Original Article

Effects of Bevacizumab on Bcl-2 Expression and Apoptosis in Retinal Pigment Epithelial Cells under Oxidative Stress

Sukjin Kim, Young Jun Kim, Na Rae Kim, Hee Seung Chin

Department of Ophthalmology and Inha Vision Science Laboratory, Inha University School of Medicine, Incheon, Korea

Purpose: To evaluate the effects of bevacizumab on expression of B-cell leukemia/lymphoma (Bcl)-2 and apoptosis in retinal pigment epithelial (RPE) cells under oxidative stress conditions.

Methods: RPE cells were treated with H_2O_2 (0, 100, 200, 300, and 400 µM) and bevacizumab at or above the doses normally used in clinical practice (0, 0.33, 0.67, 1.33, and 2.67 mg/mL). Cell apoptosis was measured using flow cytometry with annexin V-fluorescein isothiocyanate. The expression of Bcl-2 mRNA was determined using reverse transcription polymerase chain reaction.

Results: Under low oxidative stress conditions (H_2O_2 100 µM), cell apoptosis was not significantly different at any concentration of bevacizumab, but Bcl-2 mRNA expression decreased with increasing concentration of bevacizumab (0.33, 0.67, 1.33, and 2.67 mg/mL). Under moderate oxidative stress conditions (H_2O_2 200 µM), Bcl-2 mRNA expression decreased with increasing concentration of bevacizumab (0.33, 0.67, 1.33, and 2.67 mg/mL), but cell apoptosis increased only at 2.67 mg/mL of bevacizumab. Under high oxidative stress (300 µM) conditions, cell apoptosis increased at high concentrations of bevacizumab (1.33 and 2.67 mg/mL), but it did not correlate with Bcl-2 expression.

Conclusions: Withdrawal of vascular endothelial growth factor can lead to RPE cell apoptosis and influences the expression of anti-apoptotic genes such as Bcl-2 under oxidative stress conditions. Since oxidative stress levels of each patient are unknown, repeated injections of intravitreal bevacizumab, as in eyes with age-related macular degeneration, might influence RPE cell survival.

Key Words: Apoptosis, Bcl-2, Bevacizumab, Retinal pigment epithelial cell

Vascular endothelial growth factor (VEGF) has been implicated as a pro-angiogenic factor that stimulates neo-vascularization in age-related macular degeneration (AMD), and retinal pigment epithelial (RPE) is an important source of VEGF [1]. VEGF expression by RPE is a feature of tissues excised from human eyes showing AMD-related choroidal neovascularization [2]. Oxidants have been reported to increase the deposition of oxidized proteins or other oxidized compounds in Bruch's membrane in a process that might involve complement activation and inflammation, provoking VEGF-A release in patients with exudative AMD [1]. Thus, VEGF-A is induced by chronic exposure to oxidative stress, suggesting a role of this stress

in both neovascular and advanced dry AMD [3].

AMD eyes exhibited increased RPE apoptosis compared with normal eyes [3,4]. In particular, RPE apoptosis is an important feature of advanced forms of AMD; indeed, patchy loss of RPE is one of the classic features of AMD, and this process involves apoptosis of RPE cells [4]. In eyes with exudative AMD, apoptotic cells are present in choroidal neovascular membranes [5]. B-cell leukemia/lymphoma (Bcl)-2 is a key anti-apoptotic member of the Bcl-2 family that regulates the intrinsic apoptosis pathway and is comprised of important survival factors in RPE cells [5-7]. Oxidative stress activates the intrinsic apoptosis pathway, a process mediated by enhanced mitochondrial membrane permeability and decreased Bcl-2 [8].

Anti-VEGF therapy plays a significant role in the management of retinal and retinal vascular disorders and has demonstrated the best clinical outcomes of all approaches tested to date. However, VEGF-A is a known survival factor of vascular endothelial cells and the developing and mature retina [9-11]. RPE tears and choroidal atrophy in specimens from patients with anti-VEGF treated AMD raise questions about the long-term safety of anti-VEGF treatment [12].

The effects of bevacizumab, which might involve cell apoptosis and regulation of anti-apoptotic protein Bcl-2 expression, have not been fully investigated using RPE cells under oxidative stress. Our research therefore focused on evaluating the effects of bevacizumab on expression of Bcl-2 and apoptosis in RPE cells under various oxidative stress conditions and the possible complications of intravitreal bevacizumab injections in patients.

Materials and Methods

Cell culture and exposure to oxidative stress

A human retinal pigment epithelial (ARPE-19) cell line was obtained from ATCC (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 nutrient medium (Invitrogen-Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and amphotericin B. The ARPE-19 cells were used for four to six passages and plated in six-well plates at 1.5×10^5 cells per well. They were incubated for 24 hours in a humidified 5% CO_2 atmosphere at 37°C after reaching approximately 70% confluence. We next washed the cells with pH 7.4 phosphate buffered saline (PBS). The cells were serum-starved for four hours before H_2O_2 exposure and treated with H_2O_2 (100 to 400 µM) for 16 hours to induce oxidative stress before they were harvested for cell death analysis.

Enzyme-linked immunosorbent assay after exposure to oxidative stress

The cells were treated with various concentrations of H_2O_2. The supernatants were collected at baseline (0 hours) and at 16 hours, centrifuged to remove cell debris, and stored at -70°C until enzyme-linked immunosorbent assay was performed according to the manufacturer's instructions. In this analysis, a monoclonal antibody specific for VEGF-A was pre-coated onto a microplate. Standards and samples were pipetted into the wells, and any VEGF-A present was bound by the immobilized antibody. After washing any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF-A was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells, and color developed in proportion to the amount of VEGF-A bound in the initial step. The color development was then stopped, and the intensity of the color was measured. To determine the optical density of each well, we used a microplate reader at 450 and 540 nm. The concentrations of VEGF-A standard numbers 1 to 8 were 0, 15.6, 31.2, 62.5, 125, 250, 500, and 1,000 pg/mL of recombinant VEGF-A in a buffered protein base with preservatives, respectively. The level of VEGF-A protein was measured in cell-free supernatant using a human VEGF-A Quantikin ELISA kit (catalog no. DVE00; R&D Systems, Minneapolis, MN, USA).

Bevacizumab treatment and flow cytometric analysis of apoptosis after exposure to oxidative stress

The cells were washed with PBS and incubated in serum-free Dulbecco's modified Eagle's medium in the presence of H_2O_2 (100, 200, 300, and 400 µM) for 16 hours. Bevacizumab (0.33, 0.67, 1.33, and 2.67 mg/mL, respectively) was added 2 hours before H_2O_2 treatment. An annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to detect

phosphatidylserine externalization as an index of apoptosis. The cells were washed and incubated for 15 minutes at room temperature in the presence of annexin V labeled with FITC and propidium iodide (PI). In total, 10,000 cells were excited at 488 nm, and emission was measured at 530 and 584 nm to assess FITC and PI fluorescence, respectively. The cells were analyzed with a flow cytometer (BD Biosciences). The number of gated cells was plotted on a dot plot with reference to both annexin V and PI staining.

Reverse transcription polymerase chain reaction

The cells were diluted to 1×10^5 cell/mL and were incubated for 24 hours in six-well plates (Falcon, BD Biosciences). After washing the culture media twice with PBS, serum-free media was applied. H_2O_2 and bevacizumab were challenged at different concentrations (0, 100, 200, 300, 400 μM and 0.33, 0.67, 1.33, 2.67 mg/mL) and incubated for 16 hours. Total RNA was isolated using the total RNA purification kit (Invitrogen). Isolated RNA was quantified, and 1 μg of total RNA and 100 pmol of oligo dT were added to the reverse transcription (RT) premix (Bioneer, Seoul, Korea) to prepare 20 μL of cDNA. Preformed cDNA, Bcl-2 primer, and glycerol-3-phosphate dehydrogenase (GAPDH) primer were mixed with polymerase chain reaction (PCR) premix (Bioneer), and PCR was performed. The sequences of the primers are described in Table 1. Next, 10 μL of each amplicate were assessed using 1.5% agarose gel electrophoresis. Quantification of Bcl-2 mRNA content was performed using computer-assisted video densitometry (Eagle Eye II-system; Stratagene, La Jolla, CA, USA).

Statistical analysis

Data are expressed as percentage of control or mean ± standard deviation of results in three or more independent experiments. Statistical analysis was performed using SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA). The Kruskal-Wallis test was used to compare the difference between control and experimental groups. A p-value ≤0.05 was considered statistically significant.

Results

Influence of bevacizumab on apoptosis of retinal pigment epithelial cells without H_2O_2

The cells were treated with bevacizumab for 16 hours (0, 0.33, 0.67, 1.33, and 2.67 mg/mL). Cell apoptosis was not significantly different at control, clinical (0.33 and 0.67 mg/mL), or high (1.33 and 2.67 mg/mL) doses of bevacizumab without H_2O_2 (Fig. 1).

Effects of H_2O_2 on apoptosis of retinal pigment epithelial cells

The cells were cultured with H_2O_2 for 16 hours (0, 100, 200, 300, and 400 μM). Cell apoptosis was 3.26% for RPE at 100 μM H_2O_2 compared to 3.82% for the controls, but the difference was not significant. Cell apoptosis decreased at 200 μM H_2O_2 (2.65%) but increased at 300 and 400 μM H_2O_2 (10.42%, 17.99%). The means at these concentrations were significantly different than the control ($p < 0.05$) (Fig. 2A).

Vascular endothelial growth factor-A level under oxidative stress conditions

VEGF-A expression was increased after addition of 50, 100, and 200 μM H_2O_2 to RPE cells. However, after being treated with 300 and 400 μM H_2O_2, VEGF-A expression decreased. Means at these concentrations were significantly different from the control ($p < 0.05$) (Fig. 2B).

Table 1. Primer used for reverse transcription polymerase chain reaction

Target gene	Primer	Sequence	Product size (bp)
Bcl-2	Forward	5'-CTA CGA GTG GGA TGC GGG AGA TG-3'	478
	Reverse	5'-GGT TCA GGT ACT CAG TCA TCC ACA G-3'	478
GAPDH	Forward	5'-CCA CTG GCG TCT TCA CCA C-3'	481
	Reverse	5'-CCT GCT TCA CCA CCT TCT TG-3'	481

Bcl = B-cell leukemia/lymphoma; GAPDH = glycerol-3-phosphate dehydrogenase.
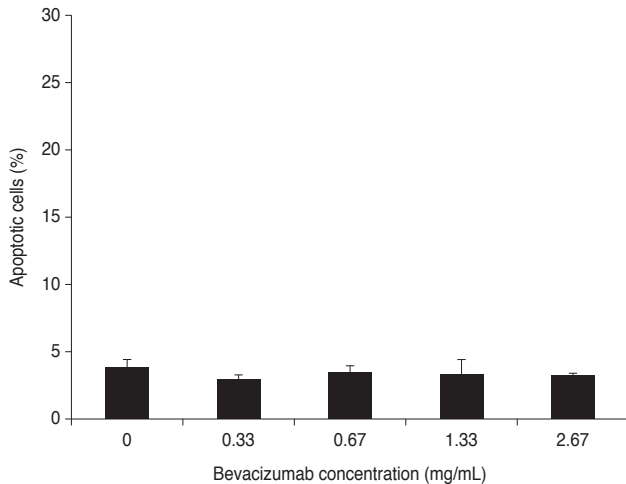
Fig. 1. Influence of bevacizumab on apoptosis of retinal pigment epithelial cells in the absence of H_2O_2. The retinal pigment epithelial cells were treated with bevacizumab for 16 hours (0, 0.33, 0.67, 1.33, and 2.67 mg/mL). Cellular apoptosis was not significantly different at control, clinical (0.33 and 0.68 mg/mL) and high (1.33 and 2.67 mg/mL) doses of bevacizumab in the absence of H_2O_2 ($p = 0.121$, 0.439, 0.221, and 0.063, respectively). Each bar shows the mean ± standard deviation of results of three or more independent experiments.

Expression of Bcl-2 mRNA after exposure to oxidative stress

The cells were cultured with H_2O_2 for 16 hours (0, 100, 200, and 300 μM). Expression of Bcl-2 mRNA decreased as oxidative stress increased ($p < 0.05$) (Fig. 2C).

Influence of bevacizumab on apoptosis of RPE cells and Bcl-2 mRNA expression under low oxidative stress

ARPE-19 cells were treated with bevacizumab (0, 0.33, 0.67, 1.33, and 2.67 mg/mL) under low oxidative stress conditions (100 μM of H_2O_2). Cell apoptosis was not significantly different at any concentration of bevacizumab at 100 μM H_2O_2. Expression of Bcl-2 mRNA decreased under low oxidative stress conditions, and the decrease was proportional to the increase in bevacizumab. There was a statistically significant difference at all concentrations compared to the result of the control. The mRNA expression of Bcl-2 was normalized to GAPDH (a housekeeping gene). Each bar shows the mean ± standard deviation of results in three or more independent experiments ($p < 0.05$) (Fig. 3).

Influence of bevacizumab on apoptosis of RPE cells and Bcl-2 mRNA expression under moderate oxidative stress

ARPE-19 cells were treated with bevacizumab (0, 0.33, 0.67, 1.33, and 2.67 mg/mL) under moderate oxidative stress conditions (200 μM of H_2O_2). As mentioned previously, cell apoptosis decreased at 200 μM H_2O_2 (2.65%) compared with the control (3.82%). Cell apoptosis increased to 3.59%, 3.50%, and 4.13% at bevacizumab concentrations of 0.33, 0.67, and 1.33 mg/mL, respectively, but the differences were not statistically significant. However, cell apoptosis increased significantly to 10.16% at a high dose of bevacizumab (2.67 mg/mL) under the same oxidative stress conditions. Expression of Bcl-2 mRNA decreased proportionally to the increase in bevacizumab dose. There was a statistically significant difference at all concentrations ($p < 0.05$) (Fig. 4).

Influence of bevacizumab on apoptosis of RPE cells and Bcl-2 mRNA expression under high oxidative stress

ARPE-19 cells were treated with bevacizumab (0, 0.33, 0.67, 1.33, and 2.67 mg/mL) under high oxidative stress conditions (300 μM H_2O_2). As mentioned previously, cell apoptosis increased at 300 μM H_2O_2 (10.42%) compared with the control (3.82%). Cell apoptosis increased to 12.65% and 10.97% at clinical doses of bevacizumab (0.33, 0.67 mg/mL) under high oxidative stress conditions, but there was no statistically significant difference compared to the control. Cell apoptosis did significantly increase to 19.83% and 24.85% at high doses of bevacizumab (1.33, 2.67 mg/mL) under the same oxidative stress. Expression of Bcl-2 mRNA decreased under high oxidative stress conditions (300 μM H_2O_2), but there was no trend of increase or decrease as the dose of bevacizumab increased (Fig. 5). At 400 μM H_2O_2, the Bcl-2 mRNA data were not able to be interpreted. We suggest that the reverse transcription-PCR results might be a result of severe cellular apoptosis under high oxidative stress conditions.

Discussion

In the present study, we tested the possible toxicity of bevacizumab on RPE cells under various oxidative stress conditions, and we examined the expression of the an-
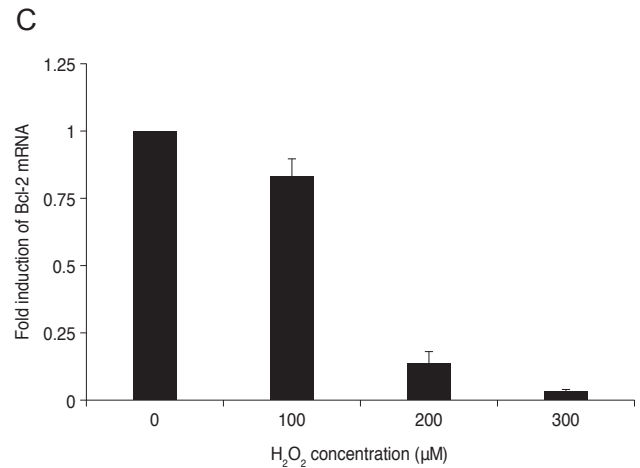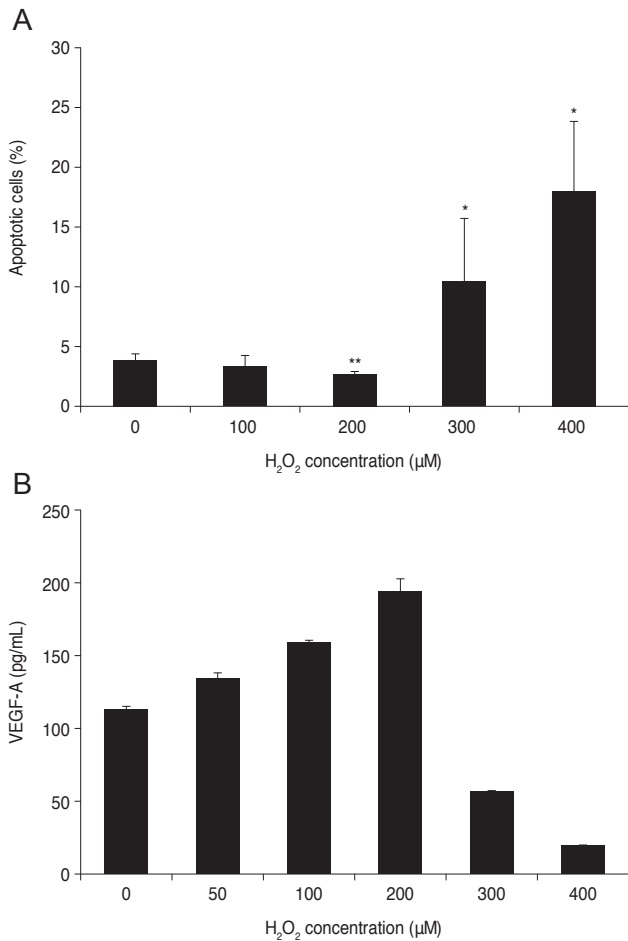
A

B

C

Fig. 2. Apoptosis and expression of vascular endothelial growth factor (VEGF)-A and B-cell leukemia/lymphoma (Bcl)-2 after treatment of retinal pigment epithelial (RPE) cells with H_2O_2. (A) Effects of H_2O_2 on apoptosis of RPE cells. The RPE cells were treated with various concentrations of H_2O_2 for 16 hours. Cell apoptosis was 3.26% for RPE at 100 μM H_2O_2 compared to 3.82% for the control, but the difference was not significant. Cell apoptosis decreased at 200 μM H_2O_2 (2.65%) but increased at 300 and 400 μM of H_2O_2 (10.42%, 17.99%). Means were significantly different than the control ($p < 0.05$). Each bar shows the mean ± standard deviation of results of three or more independent experiments. The asterisk indicates a statistically significant difference within the group (*increased, **decreased, $p < 0.05$). (B) Expression of VEGF-A after exposure to H_2O_2. VEGF-A excretion into the medium was measured using enzyme-linked immunosorbent assay. VEGF-A expression increased after addition of 50, 100, or 200 μM H_2O_2 to RPE cells. However, after being treated with 300 and 400 μM H_2O_2, VEGF-A expression decreased. Data are expressed as the mean ± standard deviation of the results of three or more independent experiments. Means were statistically significant compared to the control at all concentrations of H_2O_2 ($p < 0.05$). (C) Expression of Bcl-2 mRNA after exposure to H_2O_2. Cells were cultured with various concentrations of H_2O_2 for 16 hours (0, 100, 200, and 300 μM). Expression of Bcl-2 mRNA decreased as oxidative stress increased. Data are expressed as the mean ± standard deviation of the results of three or more independent experiments. Means were statistically significant compared with the control at all concentrations of H_2O_2 ($p < 0.05$).

ti-apoptotic protein Bcl-2 and the apoptosis of RPE cells. We used H_2O_2 to induce oxidative stress in order to replicate *in vivo* conditions that are implicated in the pathogenesis of AMD and the annexin V-FITC apoptosis kit to detect phosphatidylserine externalization as an index of apoptosis.

Intravitreal injections of anti-VEGF agents such as bevacizumab are widely used for the treatment of various retinal disorders associated with new vessels such as AMD or proliferative diabetic retinopathy in order to reduce angiogenesis [13]. Bevacizumab is a full-length, recombinant humanized immunoglobulin G antibody that binds to and inhibits all biologically active forms of VEGF-A isoforms. Despite ongoing clinical trials with intravitreal bevacizumab in many neovascular ocular disorders, its ocular safety remains an issue for research. Hypothetically, repeated injections with bevacizumab could interfere with the neuroprotective and survival actions of VEGF under oxidative stress.

In AMD patients, several studies have reported cases of RPE tears after intravitreal injections of bevacizumab for neovascular AMD [12]. Atrophy of the choriocapillaries or loss of endothelial cell fenestrations impairs nutritional support, which can lead to functional and morphologic
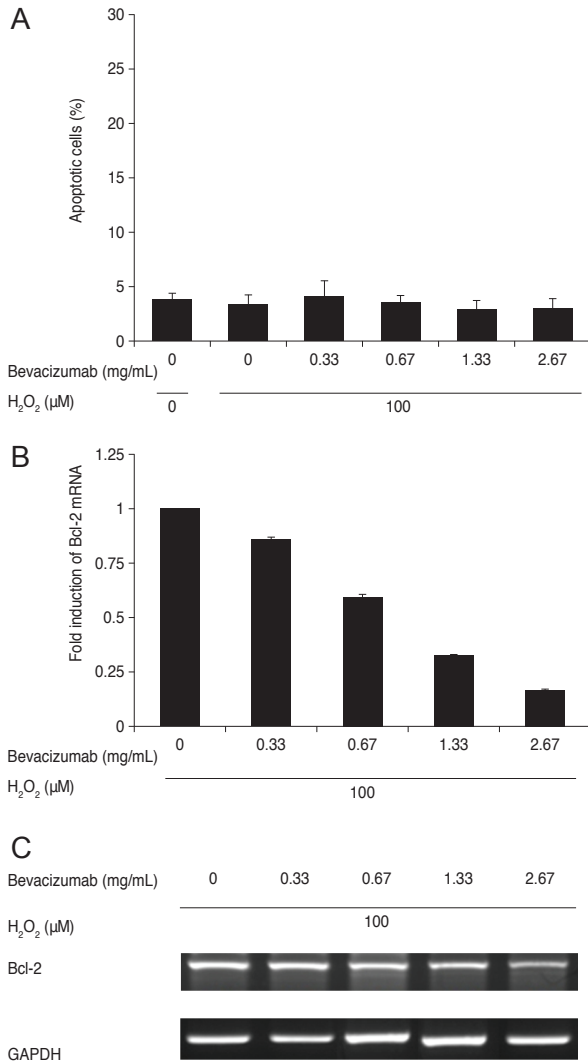
Fig. 3. Influence of bevacizumab on apoptosis of retinal pigment epithelial (RPE) cells and B-cell leukemia/lymphoma (Bcl)-2 mRNA expression under low oxidative stress (100 μM H_2O_2). (A) Influence of bevacizumab on apoptosis of RPE cells under low oxidative stress (100 μM H_2O_2). The RPE cells were treated with various concentrations of bevacizumab (0, 0.33, 0.67, 1.33, and 2.67 mg/mL) under low oxidative stress (100 μM H_2O_2). Cell apoptosis was not significantly different at all concentrations of bevacizumab less than 100 μM H_2O_2. Each bar shows the mean ± standard deviation of results of three or more independent experiments. $p < 0.05$ vs. control. (B,C) Expression of Bcl-2 mRNA with bevacizumab under low oxidative stress (100 μM H_2O_2). Expression of Bcl-2 mRNA decreased under low oxidative stress (100 μM H_2O_2), and this decrease was proportional to the increase in bevacizumab dose. There was a statistically significant difference at all concentrations. The mRNA expression of Bcl-2 was normalized to glycerol-3-phosphate dehydrogenase (GAPDH, housekeeping gene). Each bar shows the mean ± standard deviation of results of three or more independent experiments. $p < 0.05$ vs. control.
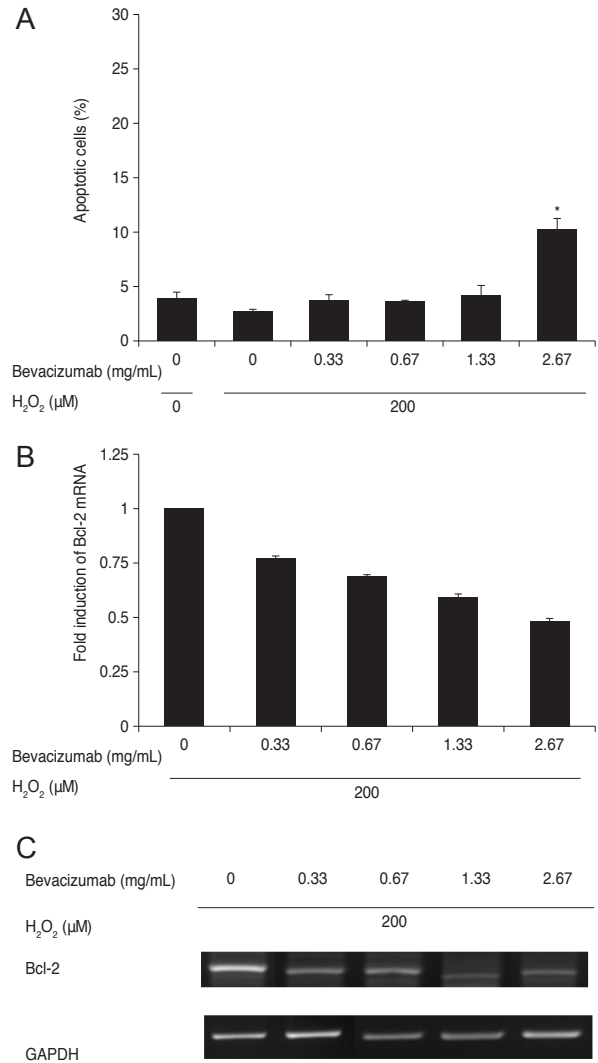
Fig. 4. Influence of bevacizumab on apoptosis of retinal pigment epithelial (RPE) cells and B-cell leukemia/lymphoma (Bcl)-2 mRNA expression under moderate oxidative stress (200 μM of H_2O_2). (A) Influence of bevacizumab on apoptosis of RPE cells under moderate oxidative stress (200 μM H_2O_2). The RPE cells were treated with various concentrations of bevacizumab (0, 0.33, 0.67, 1.33, and 2.67 mg/mL) under moderate oxidative stress (200 μM H_2O_2). Cell apoptosis decreased at 200 μM H_2O_2 (2.65%) compared with the control (3.82%). Cell apoptosis did not show significant change until a bevacizumab concentration of at least 1.33 mg/mL. Apoptosis increased to 10.16% at high doses of bevacizumab (2.67 mg/mL) under the same oxidative stress conditions. Each bar shows the mean ± standard deviation of results of three or more independent experiments. The asterisk indicates a statistically significant difference within the group (*$p < 0.05$). (B,C) Expression of Bcl-2 mRNA with bevacizumab under moderate oxidative stress (200 μM H_2O_2). Expression of Bcl-2 mRNA decreased under moderate oxidative stress (200 μM H_2O_2), and that decrease was proportional to the increase in bevacizumab dose. There was a statistically significant difference at all concentrations. The mRNA expression of Bcl-2 was normalized to glycerol-3-phosphate dehydrogenase (GAPDH, housekeeping gene). Each bar shows the mean ± standard deviation of results of three or more independent experiments. $p < 0.05$ vs. control.
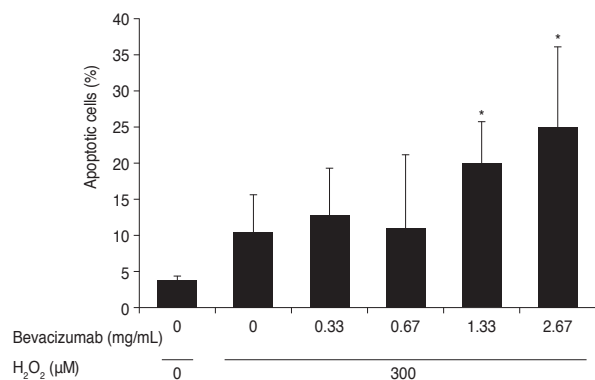
Fig. 5. Influence of bevacizumab on apoptosis of retinal pigment epithelial cells under high oxidative stress (300 μM H_2O_2). The retinal pigment epithelial cells were treated with various concentrations of bevacizumab (0, 0.33, 0.67, 1.33, and 2.67 mg/mL) under high oxidative stress (300 μM of H_2O_2). As mentioned previously, cell apoptosis increased at 300 μM H_2O_2 (10.42%) compared with the control (3.82%). Cell apoptosis increased to 12.65% and 10.97% at clinical doses of bevacizumab (0.33, 0.67 mg/mL) under high oxidative stress, but there was no statistically significant difference between the increases. Cell apoptosis significantly increased to 19.83% and 24.85% at high doses of bevacizumab (1.33, 2.67 mg/mL) under the same oxidative stress. Each bar shows the mean ± standard deviation of results of three or more independent experiments. The asterisk indicates a statistically significant difference within the group ($^*p < 0.05$).

damage to the RPE and photoreceptors, with particular adverse effects if the macular lesion is affected [14]. The development of geographic atrophy contributed to visual acuity loss after repeated anti-VEGF injections [15]. In addition, in a recent study in which a retinal neovascularization model was developed, it was suggested that caution is warranted in the treatment of patients with acute or severe retinal neovascularization using anti-VEGF drugs such as bevacizumab in order to prevent capillary nonperfusion and macular ischemia [16].

According to many recent reports, bevacizumab at concentrations at or above the dose normally used in clinical practice is not toxic to RPE cells [17-20]. Several preclinical experimental toxicity studies have reported the histopathological effects of bevacizumab on retinal cells, retinal neovascular membranes, and capillaries in paraffin-embedded sections [16,21,22] or organotypic culture [23], as well as in ultrastructural evaluations [14,17,21]. Based on *in vitro* assays, bevacizumab has little toxic effect on ganglion cells, neuroretinal cells, RPE cells, choroidal endothelial cells, or corneal epithelial cells [19,24]. However, there are limited previous preclinical studies associated with RPE under oxidative stress conditions [25].

In our study, RPE cell apoptosis did not show a significant difference at 100 μM H_2O_2 but decreased at 200 μM H_2O_2 and increased with higher oxidative stress conditions (300 and 400 μM H_2O_2) (Fig. 1A). It seems that RPE cells can tolerate up to 200 μM H_2O_2 due to cell-protective mechanisms, but higher oxidative stress conditions caused *in vitro* mitochondrial DNA damage and promoted apoptosis. Ballinger et al. [26] and Jin et al. [27] reported that exposure of RPE cells to concentrations of H_2O_2 that cause *in vitro* mitochondrial DNA damage also promotes apoptosis, and this correlates well with our results under high oxidative stress conditions.

In different cell types, including vascular smooth muscle cells [28], VEGF expression is triggered by oxidative stress. Other studies of VEGF expression are consistent with our results when H_2O_2 in concentrations up to 200 μM was added to RPE cells. However, VEGF expression decreased at 300 and 400 μM H_2O_2, suggesting that VEGF production decreases according to the decrease in live RPE cells [29].

Oxidative stress triggers multiple signaling pathways, including some that are cytoprotective and others that contribute to cell damage and eventually cell death. Among these are the pathways of the Bcl-2 family proteins, which have subcellular locations on the mitochondrial outer membrane, nuclear envelope, and endoplasmic reticulum [30]. Expression of Bcl-2 mRNA decreased as oxidative stress increased, which was consistent with another study [27].

The retina is a highly oxygen-consumptive tissue that always functions under high oxygen tension; RPE cells are therefore exposed to oxidative stress. Chronic oxidative stress can lead to impairment and death of RPE cells, implicating it as a risk factor of AMD. In our study, low to high oxidative stress conditions of RPE cells were implicated in the phase of AMD.

Oxidatively stressed RPE cells undergo apoptosis [27], and Byeon et al. [25] have reported that addition of a high concentration (2.5 mg/mL) of bevacizumab to the culture medium did not affect the survival of either control RPE cells or cells under a low level of oxidative stress (150 μM H_2O_2). However, under higher oxidative stress levels (200 or 300 μM H_2O_2), pretreatment with bevacizumab (2.5 mg/mL) induced a significantly higher level of RPE cell death [25].

Addition of clinically applicable (0.33 and 0.67 mg/mL) and high concentrations (1.33 and 2.67 mg/mL) of bevaci-

zumab did not affect the survival of RPE cells under low oxidative stress (100 μM H_2O_2); nevertheless, Bcl-2 expression decreased in a dose-dependent manner (Fig. 3). Although Bcl-2 is an important survival factor, it is sensitive to oxidative stress, and a decrease in Bcl-2 precedes cell apoptosis.

Under moderate oxidative stress conditions (200 μM H_2O_2), Bcl-2 expression decreased with increasing concentration of bevacizumab; however, cell apoptosis did not show significant change until 1.33 mg/mL of bevacizumab. It then increased to 10.16% at high doses of bevacizumab (2.67 mg/mL) under the same oxidative stress (Fig. 4).

Under high oxidative stress conditions (300 μM H_2O_2), RPE cell apoptosis increased at high bevacizumab concentrations (1.33 and 2.67 mg/mL), but this was not correlated with Bcl-2 expression (Fig. 5). This might be due to multiple survival factors in RPE cells or experimental error due to low Bcl-2 expression.

In our experiment, RPE apoptosis did not increase with increased concentration of bevacizumab in the absence of H_2O_2; however, as the oxidative stress level increased, apoptosis of RPE cells increased. The higher was the oxidative stress, the lower was the concentration of bevacizumab that induced cellular apoptosis. Although cellular apoptosis at clinically applicable levels of bevacizumab (0.33 and 0.67 mg/mL) did not show significant increase, care should be taken since Bcl-2 expression showed a significant decrease even at lower levels of oxidative stress.

To our knowledge, we have shown for the first time that bevacizumab influences Bcl-2 expression and apoptosis of RPE cells under oxidative stress. Our study has some limitations. First, our conclusions are drawn from *in vitro* studies, which are not the best approximation of *in vivo* conditions. Moreover, some previous studies have shown that non-confluent ARPE-19 cells behave quite differently in terms of oxidative stress compared to confluent monolayers [31]. Further studies are needed to examine these findings in porcine retina-RPE-choroid cultures or on mouse retinae. Second, it is difficult to determine the degree of H_2O_2 simulating oxidative stress conditions of human eyes. Third, we could not identify the additional pathway mechanisms of Bcl-2 expression. Further studies are needed to examine the intracellular pathway or cumulative effects at repeated doses. Fourth, we could not determine the additional mechanism of action of bevacizumab in our study. Bevacizumab might bind RPE cell surface receptors in addition to known VEGF receptors.

In our experiment, we demonstrated the possibility that addition of the VEGF inhibitor bevacizumab might block the protective effect of VEGF in our *in vitro* model under high oxidative stress conditions mimicking those in AMD eyes. Under increased oxidative stress conditions, high doses of bevacizumab might influence RPE cell survival. Furthermore, bevacizumab might affect the expression of anti-apoptotic genes such as Bcl-2 under all oxidative conditions. Since the oxidative stress levels of each patient are unknown, repeated injections of intravitreal bevacizumab, as in eyes with AMD, might influence RPE cell survival. Therefore close monitoring is needed in patients who receive repeated injections of intravitreal bevacizumab.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgements

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