

Effect of bevacizumab on the expression of fibrosis-related inflammatory mediators in ARPE-19 cells

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Abstract

• **AIM:** To investigate the effect of anti-vascular epithelial growth factor (VEGF) agents on the expression of fibrosis-related inflammatory mediators under normoxic and hypoxic conditions, and to further clarify the mechanism underlying fibrosis after anti-VEGF therapy.

• **METHODS:** Human retinal pigment epithelial (RPE) cells were incubated under normoxic and hypoxic conditions. For hypoxia treatment, CoCl₂ at 200 μmol/L was added to the media. ARPE-19 cells were treated as following: 1) control group: no treatment; 2) bevacizumab group: bevacizumab at 0.25 mg/mL was added to the media; 3) hypoxia group: CoCl₂ at 200 μmol/L was added to the media; 4) hypoxia+bevacizumab group: CoCl₂ at 200 μmol/L and bevacizumab at 0.25 mg/mL were added to the media. The expression of interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α were evaluated using real-time polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) at 6, 12, 24 and 48h.

• **RESULTS:** Both mRNA and protein levels of IL-1β, IL-6 and IL-8 were statistically significantly higher in the bevacizumab group than in the control group at each time point, and TNF-α gene and protein expression was only significantly higher only at 24 and 48h (*P*<0.05). Under hypoxic conditions, bevacizumab significantly increased the expression of IL-1β, IL-6, IL-8 and TNF-α at 6, 12, 24 and 48h (*P*<0.05). IL-1β, IL-8 and TNF-α peaked at 24h and IL-6 peaked at 12h after the bevacizumab treatment under both normoxic and hypoxic conditions.

• **CONCLUSION:** Treatment of ARPE-19 cells with bevacizumab can significantly increase the expression of fibrosis-related inflammatory mediators under both

normoxic and hypoxic conditions. Inflammatory factors might be involved in the process of fibrosis after anti-VEGF therapy, and the up-regulation of inflammatory factors induced by anti-VEGF drugs might promote the fibrosis process.

• **KEYWORDS:** bevacizumab; fibrosis; human retinal pigment epithelial cells; inflammatory mediators

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INTRODUCTION

In recent years, anti-vascular epithelial growth factor (VEGF) agents have been widely used in the management of choroidal neovascularization, macular edema secondary to diabetic retinopathy (DR), and retinal venous occlusive diseases^[1-4]. However, with the increasing use and long-term follow-up, the ocular complications of anti-VEGF therapy have become more observed. Ranibizumab has been shown to cause subfoveal fibrosis during the first 24mo after treatment in patients with age-related macular degeneration (AMD)^[5]. The development or progression of subfoveal fibrosis has also been found after anti-VEGF therapy in the absence of significant subfoveal hemorrhage in neovascular AMD^[6]. The post anti-VEGF therapy contraction of the neovascular membrane, which can induce tractional detachment of the retina and cause permanent vision loss, has become one of the most significant clinical problems in retinal neovascular diseases^[5,7-11].

Up to now, the mechanism of the fibrosis progression after anti-VEGF therapy has not been fully understood. Previously, our study confirmed that the process may be related with the up-regulation of connective tissue growth factor (CTGF), transforming growth factor-β₂ (TGF-β₂), basic fibroblast growth factor (bFGF) and matrix metalloproteinase-2 (MMP-2) expression^[12]. However, it is not clear whether inflammatory cytokines participate in this process. Anti-VEGF therapy could not only block VEGF, but also change the level of inflammatory mediators^[13-14], which generally act as a potent immunomodulator^[15]. Accumulated evidence has shown that the up-regulation of inflammatory mediators, such as interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor

(TNF)- α play a driving role in the process of fibrosis^[16-21]. Inflammation has been indicated as an important event in the pathogenesis of retinal or choroidal diseases^[22-25]. Therefore, we hypothesis that anti-VEGF therapy may result in changes of some inflammatory mediators in the fibrotic response. In this preliminary study, we evaluated the effect of bevacizumab on inflammatory mediators that related with fibrosis in human retinal pigment epithelial (RPE) cells under normoxic and hypoxic conditions, hoping to further clarify the mechanism of anti-VEGF agents on fibrosis progression.

MATERIALS AND METHODS

Cell Culture ARPE-19 cells (ATCC, Catalog No. CRL-2302, Rockefeller, MD, USA) were cultured in Dulbecco's modified Eagle medium/nutrient mixture F12 (DMEM/F12; Invitrogen, Carlsbad, CA, USA) (1:1) containing 10% fetal bovine serum (FBS; Invitrogen), 100 IU/mL penicillin G (Invitrogen), and 100 μ g/mL streptomycin (Invitrogen). The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The culture medium was changed every 3d. After the cells reached 90% confluence was reached, the cells were routinely passaged by dissociation in 0.05% trypsin/0.02% ethylene diamine tetraacetic acid (EDTA), and then re-plated at a split ratio ranging from 1:3 to 1:4.

Exposure to CoCl₂ and Bevacizumab ARPE-19 cells were trypsinized and inoculated into 6-well plates with the complete medium until a confluent monolayer was established. Then the complete medium was replaced with fresh 2% FBS medium. To mimic a pathological condition in which anti-VEGF therapy was used, a hypoxia environment was created by adding CoCl₂ (Sigma-Aldrich; 200 μ mol/L) to the 2% FBS medium. In group 1, the control group, ARPE-19 cells were kept in 2% FBS medium. In group 2, 0.25 mg/mL bevacizumab (Genentech Inc, San Francisco, CA, USA), roughly equal to the concentration used clinically was added to the 2% FBS medium. In group 3, the hypoxia group, the ARPE-19 cells were treated with 200 μ mol/L CoCl₂. In group 4, in addition to 200 μ mol/L CoCl₂, 0.25 mg/mL bevacizumab was mixed in the medium. The cells were collected at 6, 12, 24 and 48h for biochemical assays.

Quantitative Real-time Polymerase Chain Reaction At 6, 12, 24 and 48h total RNA was extracted from ARPE-19 cells with Trizol (Invitrogen, Carlsbad, CA, USA), and subsequently reverse transcribed into cDNA using Primescript™ RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). Real-time polymerase chain reaction (RT-PCR) was performed with ABI 7500 RT-PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR green (Takara, Dalian, China) according to the manufacturer's instructions. The initial 30s hot-start denaturation step at 95 °C was followed by 40 cycles at 95 °C for 5s and at 60°C for 34s. All expression data were normalized to those for GAPDH. The relative gene expression levels

Table 1 Sequence of primers used for the RT-PCR

Target	Sequence of primer
GAPDH	Forward-5'-ATGCTGGCGCTGAGTACGT-3'
	Reverse-5'-AGCCCCAGCCTTCTCCAT-3'
IL-1 β	Forward-5'-CCTGTCCTGCGTGTGAAAGA-3'
	Reverse-5'-GGGAAGTGGGAGACTCAA-3'
IL-6	Forward-5'-TGGCTGAAAAGATGGATGCT-3'
	Reverse-5'-TCTGCACAGCTCTGGCTTGT-3'
IL-8	Forward-5'-TTGGCAGCCTTCTGATTTC-3'
	Reverse-5'-TGGTCCACTCTCAATCACTCTCA-3'
TNF- α	Forward-5'-TGTAGCCCATGTTGTAGCAAACC-3'
	Reverse-5'-GAGGACCTGGGAGTAGATGAGGTA-3'

were calculated using the comparative Ct ($\Delta\Delta$ Ct) method; the relative expression was calculated as $2^{-\Delta\Delta Ct}$, and Ct represented the threshold cycle (Table 1).

Enzyme-linked Immunosorbent Assay ARPE-19 cells were plated into 6-well plates and treated as previously described. The supernatants in the four groups were collected from cell cultures and centrifugated at 3500 rpm for 10min. The concentration of IL-1 β , IL-6, IL-8 and TNF- α in each group was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). Each assay was performed according to the manufacturer's instructions. Optical density was determined at 450 nm using an absorption spectrophotometer within 15min. Concentrations were measured in pg/mL. The standard curves for each cytokine were generated by using the reference cytokine concentrations in the manufacturer's instructions.

Statistical Analysis The results were expressed as mean \pm SD. Differences between experimental groups were evaluated by analysis of variance (ANOVA) with LSD *t*-test. SPSS 17.0 for Windows was used for statistical analysis. Two-tailed test results were considered significant at $P < 0.05$.

RESULTS

Detection of IL-1 β , IL-6, IL-8, and TNF- α mRNA Expression by Real-time Polymerase Chain Reaction By RT-PCR, the expressions of IL-1 β , IL-6 and IL-8 in group 2 were found to be significantly higher than in group 1 at 6, 12, 24 and 48h, while TNF- α was significantly higher at 24 and 48h ($P < 0.05$). Significant differences were also found between groups 3 and 4. Bevacizumab significantly increased the secretion of IL-1 β , IL-6, IL-8 and TNF- α under hypoxic conditions ($P < 0.05$) (Figures 1A-4A). Both under normoxic and hypoxic conditions, IL-1 β , IL-8 and TNF- α increased obviously at 24h than other time points, and IL-6 increased obviously at 12h compared with other time points after the treatment of bevacizumab ($P < 0.05$) (Figures 1A-4A).

Protein Expressions of IL-1 β , IL-6, IL-8, and TNF- α By ELISA, the protein expressions of IL-1 β , IL-6 and IL-8 in group 2 were found to be significantly higher than in group 1

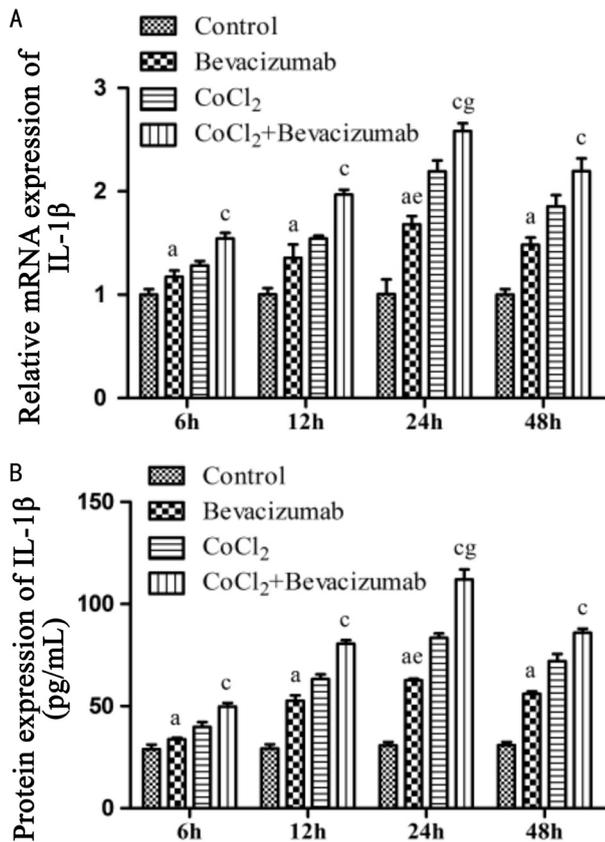


Figure 1 Expression of IL-1β in ARPE-19 cells given different treatments The expressions of IL-1β in bevacizumab group were significantly higher than in the control group at 6, 12, 24 and 48h (^a*P*<0.05). Significant differences were also found between the hypoxia (CoCl₂ treatment/no bevacizumab) group and hypoxia+bevacizumab group (^c*P*<0.05). The expression of IL-1 β peaked at 24h after the treatment of bevacizumab under normoxic and hypoxic conditions, compared with the other time points (^{e,g}*P*<0.05).

at 6, 12, 24 and 48h and TNF-α was statistically significantly higher at 24 and 48h (*P*<0.05). IL-1β, IL-6, IL-8 and TNF-α secretion were significantly higher after bevacizumab and CoCl₂ treatment (group 4) than after CoCl₂ treatment alone (group 3) (*P*<0.05) (Figures 1B-4B). IL-1β, IL-8 and TNF-α increased obviously and reached a peak at 24h and IL-6 peaked at 12h after the treatment of bevacizumab under normoxic and hypoxic conditions. There were significant differences in the level of inflammatory mediators between the peak time and other time points in the groups related to bevacizumab (*P*<0.05) (Figures 1B-4B).

DISCUSSION

Inflammation involves in the pathogenesis of many retinal or choroidal diseases, such as proliferative vitreoretinopathy (PVR), proliferative diabetic retinopathy (PDR), and neovascular AMD^[26-32]. The post-bevacizumab fibrotic phenomenon has been observed in retinal and choroidal neovascular diseases^[5,8,33-36]. However, the mechanisms underlying the transition from angiogenesis to fibrosis have not been fully elucidated. In order to further evaluate the mechanisms

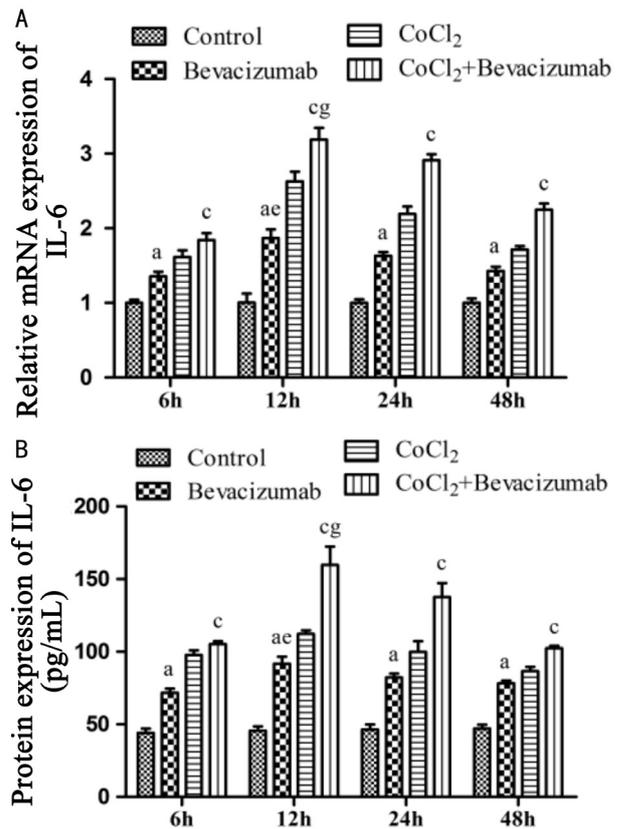


Figure 2 Expression of IL-6 in ARPE-19 cells given different treatments The expressions of IL-6 in the bevacizumab group were significantly higher than in the control group at 6, 12, 24 and 48h (^a*P*<0.05). Significant differences were also found between the hypoxia group and hypoxia+bevacizumab group (^c*P*<0.05). IL-6 peaked at 12h after bevacizumab treatment under normoxic and hypoxic conditions (^{e,g}*P*<0.05).

underlying the angiofibrotic switch, we investigated the effect of bevacizumab on the expressions of proinflammatory mediators related to fibrosis at the cellular level. The results showed that bevacizumab can up-regulate the expressions of IL-1β, IL-6, IL-8 and TNF-α in ARPE-19 cells under both normoxic and hypoxic conditions.

IL-1β is a major proinflammatory cytokine that plays an important role in acute and chronic inflammatory diseases^[37]. A number of studies have shown that IL-1β is related to neovascular fibrosis. It is likely that IL-1β initiates the endothelial to mesenchymal transformation and can induce the epiretinal membrane formation in eyes^[18,38]. Moreover, an *in vitro* model of retinal membrane contraction, as occurs in PVR, is modulated by IL-1β^[39]. In our study, the expression of IL-1β was up-regulated after bevacizumab treatment under normoxic and hypoxic conditions, suggesting that IL-1β may participate in the process of fibrosis and that bevacizumab may play a driving role in the process.

It has been reported that the concentration of IL-6 correlates well with that of IL-8^[40-41]. Intravitreal injection of bevacizumab (IVB) can induce elevations of intraocular

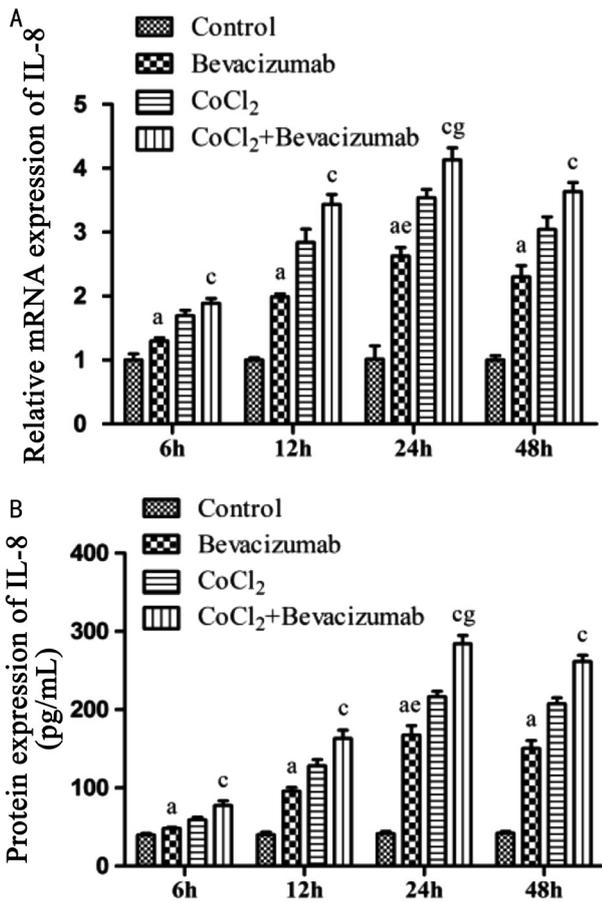


Figure 3 Expression of IL-8 in ARPE-19 cells given different treatments Secretion of IL-8 from ARPE-19 cells after treatment with 0.25 mg/mL bevacizumab under normoxic and hypoxic conditions at 6, 12, 24 and 48h (^{a,c}*P*<0.05). IL-8 peaked at 24h after bevacizumab treatment under normoxic and hypoxic conditions (^{c,s}*P*<0.05).

IL-6 immediately and subsequent up-regulation of IL-8 and TGF- β_2 ^[40]. IL-6 and IL-8 levels are also evaluated in this study. The two kinds of inflammatory mediators were significantly higher after bevacizumab treatment, which is consistent with the results of previous studies. It may be postulated that IVB aggravates IL-6 and IL-8, and so mediates more inflammatory changes and the formation and contraction of fibrovascular tissue and alters the intraocular environment leading to complications.

TNF- α , a proinflammatory cytokine, generally acts as a potent immunomodulator and plays an important role in neovascularization and fibroplasia^[15]. It may alter the attachment and migration of RPE cells to favor provisional extracellular matrix-molecules through changes in integrin expression. TNF- α is also a major regulator of RPE activation responses, including cell attachment, spreading, chemotaxis, and migration^[20]. In the current *in vitro* experimental study, results showed that treatment of ARPE-19 cells with bevacizumab at clinical doses significantly increased the secretion of TNF- α under hypoxic conditions. However, under normoxic conditions, TNF- α levels were only significantly higher at 24

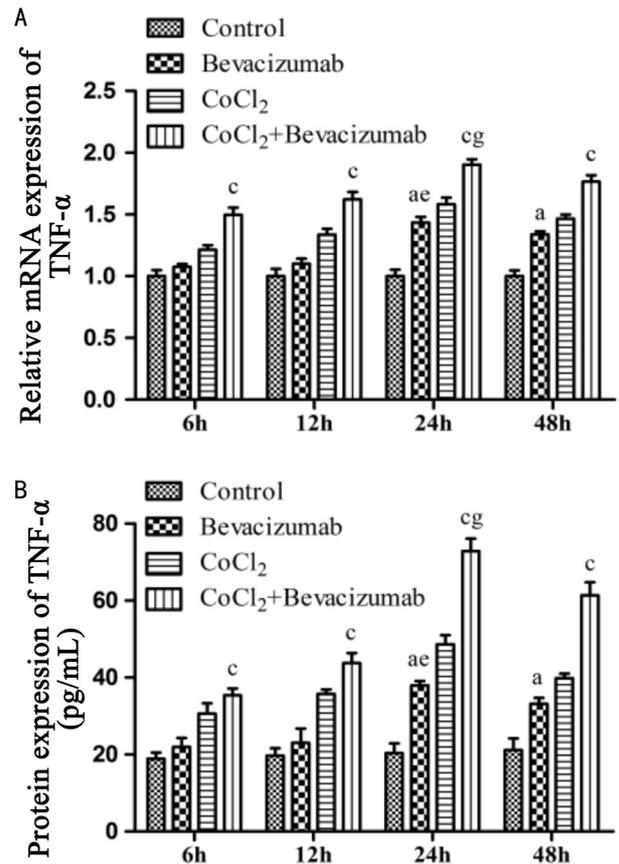


Figure 4 Expression of TNF- α in ARPE-19 cells given different treatments Under normoxic conditions, the expression of the TNF- α gene and protein were significantly higher than in the control group only at 24 and 48h (^a*P*<0.05). Under hypoxic conditions, bevacizumab significantly increased the secretion of TNF- α at 6, 12, 24 and 48h (^c*P*<0.05). TNF- α peaked at 24h after the bevacizumab treatment under normoxic and hypoxic conditions (^{c,s}*P*<0.05).

and 48h after bevacizumab treatment. These results show that the effects of bevacizumab on different inflammatory factors and even on the same factor under normoxic and hypoxic conditions may be different.

Neovascular diseases, such as PDR and AMD, are associated with hypoxia, and fibrosis is the inevitable outcome of neovascularization. Until now, the mechanism of the fibrosis progression after anti-VEGF therapy has not been fully understood. In our research, both bevacizumab and hypoxia can promote the expression of fibrosis related inflammatory factors. The effects of hypoxia on fibrosis related inflammatory factors were more obvious than those of bevacizumab. Hypoxia and bevacizumab have synergistic effects on the promotion of fibrosis related inflammatory factors. This indicates that bevacizumab may accelerate the original course of fibrosis. However, the levels of proinflammatory mediators showed a transient increase with different peak times. The impacts of bevacizumab on inflammatory mediators did not last long. It was here deduced that the increased proinflammatory mediators may participate in the process of fibrosis.

In summary, the effect of bevacizumab on the expressions of inflammatory mediators related to fibrosis in ARPE-19 cells under normoxic and hypoxic conditions was investigated for the first time in this study. The up-regulation of IL-1 β , IL-6, IL-8 and TNF- α in ARPE-19 cells after bevacizumab treatment at clinical doses may be one of the mechanisms underlying fibrosis. And bevacizumab may accelerate the original course of fibrosis. The development of anti-fibrotic agents may allow a broader range of patients to benefit from pharmacologic treatment of neovascular diseases.

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Conflicts of Interest: Chu SJ, None; Zhang ZH, None; Wang M, None; Xu HF, None.

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