

Induction of vascular endothelial growth factor receptor expression in human umbilical vein endothelial cells after repeated bevacizumab treatment *in vitro*

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Abstract

• **AIM:** To investigate the mechanism underlying the loss of responsiveness to anti-vascular endothelial growth factor (VEGF) treatment after repeated injections for choroidal neovascularization, VEGF and VEGF receptor (VEGFR) expressions were evaluated following repeated bevacizumab treatments in hypoxic human umbilical vein endothelial cells (HUVECs) *in vitro*.

• **METHODS:** HUVECs were incubated under hypoxic conditions in two media of different bevacizumab concentrations (1.0 or 2.5 mg/mL) for 17h, and then in a new medium without bevacizumab for 7h. This procedure was repeated twice more. A culture with an identical volume of excipients served as the control. Cytotoxicity and cell proliferation were assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide and Ki-67 assays, respectively. Levels of VEGF and VEGFR were assessed using enzyme-linked immunosorbent assay and Western blot respectively.

• **RESULTS:** Cytotoxic effects were not reported for either bevacizumab concentration. Cell proliferation was not reduced after anti-VEGF treatments. VEGF level after single treatment was significantly higher than that of the control and after repeated treatments. Phosphorylated VEGFR-2 expression increased significantly after single

and repeated bevacizumab treatments compared with the control. The 1.0 mg/mL bevacizumab induced significantly higher expressions of VEGFR-2 than the 2.5 mg/mL in single and repeated treatment groups.

• **CONCLUSION:** Bevacizumab treatment of HUVECs elevated VEGFR expression in both single and repeated treatments, indicating a mechanism for the reduced efficacy of anti-VEGF therapy in ocular neovascular disorders.

• **KEYWORDS:** vascular endothelial growth factor; vascular endothelial growth factor receptor; choroidal neovascularization; bevacizumab; repeated treatments

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INTRODUCTION

Exudative age-related macular degeneration (ARMD) complicated with choroidal neovascularization (CNV) is the leading cause of visual loss in developed countries^[1-3]. CNV is thought to result from the orchestrated interaction of various cellular components and a number of growth factors^[4], with vascular endothelial growth factor (VEGF) being a key factor.

The introduction of therapies targeting VEGF has changed the treatment paradigm of neovascular ARMD^[5-6]; however, anti-VEGF treatment has some limitations, including the necessity of repeated injections and non-responders in clinical practice^[7]. Some patients who initially respond to anti-VEGF treatment lose responsiveness following repeated injections^[8-10]. Although the exact mechanism underlying this tolerance (also known as tachyphylaxis) is still unknown, reduction of therapeutic effects is known to develop in numerous ways and for different reasons including vascular structural changes^[9], systemic immune responses^[11], up-regulation of other angiogenic signaling pathways as a compensatory mechanism^[4], and increased expression of VEGF or VEGF receptors (VEGFRs)^[12].

Repeated anti-VEGF treatments under hypoxic conditions and comparison between single and repeated anti-VEGF treatments would be essential to elucidate these mechanisms. A previous study has shown that a single treatment of anti-VEGF eliminates VEGF and reduces VEGFR expression in a non-hypoxic condition^[13]. These laboratory observations cannot explain the changes in responsiveness to repeated anti-VEGF treatments under hypoxia. This is because repeated injections are performed in clinical settings where choroidal hypoxia is suggested to be the mechanism for exudative ARMD, based on choroidal thinning and presence of the watershed zone^[14-15].

We performed an *in vitro* study using human umbilical vein endothelial cells (HUVECs) with single and repeated anti-VEGF treatments under hypoxic conditions to observe changes in VEGF and VEGFR expression.

MATERIALS AND METHODS

Cell Culture Under Hypoxic Condition HUVECs (Science Cell Research Labs, San Diego, CA, USA) between passages 4 and 9 were cultured in M199 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (Invitrogen Life Technologies, Scotland, UK), 1% penicillin/streptomycin (Invitrogen Life Technologies), 0.1% heparin (Sigma-Aldrich), and 0.1% endothelial cell growth supplement (Sigma-Aldrich), and maintained at 37°C in a humidified 5% CO₂ condition. HUVECs were subjected to a hypoxic condition when 70%-90% confluence was achieved.

To achieve the hypoxia condition, tissue-culture plates with HUVECs were placed in a hypoxic chamber (Modular Incubator Chamber, Billups-Rothenberg, Del Mar, CA, USA) and flushed with a gas mixture comprising 1% O₂, 5% CO₂, and 94% N₂ for 5min at 10 L/min. The chambers were then closed and placed in a CO₂ incubator at 37°C. The chamber gas was replaced daily. The medium was pre-equilibrated with a 5% O₂ gas mixture overnight before the hypoxic experiments were performed.

Bevacizumab Treatment First, HUVECs were treated with 1.0 mg/mL and 2.5 mg/mL bevacizumab (Avastin[®]; Genentech, Inc. San Francisco, CA, USA) for 17h under hypoxia with culture medium, and then the dish was washed out five times with phosphate buffered saline (PBS) and replaced with a new culture medium for 7h under the same hypoxic condition. Second, this procedure was repeated twice more. Third, for the control, the same volume of excipients^[16] was added to the HUVECs, and the cells incubated for 17h under hypoxia; the dish was then washed out five times with PBS and incubated in new medium for 7h under the same hypoxic condition.

Assays Each assay was performed immediately to prevent exposure to normal O₂ when the testing condition was accomplished. The number of replicates varied from 6 to 14. Potential cell metabolic activity reduction after bevacizumab treatment on HUVECs were evaluated using an MTT

[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The cells were washed twice with PBS and then subjected to the MTT assay as previously described^[17]. Optical density (OD) was measured at 540 nm.

Secreted VEGF concentrations were quantified using a human VEGF enzyme-linked immunosorbent assay kit (Quantikine, Human VEGF immunoassay, R&D System Inc., Minneapolis, MN, USA) in accordance with the manufacturer's instructions. ODs were measured at 450-570 nm, and the VEGF was calculated as the protein-adjusted amount of VEGF (pg/mg protein).

Activated VEGFR-2 expression was assessed using Western blot. Proteins were isolated from HUVEC lysates using Tripure (Roche Diagnostics, Basel, Switzerland), quantified using a spectrophotometer (Jenway, 6405 UV/vis, Essex, UK), and then equal amounts of the protein was loaded on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis with a 5% stacking gel. After electrophoresis, the proteins were blotted onto a Hybond nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA), using a mini-transblot electrophoretic transfer cell (Amersham Biosciences). Immunodetection of phosphorylated (activated) VEGFR-2 and beta-actin were accomplished using enhanced chemiluminescence (ECL kit, Amersham Biosciences). The relative intensity of each protein on the blotting analysis was measured using a computerized software program (Bio-Rad, Richmond, CA, USA) and normalized with beta-actin bands to compare the expression of proteins between different treatment conditions and concentrations.

Cell proliferation in the different culture conditions was determined using a monoclonal antibody that reacted with nuclear antigen Ki-67 of proliferating human cells in the G1-M phase, but not in the G0 phase. A primary rabbit anti-human Ki-67 antibody (Dako, Carpinteria, CA, USA) was utilized for cytopsin preparation at a 1:40 dilution for 1h at room temperature in a moist chamber. Excess antibodies were removed by washing the slides with Trisaminomethane-buffered saline (TBS, Sigma-Aldrich). Negative controls with TBS and an irrelevant antibody were established alongside the test slides. Fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit antibodies (Dako, Glostrup, Denmark) in a 1:20 dilution were utilized as the secondary antibodies and incubated for 30min. The slides were mounted with glycerol/PBS. The proportion of positively labeled cytopsin cells was determined by analyzing 200 cells per slide under a fluorescent microscope.

Statistical Analysis We compared MTT, VEGF secretion, VEGFR-2 expression, and Ki-67 between the control and each bevacizumab treatment condition. We also compared VEGF secretion and VEGFR-2 expression between 1.0 mg/mL and 2.5 mg/mL bevacizumab in the single, and repeated

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bevacizumab treatment groups. Wilcoxon rank sum tests were used for statistical analyses that were performed using the Statistical Package for Social Sciences (SPSS 12.0 Inc., Chicago, IL, USA). P values <0.05 were considered statistically significant.

RESULTS

The MTT assay revealed that cell metabolic activity was not affected by each bevacizumab treatment condition compared with the control (all $P>0.05$). The MTT assay was performed in highly confluent cell cultures with slow replication rates. This ensured that the effects of bevacizumab on cell proliferation, apoptosis, and cell morphology did not mask its potential toxic effects.

The amount of VEGF secretion with single 1.0 mg/mL and 2.5 mg/mL bevacizumab treatment was higher than the control and each repeated bevacizumab treatment conditions (all $P<0.05$, Figure 1), and there was no difference between 1.0 mg/mL and 2.5 mg/mL bevacizumab treatments ($P=0.5$, Figure 1). VEGF secretion with 1.0 mg/mL and 2.5 mg/mL repeated bevacizumab treatments was similar to the controls (all $P>0.05$, Figure 1) and similar between both concentrations ($P=0.3$, Figure 1).

The VEGFR-2 expression level for each single bevacizumab treatment was significantly higher than that the control ($P=0.010$ in 1.0 mg/mL and $P=0.020$ in 2.5 mg/mL, Figure 2), and the repeated bevacizumab treatment at each concentration also resulted in a higher expression compared with the control ($P<0.001$ in 1.0 mg/mL and $P=0.010$ in 2.5 mg/mL, Figure 2). When comparing between each bevacizumab concentration, 1.0 mg/mL significantly induced higher VEGFR-2 expression than 2.5 mg/mL bevacizumab in both single ($P=0.03$, Figure 2) and also repeated bevacizumab treatment conditions ($P=0.02$, Figure 2).

Ki-67 expression, an indicator of the number of proliferating cells, was not significantly different between each treatment group and the control (all $P>0.05$).

DISCUSSION

Exudative ARMD is a chronic disease that requires long-term management^[16-17]. Monthly injections of ranibizumab over a 2-year period have been shown to be ineffective at preventing CNV recurrence and lead to the development of a loss of responsiveness in some patients^[8-10]. The reduction in anti-VEGF treatment response challenges long-term treatment efficacy. Pathophysiological mechanisms have yet to be elucidated with ongoing research into effective treatments for the prevention of this progressive condition.

Angiogenesis is a highly complex and coordinated process requiring multiple intercellular mediators and receptors. VEGF is the most important activating factor of angiogenesis. It mediates a wide range of responses primarily in the vascular endothelial cells, from normal physiological functions to

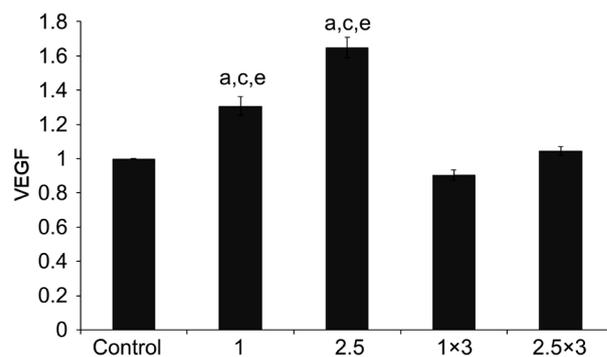


Figure 1 Results of secreted VEGF levels under hypoxic conditions The values were calculated from separate experiments. 1: 1.0 mg/mL bevacizumab single injection; 2.5: 2.5 mg/mL bevacizumab single injection; 1×3: 1.0 mg/mL bevacizumab repeated injection; 2.5×3: 2.5 mg/mL bevacizumab repeated injection. ^a $P<0.05$ vs control, ^c $P<0.05$ vs 1.0 mg/mL in repeated treatments, ^e $P<0.05$ vs 2.5 mg/mL in repeated treatments.

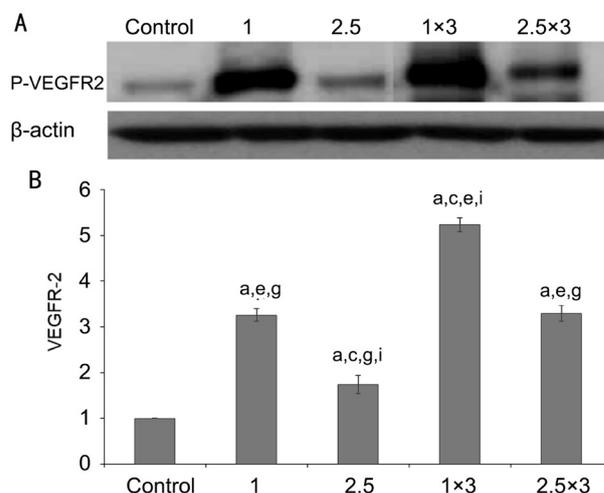


Figure 2 Expression of VEGFR-2 under hypoxic conditions were evaluated by Western blot (A) and statically compared to each groups using relative intensity of each label from separate experiments (B) 1: 1.0 mg/mL bevacizumab single injection; 2.5: 2.5 mg/mL bevacizumab single injection; 1×3: 1.0 mg/mL bevacizumab repeated injection; 2.5×3: 2.5 mg/mL bevacizumab repeated injection. ^a $P<0.05$ vs control, ^c $P<0.05$ vs 1.0 mg/mL in single treatment, ^e $P<0.05$ vs 2.5 mg/mL in single treatment, ^g $P<0.05$ vs 1.0 mg/mL in repeated treatments, ⁱ $P<0.05$ vs 2.5 mg/mL in repeated treatments.

pathological disease progression. VEGF is not only an angiogenic factor but also a trophic factor for cell survival to prevent hypoxic damage^[18-20]. Accordingly, depletion of VEGF by anti-VEGF treatment may induce compensatory physiological responses. VEGF and VEGFR levels following anti-VEGF treatment would be a key indicator of treatment outcome for intraocular neovascular diseases.

In our study, secretion of VEGF increased after a single treatment of bevacizumab; however, VEGF secretion returned to control levels with repeated treatment. In contrast, VEGFR expression was significantly elevated after a single and repeated treatment, and 1.0 mg/mL bevacizumab treatment

induced higher VEGFR-2 expression than 2.5 mg/mL concentration in both a single and repeated treatment was. A previous study reported that VEGFR expression decreases in HUVECs after treatment with various anti-VEGF agents under non-hypoxic conditions^[13]. This discrepancy may be due to the different *in vitro* HUVEC culture conditions including hypoxic condition versus normal oxygen status between studies.

The present findings may be considered a physiological mechanism for HUVEC survival in order to compensate the depletion of VEGF under hypoxic conditions. We suggest that increased secretion of VEGF after a single treatment accounts for a prompt response to VEGF ablation, and increased expression of VEGFR accounts for a delayed response for the maintenance of HUVEC cell survival. Although these two stages of responses are postulated to compensate for anti-VEGF treatment, the effects of anti-VEGF treatment may be different at each stage.

Increased secretion of VEGF may be blocked by repeated treatments or higher doses of anti-VEGF. However, up-regulated VEGFR will increase sensitivity to VEGF and diminish responsiveness to anti-VEGF therapy. This may comprise a potential mechanism for tolerance to anti-VEGF treatment, at least in some patients.

Our experiments demonstrated there was no significant change in cell metabolic activity based on MTT assay and cell proliferation based on Ki-67 expression after treatment with 1.0 and 2.5 mg/mL bevacizumab. These findings suggest that our experimental anti-VEGF treatment concentrations did not achieve anti-proliferative effects on HUVECs. Anti-VEGF therapeutic effects would be compensated by up-regulated VEGF secretion and VEGF receptor expression, as observed in our study.

We also recorded an increase in the expression of VEGFR with the lowest treatment dose of bevacizumab. We propose that suboptimal treatment may result in a more rapid loss of responsiveness to anti-VEGF treatment due to the up-regulation of VEGFR under hypoxic conditions. Therefore, achieving optimal therapeutic concentrations may be essential for improving treatment outcomes and preventing tolerance in exudative ARMD.

Our study has several limitations. First, the repeated treatment interval was much shorter than in clinical practice. Expression changes of other cytokines related to angiogenesis under the hypoxic condition were not investigated. As mentioned earlier, neovascularization is the result of interactions among numerous cells and cytokines. Finally, the pathway for the upregulated VEGF and VEGFR-2 expression was not evaluated in the current experiment.

However, we demonstrated that the response to anti-VEGF treatment may vary and be occasionally paradoxical according

to treatment dose and repetition. Further studies are needed to find the feedback mechanism for VEGFR-2 expression related to VEGF level under a hypoxic condition.

In summary, bevacizumab treatment of hypoxic HUVECs potentially increased VEGFR expression to maintain cellular metabolism under hypoxia. Our results may partially explain reduced responsiveness after repeated anti-VEGF treatment for neovascular ARMD in clinical settings.

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