ORIGINAL RESEARCH

Bevacizumab inhibits proliferation of choroidal endothelial cells by regulation of the cell cycle

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Background: The purpose of this study was to evaluate cell cycle changes in choroidal endothelial cells treated with varying doses of bevacizumab in the presence of a range of concentrations of vascular endothelial growth factor (VEGF). Bevacizumab, a drug widely used in the treatment of neovascular age-related macular degeneration, choroidal neovascularization, and proliferative diabetic retinopathy, neutralizes all isoforms of VEGF. However, the effect of intravitreal administration of bevacizumab on the choroidal endothelial cell cycle has not been established.

Methods: Monkey choroidal endothelial (RF/6A) cells were treated with VEGF 50 ng/mL and escalating doses of bevacizumab 0.1–2 mg/mL for 72 hours. Cell cycle changes in response to bevacizumab were analyzed by flow cytometry and propidium iodide staining. Cell proliferation was measured using the WST-1 assay. Morphological changes were recorded by bright field cell microscopy.

Results: Bevacizumab inhibited proliferation of choroidal endothelial cells by stabilization of the cell cycle in G0/G1 phase. Cell cycle analysis of VEGF-enriched choroidal endothelial cells revealed a predominant increase in the G2/M population (21.84%, $P < 0.01$) and a decrease in the G0/G1 phase population (55.08%, $P < 0.01$). Addition of escalating doses of bevacizumab stabilized VEGF-enriched cells in the G0/G1 phase (55.08%, 54.49%, 56.3%, and 64% [$P < 0.01$]) and arrested proliferation by inhibiting the G2/M phase (21.84%, 21.46%, 20.59%, 20.94%, and 16.1% [$P < 0.01$]). The increase in GO/G1 subpopulation in VEGF-enriched and bevacizumab-treated cells compared with VEGF-enriched cells alone was dose-dependent.

Conclusion: Bevacizumab arrests proliferation of VEGF-enriched choroidal endothelial cells by stabilizing the cell cycle in the G0/G1 phase and inhibiting the G2/M phase in a dose-dependent fashion.

Keywords: bevacizumab, age-related macular degeneration, vascular endothelial growth factor

Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness among patients over the age of 55 years in developed nations. Exudative AMD, a less common form of age-related macular degeneration, is characterized by formation of a choroidal neovascular membrane fibrovascular complex that emanates from the choriocapillaris through a defective Bruch's membrane. Exudation, hemorrhage, and subsequent detachment involving the neurosensory retina and retinal pigment epithelium are often associated with the neovascular process. Vascular endothelial growth factor (VEGF), a diffusible cytokine that induces epithelial cell proliferation and leakage,

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has been implicated as an important factor in this process.[1,2] Inhibition of VEGF has become a widely accepted treatment for exudative AMD.

Bevacizumab, an anti-VEGF monoclonal antibody, is used intravitreally to inhibit choroidal endothelial cell proliferation associated with choroidal neovascularization. In vivo, the cell cycle of choroidal endothelial cells is under the influence of numerous growth factors present in the choroid, including vascular endothelial growth factor.[3] Neither the amount of bevacizumab needed to inhibit proliferation of choroidal endothelial cells, nor the mechanism behind binding of bevacizumab to VEGF after intravitreal administration is known. In vitro, bevacizumab decreases VEGF-mediated monkey choroidal endothelial cell (RF/6A) proliferation.[4]

In this report, we investigated the effect of escalating doses of bevacizumab on the cell cycle dynamics of VEGF-enriched proliferating choroidal endothelial cells. Our findings confirm RF/6A cell cycle stabilization in the G0/G1 phase after treatment with bevacizumab and subsequent inhibition of proliferation in a cell culture model.

Materials and methods
Cell culture
RF/6A cells were obtained from the American Type Culture Collection (CRL-1780, Manassas, VA, USA) and were cultured in Eagle's minimal essential medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA), penicillin 100 U/mL, and streptomycin 100 µg/mL.

RF/6A cells were treated with human VEGF$_{165}$ (Pepro Tech, Rocky Hill, NJ) 50 ng/mL and/or 0.1, 1, 1.5 or 2 mg/mL of bevacizumab (Avastin®, Genentech, South San Francisco, CA, USA), a recombinant humanized monoclonal antibody that inhibits the biologic activity of human VEGF for 72 hours.

Cell growth assays
For RF/6A cell growth assays, cells were plated at a density of 3000 cells/well in 96-well plates. Cellular proliferation was assessed according to the manufacturer's instructions with a 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.3-benzene disulfonate (WST-1) kit (Roche, Mannheim, Germany). The colorimetric assay is based on cleavage of the tetrazolium salt, WST-1, by mitochondrial dehydrogenases in viable cells. WST-1 solution (100 µL/well) was added to cells in 96-well plates followed by incubation for 2 hours at 37°C. The plate was read on a spectrophotometer at 440 nm with a reference wavelength at 690 nm. Cells were treated with VEGF 50 ng/mL and/or different doses of bevacizumab (0.1, 1, 1.5, 2 mg/mL) for 72 hours.

Cell morphology
Before exposure of the choroidal endothelial cells to bevacizumab, cellular morphology was recorded with bright field microscopy. Subsequently, cell morphology was assessed with an Olympus IX51 microscope 72 hours after incubation, with respective concentrations of VEGF 50 ng/mL, bevacizumab 0.1, 1, 1.5, 2 mg/mL, and VEGF plus bevacizumab. Cells treated with hydrogen peroxide 1 mM served as negative controls.

Cell cycle analysis
Choroidal endothelial cell cycle changes were assessed with flow cytometric evaluation of cells stained with propidium iodide (Sigma-Aldrich). For the flow cytometry assays, 100,000 cells/well were plated in six-well plates. The cells were allowed to attach for 24 hours. The cells were treated with varying concentrations of bevacizumab (0.1, 1.0, 1.5, 2 mg/mL) and/or VEGF 50 ng/mL and incubated for another 72 hours. For flow cytometry assays using propidium iodide only, the cells were trypsinized and the pellet was resuspended in 70% ethanol (Sigma-Aldrich) solution for 10 minutes at 4°C. After centrifugation and subsequent washes with Hank's Balanced Salt Solution (Invitrogen), the pellet was resuspended in 10 µg/mL of propidium iodide solution and incubated at room temperature for 30 minutes in the dark.

Stained cells were analyzed on a Beckman Coulter (Fullerton, CA, USA) flow cytometer using excitation at 488 nm and emission at 600 nm for propidium iodide. Ten thousand cells were counted per sample, and the data were processed using standard software (Beckman Coulter or WINMDi 2.9). For each histogram obtained, gating was established to determine specific subpopulations based on their position in the cell cycle. Gates for flow cytometry analysis were established on control samples and were applied uniformly throughout for the other treatment groups. Serum-starved cells served as controls.

Statistical analysis
All experiments were done in at least triplicate. Statistical analysis amongst treatment groups was performed with analysis of variance (GraphPad, La Jolla, CA, USA). For the proliferation assays, two-tailed t-test analysis was used to determine P values. Trend lines were determined using Excel (Microsoft, Redmond, WA, USA).

Results

Cell cytotoxicity: cell growth assays

Proliferation rates in the treatment groups were quantified as percentages of control proliferation values (which were considered 100%). Compared with controls, VEGF 50 ng/mL produced a 7.7% increase in proliferation of RF/6A cells ($P = 0.04$). Treatment with VEGF 50 ng/mL and bevacizumab 0.1 mg/mL produced a 24% decrease in RF/6A cell proliferation rates compared with controls ($P = 0.03$). At higher concentrations, bevacizumab (1 mg/mL and 2 mg/mL) induced a 12.1% and 10.2% decrease, respectively, in proliferation of RF/6A cells enriched with VEGF 50 ng/mL compared with controls ($P = 0.02$ and $P = 0.02$, respectively, Figure 1). Bevacizumab alone produced a decrease in RF/6A cell proliferation concentrations at doses of 0.1, 1, and 2 mg/mL (3.69%, 4.81%, and 5.42%, respectively, compared with control proliferation rates, $P = 0.06$, $P = 0.05$, and $P = 0.05$, respectively).

Cell cytotoxicity: morphology

Cellular changes after treatment with bevacizumab (0.1, 1, 1.5, 2 mg/mL) were assessed by bright field microscopy. The morphology of cells treated with bevacizumab and/or VEGF was unchanged compared with controls (Figure 2A and B). Cells maintained their polygonal shape and tight intercellular contacts with all treatments. Bright field microscopy of RF/6A cells in culture after 72 hours did not show cell membrane damage, a shrunken cytosol, or nuclear changes in the controls, VEGF, bevacizumab alone, or VEGF plus bevacizumab groups at any of the concentrations tested (0.1, 1, 1.5, 2 mg/mL bevacizumab, and VEGF 50 ng/mL). In comparison, in negative controls, represented by cells treated with 1 mM hydrogen peroxide treatment for 72 hours, we observed cellular debris and a few cells with shrunken cytoplasm.

Cell cycle analysis: flow cytometry

In cells under serum-starved conditions (representing the normal choroidal endothelial cell milieu), the percentage of cells in G0/G1 phase was 70.39% (controls), in G2/M phase was 14.56%, and in S phase was 11.32% (Table 1). In RF/6A cells treated with VEGF 50 ng/mL, a decrease in the G0/G1 phase population (55.08%) was observed. In VEGF-treated RF/6A cells, the percentage of cells in S phase was 14.9%. VEGF 50 ng/mL increased the percentage of RF/6A cells in G2/M phase (21.84% versus 14.56% in controls, Figure 3A and B).

Compared with controls, a negative linear trend line was observed for the G0/G1 subpopulations of VEGF-enriched cells ($r^2 = 1$; $y = -15.317x + 85.713$). Compared with controls, a positive linear trend was observed for the G2/M subpopulations of VEGF-enriched cells ($r^2 = 1$; $y = 7.28x + 7.28$).

Addition of bevacizumab (0.1, 1, 1.5, 2 mg/mL) to VEGF-enriched cells (50 ng/mL) produced an increased percentage of cells in the G0/G1 state (55.08%, 54.49%, 56.3%, and 64%, respectively). VEGF 50 ng/mL and bevacizumab (0.1, 1, 1.5, 2 mg/mL) produced dose-dependent decreased percentages of cells in the G2/M state compared
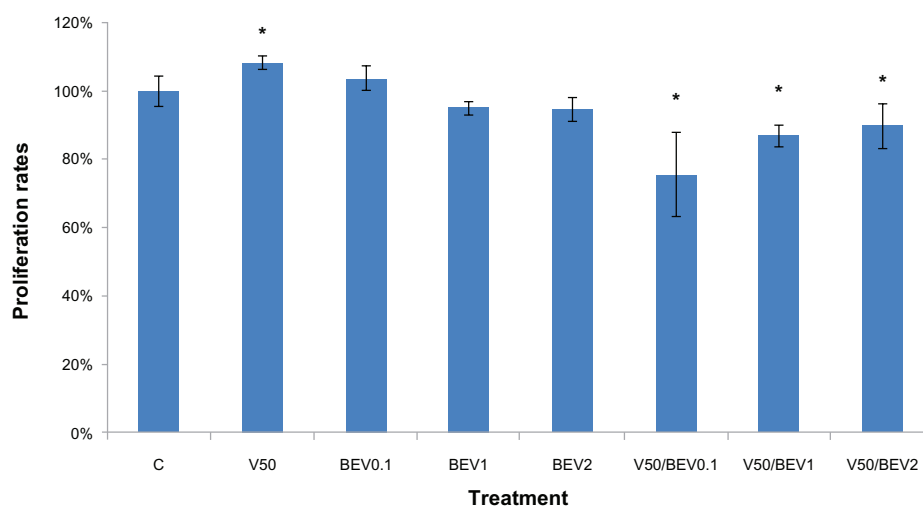

Figure 1 Proliferation of RF/6A cells in response to VEGF and bevacizumab.
Notes: Effect of different concentrations (0.1–2 mg/mL) of bevacizumab and/or VEGF 50 ng/mL on proliferation of RF/6A cells. Increasing concentrations of bevacizumab 1–2 mg/mL produce a significant decrease in cell proliferation rate compared with controls. VEGF increases RF/6A proliferation rates as compared with controls. Cell proliferation was determined by the WST-1 assay. Proliferation rates are expressed as percentages of control values. *$P < 0.05$.
Abbreviations: BEV, bevacizumab; VEGF, vascular endothelial growth factor.
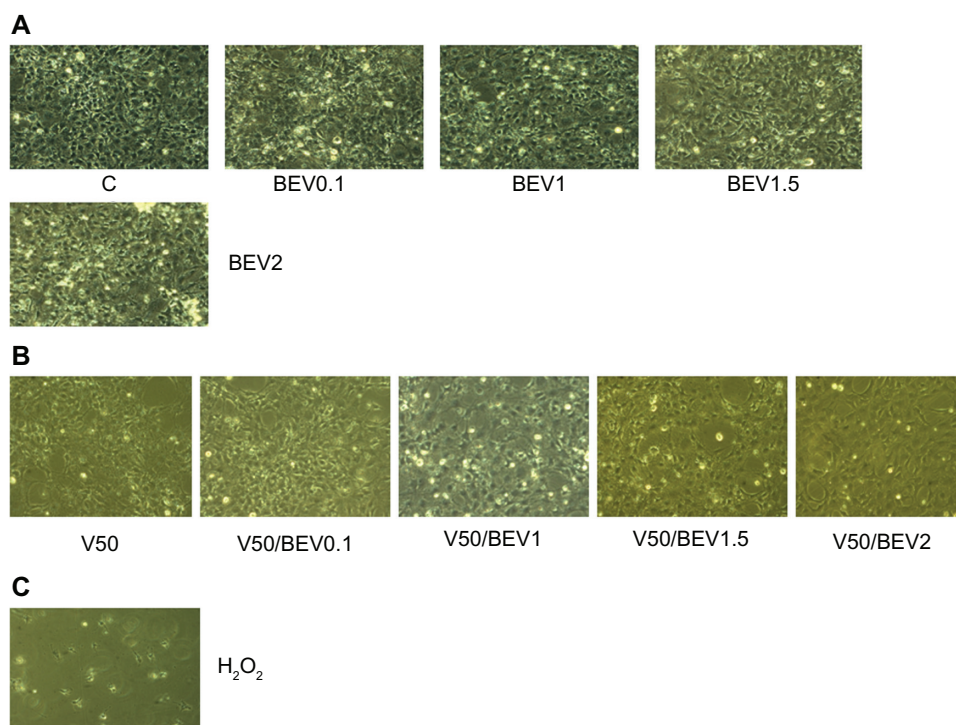
Figure 2 Morphology of RF/6A cells treated with bevacizumab and/or VEGF. (**A**) Effect of different concentrations of bevacizumab alone on RF/6A cell morphology. (**B**) Effect of different concentrations of bevacizumab and VEGF on RF/6A cell morphology. Photomicrographs showing no observable differences in morphology amongst different treatment groups and controls. Bright field images were taken at a 20 × magnification. (**C**) Hydrogen peroxide-treated negative controls cells showed cellular remnants and a few viable cells.
Abbreviations: BEV, bevacizumab; VEGF, vascular endothelial growth factor.

with controls (21.46%, 20.59%, 20.94%, and 16.1%, respectively). The highest increase in G0/G1 phase subpopulation occurred in cells treated with bevacizumab 2 mg/mL plus VEGF, where the G0/G1 phase population was 64% compared with 52% for VEGF-enriched cells (Figure 4A and B). The increase in the G0/G1 subpopulation in VEGF-enriched and bevacizumab-treated cells compared with VEGF-enriched cells was dose-dependent. VEGF 50 ng/mL and bevacizumab (0.1, 1, 1.5, 2 mg/mL) did not alter the S phase population, with the exception of the VEGF and bevacizumab 2 mg/mL treatment groups (15.75%, 16.03%, 16.66%, and 13.5%, respectively, Figure 4A and B). VEGF 50 ng/mL and bevacizumab (0.1, 1, 1.5, 2 mg/mL) produced a positive linear increase in the subpopulation of G0/G1 cells compared with VEGF-enriched cells ($r^2 = 0.2854$; $y = 1.4143x + 50.4$). Treatment with VEGF 50 ng/mL and bevacizumab (0.1, 1, 1.5, 2 mg/mL) produced a linear decrease in G2/M cell subpopulation percentages ($r^2 = 0.455$; $y = -0.9608x + 24.256$). VEGF 50 ng/mL and bevacizumab (0.1, 1, 1.5, 2 mg/mL) did not determine changes in S phase population percentages compared with control percentages ($r^2 = 0.0473$; $y = -0.164x + 16.308$).

Discussion

AMD is the leading cause of severe vision loss and blindness in individuals over the age of 50 years, and affects 15 million North Americans. The exudative (or neovascular) variety accounts for approximately 10% of individuals with wet AMD. Exudative AMD is characterized by the presence of a neovascular complex resulting from a break in Bruch's membrane. The fibrovascular complex proliferates within the inner aspect of Bruch's membrane and destroys the

Table 1 Cell cycle analysis of RF/6A choroidal endothelial cells treated with bevacizumab and VEGF

Cell cycle phases		
Treatments	**G0/G1**	**S + G2/M**
VEGF (50 ng/mL)	55.08	36.74
VEGF + BEV (0.1 mg/mL)	55.08	37.21
VEGF + BEV (1 mg/mL)	54.49	39.62
VEGF + BEV (1.5 mg/mL)	56.3	37.6
VEGF + BEV (2 mg/mL)	64	29.6

Notes: Effect of different concentrations of bevacizumab and VEGF on cell cycle as quantified by flow cytometry. Gating was performed on DNA content plot graphs to measure percentages of cells in G0/G1, S, and G2/M subphases.
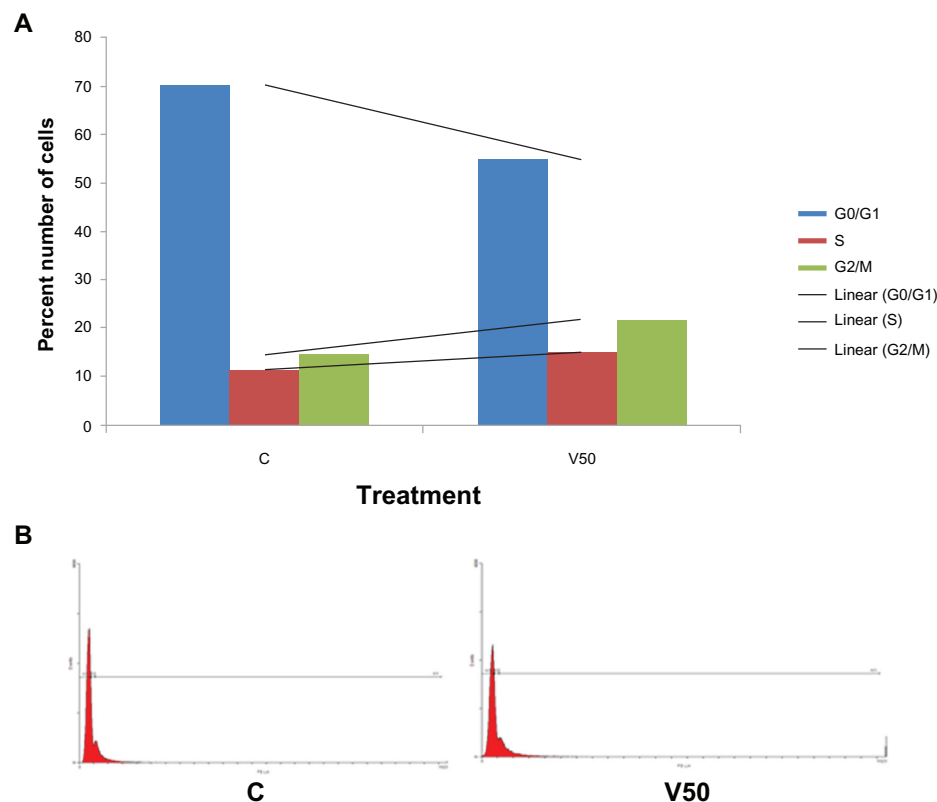**Abbreviations:** BEV, bevacizumab; VEGF, vascular endothelial growth factor.

Figure 3 Cell cycle analysis of RF/6A choroidal endothelial cells under control or VEGF-enriched conditions. (**A**) Effect on cell cycle as quantified by flow cytometry. Flow cytometry analysis of control RF/6A or VEGF-treated cells. Cells were stained with propidium iodide prior to flow cytometry analysis. For analysis purposes, gating was performed on DNA content plot graphs to measure percentages of cells in G0/G1, S, and G2/M subphases. Gates were established on control samples and were maintained constant for the other treatment groups. (**B**) Representative DNA histograms showing phase cell cycle changes in response to treatment.
Abbreviation: VEGF, vascular endothelial growth factor.

normal architecture of the choriocapillaris, Bruch's membrane, retinal pigment epithelium, and outer retina.[1,3,5,6]

Histologically in exudative AMD, accumulation of drusen with concomitant thickening of the collagenous layers within Bruch's membrane creates a hydrophobic barrier impeding the passage of fluid and nutrients between the choroid and outer retina; therefore, an area of relative ischemia develops.[1,3,5,6] Ischemia causes vasodilation of existing blood vessels and increased vascular permeability; in combination with an influx of growth-promoting and growth-inhibiting cytokines, choroidal endothelial cells proliferate and fracture in Bruch's membrane, providing a path for ingrowth of neovascular complexes.[1,3] Although many cytokines are currently under investigation, VEGF appears to be the most important factor in initiating choroidal endothelial cell proliferation.[3,4,7–9]

Anti-vascular endothelial growth factor therapies are currently the mainstay of treatment for exudative AMD. Pegaptanib, ranibizumab, and bevacizumab are recognized modalities for inhibiting the effect of VEGF. Pegaptanib is

an oligonucleotide aptamer that antagonizes the effect of VEGF and was one of the first compounds used to treat exudative AMD. Bevacizumab is a full-length monoclonal antibody to VEGF that is extensively used as an off-label treatment for macular edema and exudative AMD. Anti-VEGF agents interfere with VEGF and inhibit the process of angiogenesis.[10–14]

Traditionally, the cell cycle is divided into four distinct phases, ie, G0, G1, S, G2, and M. Each phase involves numerous cytokines and cell signaling to serve as a series of checks and balances to ensure proper regulation of the cell cycle, thus ensuring balance of cell lines. The ubiquitin-proteasome system, through ubiquitination and deubiquitination, manages cell cycle progression between each phase, ensuring a unidirectional pathway to the cell cycle.[15,16]

A key regulating factor, known as hypoxia inducible factor (HIF)-1α, modulates angiogenesis by promoting mitogenic and migratory activities of endothelial cells. Two angiogenic factors, VEGF and angiopoetin-2, are dependently regulated by HIF-1α; in normal conditions, HIF-1α is
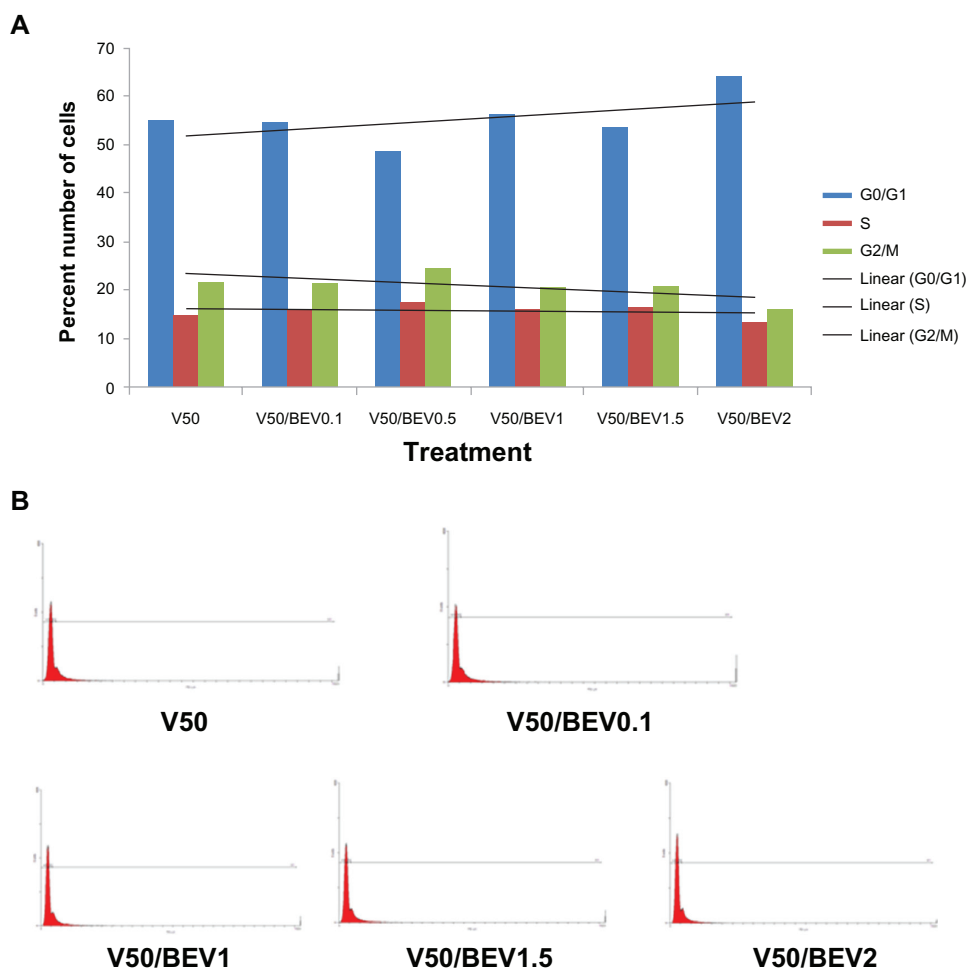
A

B

Figure 4 Cell cycle analysis of RF/6A choroidal endothelial cells treated with bevacizumab and VEGF. (**A**) Effect of different concentrations of bevacizumab and VEGF on cell cycle as quantified by flow cytometry. Flow cytometry analysis of RF/6A cells treated with bevacizumab. Cells were stained with propidium iodide prior to flow cytometry analysis. For analysis purposes, gating was performed on DNA content plot graphs showing percentage of cells G0/G1, S, and G2/M phases. Gates were established on control samples and were maintained constant for the other treatment groups. (**B**) Representative DNA histograms showing phase cell cycle changes in response to treatment.
Abbreviations: BEV, bevacizumab; VEGF, vascular endothelial growth factor.

constantly degraded to modulate vascular growth.[16] In stress conditions, such as hypoxia, ubiquitination of HIF-1α enables heterodimerization with HIF-1β and subsequent attachment to hypoxic responsive elements within the promoter regions of target genes. HIF-1α directly regulates hypoxia-induced activity of nitric oxide synthase, through binding sites with affinity to nitric oxide synthase in a wide range of cell types, including cardiac myocytes, endothelial, glioblastoma, and hepatoma cells. In turn, the nitric oxide produced promotes endothelial cell proliferation by inhibiting caspase-induced apoptosis.[15,16]

This study investigated the effect of increasing doses of bevacizumab on the cell cycle of VEGF-enriched proliferating choroidal endothelial cells. In our model, serum-starved choroidal endothelial cells served as control cells and represent stable choroidal endothelium; VEGF-enriched culture conditions represent active neovascularization. Addition of

VEGF increased proliferation rates of choroidal endothelial cells to 107.7%. In the presence of bevacizumab, proliferation rates of VEGF-enriched cells decreased in a dose-independent fashion (range 10.2%–24%). Flow cytometric assays of untreated RF/6A cells demonstrate 60% in the G0/G1 phase, 20% in the S phase, and 20% in the G2/M phase.[17] In our study, flow cytometry results showed a significant shift in the proportion of cells from the G2/M phase to G0/G1 phase after addition of bevacizumab to VEGF-enriched cells. Compared with VEGF-enriched only cells, the percentage of cells in the "resting" phase increased, while the percentage of cells in the "active" phase decreased in a dose-dependent fashion. The highest increase in the G0/G1 phase subpopulation occurred in VEGF-enriched cells treated with bevacizumab 2 mg/mL. Brar et al[4] also reported a 29.7% increase in cell growth with addition of VEGF 50 ng/mL. Although some studies exhibited VEGF-induced RF/6A

cellular proliferation, the impact on cell cycle control was not evaluated.[8,9,18,19] Our study demonstrated a reduced rate of proliferation, with a higher percentage of cells in G0/G1 and a lower percentage of cells in G2/M upon addition of various concentrations of bevacizumab to VEGF-enriched cells. In ARPE-19 cells, bevacizumab produced a G1/S cell cycle arrest.[20]

Cell cycle inhibitors, such as p27 KIP, may be involved in the mechanism behind this effect.[21] In fact, the addition of rosmarinic acid, a VEGF antagonist, had a similar effect on choroidal endothelial cells and produced G2/M cell cycle arrest with upregulation of p27 KIP.[21] In summary, bevacizumab inhibits G2/M phase and arrests proliferation of VEGF-enriched choroidal endothelial cells by stabilizing the cell cycle in G0/G1 phase in a dose-dependent manner.

Disclosure

The authors report no conflicts of interest in this work.

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