0kg, 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 lenth and area of corneal neovascularization, slit-lamp photographs in a standardized magnification were taken on day 1, 3, 7, 14 and 21 after surgery. Photographs were digitized, and the lenth of the corneal neovascularition (mm) determined, the area of the CNV (mm^2) were determined using the rule $A = C/12 \times 3.1416[r^2-(r-L)^2]$. All values were presented as means and standard errors.

Gene transfection After surgery, 40 rabbits were at once divided into 4 random groups , 10 per each group: group 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), group B: by plasmid pcDNA$_4$, (pcDNA$_4$ 20.0μL, Effectene™ reagent 230.0μL, Enhancer 40.0μL, the total was 290.0μL); group C: by Effectene™, (Effectene™ lipofectine transfection 290.0μL); and group D: by normal saline (the 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, immunohistochemistry was used to detected the expression of VEGI protein in cornea at 1, 3, 7, 14 and 21 days.

Histology and immunohistochemistry At day 1, 3, 7, 14 and 21 after gene transfection, 2 rabbits were random chosen from each group 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, embeded in paraffin and serially sectioned at 4Um, dewaxed, and rehydrated using routine procedures mmunolocalization. The sections were blocked for endogenous peroxidase (10g/L H_2O_2 in PBS for 10 minutes), treated with acid, and labeled with a biotin-conjugated mouse anti-VEGI antibody (was used at 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 means± 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 group A was 6.3 days, in group B was 3.1 days, group C was 3.2 days, and in group D was 3.2 days. Difference between groups A and B, C, D was meaning (F=39.838, P=0.000), while difference in groups B, C and D was meaningless (F=0.064, P=0.938).

On the 1st day after surgery, there were no CNV in any corneas of 4 groups; on the 3rd day after surgery, we still couldn't found CNV growing in group A, while there were some CNV in the corneas of other 3 groups. On the 7th day postoperatively, CNV could be seen in every cornea, yet the lenth and areas of CNV, the corneal edema and opacity were of varying degrees in group A from groups B, C and D. In group A, there were less growth of CNV, and CNV were found to grow only around the sutured silks (Figure 1); in groups B ,C and D, the CNV were longer, thick and the corneas were more edemous, the lenth of the longest CNV was 2.9mm, the clock point was 0.4 to 1.3. On the 14th day postoperatively, all the CNV were longer. But in group A,

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

Groups	Average length of the longest vessel (mm)			Average area of CNV (mm^2)		
	7d	14d	21d	7d	14d	21d
A	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	2.0±0.5[b]	5.8±0.3[b]	8.0±0.6[b]	26.9±2.2[b]	52.7±0.6[b]	46.1±1.1[b]
C	2.3±0.3[d]	5.7±0.3[d]	7.7±0.5[d]	29.0±2.9[d]	52.7±0.8[d]	46.5±1.4[d]
D	2.3±0.4[f]	5.7±0.5[f]	8.0±0.5[f]	30.0±3.9[f]	52.6±1.4[f]	45.7±1.5[f]

[b]P<0.01, group A *vs* group B; [d]P<0.01, group A *vs* group C; [f]P<0.01, group A *vs* group D

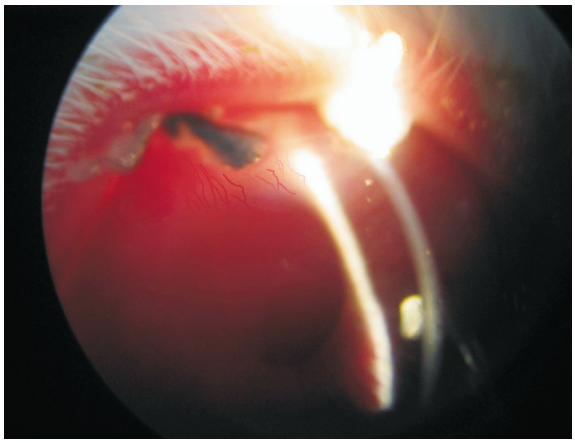
CNV became sparser and the cornea became more transparent. The lenth of the longest CNV in group A was 4.0mm while that was 6.4mm in groups B, C and D, the clock point was 3.2. On the 21st day after surgery, part of the CNV were extincted, there were much less CNV in group A than in groups B, C and D. The length of the longest CNV in group B, C and D was 8.8mm, the clock point was 1.5. Statistic showed that length and average area of CNV in each period in group A was meaningful different from those in grouos B, C, and D, while differences in groups B, C and D were meaningless (Table 1).

Immunohistochemistry Results Immunohistochemistry study revealed the following: in group 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 cellula columnoepithelialis of basal membrane (Figure 2). In the contrast groups 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 propotional contribution. Positive ion liposome is a kind of phospholipids molecule with positive charge，which could transfect the exogenous gene into the recipient cells by parcelling 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 payed more and more attention[10].



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

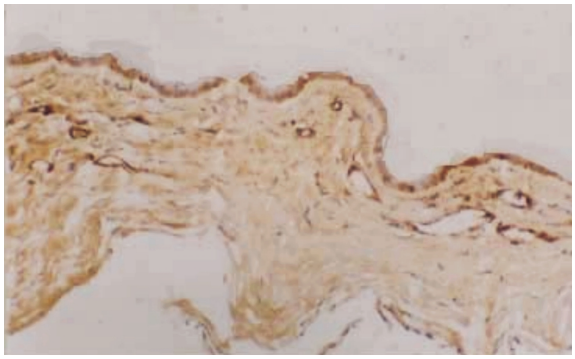


Figure 2 Seven days after VEGI gene transfection, there were lots of yellow–brown stained VEGI positive cells in the the tubal wall of the CNV, the inner– and inter–kytoplasm in cellula columnoepithelialis of basal membrane under the microscope×200

Many studies have proved that VEGI was a type of trans-membranous 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]. But 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. Results have shown that on every time of experiment, the secreted VEGI protein could be seen in immunohistochemistry test and the CNV had been obviously inhibited in VEGI transfection group compared with the control groups.

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

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