Autocrine Vascular Endothelial Growth Factor Enhances Survival of Retinal Pigment Epithelium Cells under Oxidative Stress *via* the VEGF R-2/PI3K/Akt

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Autocrine Vascular Endothelial Growth Factor Enhances Survival of Retinal Pigment Epithelium Cells under Oxidative Stress *via* the VEGF R-2/PI3K/Akt

Directed by Professor Sung Chul Lee

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<ABSTRACT>

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Purpose: Vascular endothelial cell growth factor (VEGF) is strongly induced by oxidative stress in retinal pigment epithelium (RPE) cells and VEGF-A is a survival factor for various cell types, thus, we examine whether the autocrine VEGF signal pathway in RPE cells is involved in the mechanism of adaptive response to oxidative stress.

Methods: ARPE-19 cells were treated with hydrogen peroxide and cell death was measured with flow cytometric analysis with annexin V-fluorescein isothiocyanate. Survival analysis was performed with pretreatment of VEGF-A neutralizing antibodies, VEGF receptor tyrosine kinase inhibitor (SU5416) or VEGF-A receptor neutralizing antibodies (anti-VEGF R1, anti-VEGF R2). Expression of VEGF-A, VEGF R1, VEGF R2 and soluble VEGF R1 was determined by semiquantitative RT-PCR or Western blot. Phosphorylation of VEGF R2 was detected

with immunoprecipitation and immunoblot.

Results: Hydrogen peroxide-induced cell death was promoted by pretreatment with VEGF-A neutralizing antibody and anti-VEGF R2 neutralizing antibody, but not with use of anti-VEGF R1 neutralizing antibody. Phosphorylation of VEGF R2 in RPE cells was induced by hydrogen peroxide and pretreatment with anti-VEGF-A neutralizing antibody inhibited phosphorylation. Phosphorylation of Akt under oxidative stress was abrogated by pretreatment with either neutralizing antibodies against VEGF-A or SU5416.

Conclusions: Autocrine VEGF-A enhanced RPE cell survival under oxidative stress; the autocrine VEGF-A/VEGF R2/PI3K/Akt pathway is involved. Neutralization of VEGF-A signaling, as in age-related macular degeneration eyes, may influence RPE cell survival.

Key words : age-related macular degeneration; vascular endothelial growth factor; retinal pigment epithelium; oxydative stress; Apoptosis; VEGF R2; PI3K; Akt; soluble VEGF R1

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I. INTRODUCTION

VEGF-A is a potent endothelial cell mitogen and recent studies have shown that VEGF-A acts as an autocrine growth and survival factor in VEGF-A-producing cells.[1-4] Substantial evidence indicates that VEGF-A is a major mediator of angiogenesis and vascular leakage in exudative age-related macular degeneration (AMD).[5-9] Inhibition of VEGF-A activity has been a central theme in many therapies under investigation. Several VEGF-A inhibitors have been developed and are now used clinically; these include a VEGF-A neutralizing oligonucleotide aptamer, a humanized monoclonal antibody Fab fragment (ranibizumab), and a VEGF-A receptor analog (soluble VEGF receptor 1; s VEGF R1). RNA interference (RNAi) has recently emerged as a potentially exciting therapeutic modality, and the first clinical application of an RNAi, a trial involving siRNA targeting VEGF-A or its receptor for treatment of AMD by intravitreal injection, is currently underway.[3, 9,10] Another method for

VEGF-A signal blocking is to employ a receptor tyrosine kinase (RTK) inhibitor to interrupt VEGF-A signaling. Many RTK inhibitors are under evaluation for treatment of exudative AMD.[6]

In normal eyes, VEGF-A receptors are localized to the choriocapillary endothelium opposite retinal pigment epithelium (RPE) cells. And tonic VEGF-A expression in RPE may be trophic for the choriocapillaries and is possibly required for maintenance of the choriocapillaris fenestrae.[6, 11] However, VEGF-A levels are significantly higher in patients with neovascular AMD than in healthy control patients, but the precise trigger and outcomes of enhanced VEGF-A expression remain unclear.[12, 13]

VEGF-A expression is increased in the RPE cells of the macula in patients with AMD, a condition associated with a high risk of choroidal neovascularization (CNV) development.[7] Also, VEGF-A is present in fibroblastic cells and transdifferentiated RPE cells in surgically removed CNV specimens.[11, 14] The presumed principal source of VEGF-A in exudative AMD is the RPE, and oxidants have been reported to increase the deposition of oxidized proteins or other oxidized compounds in Bruch's membrane, in a process that may involve complement activation and inflammatory processes, provoking pro-angiogenic VEGF-A release from the RPE in patients with exudative AMD.[6-8, 13, 14] In addition, oxidant compounds *per se* have been shown to stimulate VEGF-A release from the RPE.[13, 15] However, the function of VEGF-A secretion from RPE under oxidative stress is teleologically

inexplicable.

Cellular damage resulting from oxidative stress in RPE cells and photoreceptors may play a causative role in aging of the RPE.[5] Oxidative stress-induced RPE cell apoptosis has been proposed as a major pathophysiological mechanism of AMD.[5, 16, 17] In particular, RPE cell apoptosis is an important feature of the advanced form of dry AMD.[5, 18] Thus, oxidative stress induces VEGF-A expression from the RPE and also RPE death, suggesting a role for such stress in both neovascular and advanced dry AMD.

Although current treatments that target VEGF-A have demonstrated the best clinical outcomes of all approaches trailed to date, concern about broad inhibition of VEGF-A activity in AMD eyes remains.[6] VEGF-A is a known survival factor for the developing and mature retina, stimulating both endothelial and neural cells.[6] Inhibition of VEGF-A has been reported to lead to geographic atrophy and poor visual outcome in some neovascular AMD patients.[6] Also, RPE tears and choroidal atrophy in specimens from treated AMD patients raise questions about the long-term safety of anti-VEGF-A treatment.[19]

It has been suggested that the presence of both VEGF-A receptors and neuorpilin-1 on transdifferentiated RPE cells, and RPE cell death caused by VEGF-A chimeric toxin, imply the presence of functional VEGF-A receptors on human RPE cells.[20-25] Thus, we are of the view that an investigation of the relationship between VEGF-A expression and RPE cell activities, especially

under conditions of oxidative stress, may help to explain the pathogenesis of exudative or dry AMD.

As VEGF-A is an autocrine survival factor for various cell types and as VEGF-A is strongly induced by oxidative stress in RPE cells, we examined whether the autocrine VEGF-A signal pathway was involved in the mechanism of adaptive response to oxidative stress. [7, 13, 15, 26- 28]

II. MATERIALS AND METHODS

1. Chemical reagents and cell culture media

Dulbecco's modified Eagle's medium (DMEM), F-12 nutrient mixture, fetal bovine serum (FBS), HEPES buffer, amphotericin B, and gentamicin were purchased from Hyclone Laboratories, Inc. (Logan, UT). VEGF R1 (Flt-1) neutralizing antibodies (#AF321), VEGF R2 (Flk-1/KDR) neutralizing antibodies (#MAB3572), and recombinant human $VEGF_{165}$ were purchased from R&D Systems, Inc. (Minneapolis, MN). Recombinant PlGF (Placental growth factor, #P1588) was purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO). Anti-VEGF neutralizing antibodies (#PC315) and LY294002 (#440202) and SU5416 (#676487) were obtained from Calbiochem (San Diego, CA). Horseradish peroxidase (HRP)-conjugated secondary antibody was the product of Dako (Glostrup, Denmark).

2. Cell culture

The ARPE-19 cell line was obtained from ATCC (Manassas, VA), and maintained in DMEM with Ham's F-12 nutrient medium (DMEM F-12; Gibco, Carlsbad, CA). ARPE-19 cells were used within 10 passages. Cells were plated in six-well plates at $1.5x10^5$ cells per well and incubated at 37°C under 5% (v/v) CO_2 to reach 70% confluence before exposure to H_2O_2. Cells were serum-starved for 16 hours before H_2O_2 exposure. Serum-starved cells were

treated with H_2O_2 to induce oxidative stress, for 16 hours, before harvesting for cell death analysis.

3. Flow cytometric analysis of apoptosis

Cells were washed with PBS and incubated in serum-free DMEM in the presence of H_2O_2 (200-300 μM) for 16 hours. Anti-VEGF-A-neutralizing antibody or other neutralizing antibodies (anti-VEGF R1 or anti-VEGF R2) were added 2 hours before H_2O_2 treatment. An annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (BD Biosciences, Franklin Lakes, NJ) was used to detect phosphatidylserine externalization, as an index of apoptosis. Cells were washed and incubated for 15 minutes at room temperature in the presence of annexin V labeled with FITC and propidium iodide. In total, 10,000 cells were excited at 488 nm, and emission was measured at 530 and 584 nm to assess FITC and propidium iodide (PI) fluorescence, respectively. Cells were analyzed with a flow cytometer (FACSn; BD Biosciences). Gated cell numbers were plotted on a dot plot with reference to both annexin V and propidium iodide staining.

4. Semiquantitative RT-PCR

RNA isolation and semiquantitative RT-PCR were performed as described previously.[29] Primer sequences specific for amplification of genes encoding VEGF-A, VEGF R, soluble VERF R1, membrane-bound VEGF R1 (mb VEGF

R1), and VEGF R2 were designed from available human gene sequences (Table 1).

Table 1. Primer used for semiquantitative RT-PCR

Target Gene	Primer	Sequence	Product size (bp)
VEGF	forward	5'-ATG GCA GAA GGA GGG CAG CAT-3'	255
	reverse	5'-TTG GTG AGG TTT GAT CCG CAT CAT-3'	255
VEGF R1	foward	5'-GTAGCTGGCAAGCGCT CTTACCGGCTC-3'	316
	reverse	5'-GGATTTGTCTGCTGCCC AGTGGGTAGAGA-3'	316
mb VEGF R1	forward	5'-CCA CCT TGG TTG CTG AC – 3'	587
	reverse	5'-TGG AAT TCG TGC TGC TTC CTG GTC C – 3'	587
s VEGF R1	forward	5'-CCA GGA ATC ACA CAG G -3'	393
	reverse	5'-CAA CAA ACA CAG AGA AGG-3'	393
VEGF R2	foward	5'-TCT GGT CTT TTG GTG TTT TG-3'	497
	reverse	5'-TGG GAT TAC TTT TAC TTC TG-3'	497
GAPDH	foward	5'-GCC AAG GTC ATC CAT GAC AAC-3'	511
	reverse	5'-GTC CAC CAC CCT GTT GCT GTA-3'	511

s VEGF R1 = soluble VEGF R1; mb VEGF R1 = membrane-bound VEGF R1

5. Western immunoblot analysis

Adherent cells were washed with ice-cold PBS and lysed with cell lysis buffer (20 mM HEPES [pH 7.2], 10% [v/v] glycerol, 10 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1% [v/v] Triton X-100; Sigma-Aldrich) on ice for 30 minutes. Lysates were sonicated, centrifuged for 10 minutes at 12,000 *g*, and cell homogenate fractions stored at -70°C before use.

Protein concentrations in supernatant fractions were determined by the Bradford assay. Equal amounts of protein (30 μg) were boiled in Laemmli sample buffer and resolved by 8% (w/v) SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon; Millipore, Billerica, MA), probed overnight with primary antibodies diluted in TBST, and washed three times with TBST. Anti-VEGF R2 antibody (#2479), anti-β-Actin antibody (#4967), anti-phosphor-Akt (Ser473) antibody (#9271), and anti-Akt antibody (#9272) were all obtained from Cell Signaling Technology (Beverly, MA). Anti-VEGF R1 antibody (#ab32152) was the product of Abcam (Cambridge, UK). Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence.

6. Immunodetection of VEGF R2 phosphorylation

After overnight serum starvation, equal numbers of ARPE-19 cells were

stimulated with H_2O_2 (800 μM) for 15 minutes in the absence or presence of anti-VEGF antibody (4 μg/mL). Recombinant human $VEGF_{165}$ (20 ng/mL) was treated as a positive control. Equal amounts of cell lysate were immunoprecipitated with antibody to VEGF R2 (NEF) immobilized to protein-A-Sepharose, subjected to SDS-PAGE, immunoblotted with phosphotyrosine-specific antibody (anti-p-VEGF R2 (Tyr 996)-R, #sc-16629-R; Santa Cruz Biotechnology, CA) and subsequently reprobed with antiserum to VEGF R2. Protein expression was quantified by densitometry.

7. Immunocytochemistry

Cells were fixed for 5 minutes in 3.7% (v/v) formaldehyde and permeabilized with 0.5% (v/v) Triton X-100 for 8 minutes. Single or double-labeled immunofluorescence analysis was performed. As controls, samples were run without primary antibody or after addition of an irrelevant IgG, to assess nonspecific binding of secondary antibody. In all experiments, samples were incubated with anti-VEGF R1 or anti-VEGF R2 antibody for 2 hours at room temperature, followed by 1 hour incubation with FITC-conjugated secondary antibody. Anti- VEGF R2 antibody (#2479, Cell Signaling Technology), and anti-VEGF R1 antibody (#AF321, R&D Systems) were used. After washing with PBS, samples were examined by confocal microscopy (TSE SPE instrument; Leica Microsystems, Wetzlar, Germany).

8. ELISA

Cells were treated with various concentration of H_2O_2 at baseline (0 hours) and 16 hours. Supernatants were collected, centrifuged to remove cell debris, and stored at -70°C prior to ELISA analysis (using R&D Systems kits) according to the manufacturer's instructions. VEGF-A levels were adjusted to reflect total protein concentration. The level of VEGF-A protein was measured in cell-free supernatant using a human VEGF-A Quantikine ELISA kit (R&D Systems).

III. RESULTS

1. Relevance of autocrine VEGF-A to RPE cell viability under oxidative stress conditions

As VEGF-A functions as a survival factor for various cell types and is strongly induced by oxidative stress in RPE cells, we examined whether survival of RPE cells under oxidative stress was related to stress-induced VEGF-A synthesis.[4, 30] Pre-treatment of VEGF-A-neutralizing antibodies to culture medium inhibited the ability of RPE cells to survive oxidative stress caused by H_2O_2 (Figure 1 A, B). Furthermore, apoptosis of RPE cells under oxidative stress conditions was rescued by concomitant supplementation with recombinant human $VEGF_{165}$ (Figure 1 C). This indicated that autocrine VEGF-A-mediated survival signals prohibited entry into the death pathway under conditions of oxidative stress.

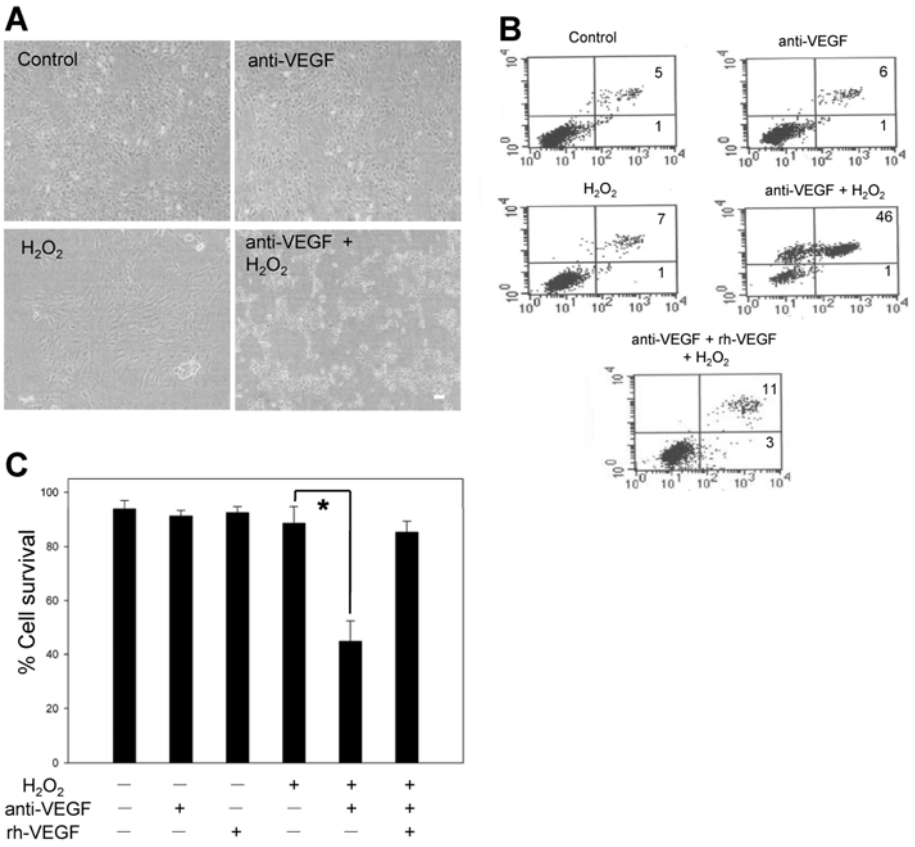

Figure 1. Autocrine VEGF-A protects against H_2O_2-induced cell death in ARPE-19 cells.

A. Immortalized retinal pigment epithelium (RPE) cell line, ARPE-19 cells were cultured with 10% fetal bovine serum and DMEM:F12 medium. When APRE-19 cells were 70% confluent, anti-VEGF antibody was treated 2 hours before 300 μM of H_2O_2 treatment. After 16 hours H_2O_2 treatment, photographs were taken by inverted microscopy (Nikon2, Tokyo, Japan) (bar=100 μm).

B. ARPE-19 cells were incubated with 200 μM H_2O_2 for 16 hours and cells were next analyzed using annexin V-fluorescein isothiocyanate and propidium iodide staining as described in Materials and Methods. LL: viable and undamaged cells (annexin V^-, PI^-); RL: cells undergoing early apoptosis

(annexin V^+, PI^-); and, RU: necrotic or late apoptotic cells (annexin V^+, PI^+). Each panel shows a typical flow cytometric histogram of 10,000 cells/sample from a representative experiment (rh VEGF; recombinant human $VEGF_{165}$). C. ARPE-19 cells were incubated with 200 μM H_2O_2 for 16 hours; cell survivals were then analyzed by FACS. Each bar shows a mean ± standard deviation for 9-12 wells from three independent experiments. * $P<0.001$ compared with control.

2. Expression of VEGF-A, VEGF R2, and VEGF R1, and regulation by oxidative stress

The concentration of secreted VEGF-A increased in a dose-dependent manner when H_2O_2 was added to RPE cells (Figure 2 B). Gene expression analysis indicated that expression of all of VEGF-A, VEGF R1, and VEGF R2 was induced by H_2O_2 (Figure 2 C). Immunohistocytochemistry showed that both VEGF R1 and VEGF R2 protein expression was induced by H_2O_2 stimulation (Figure 2 D).

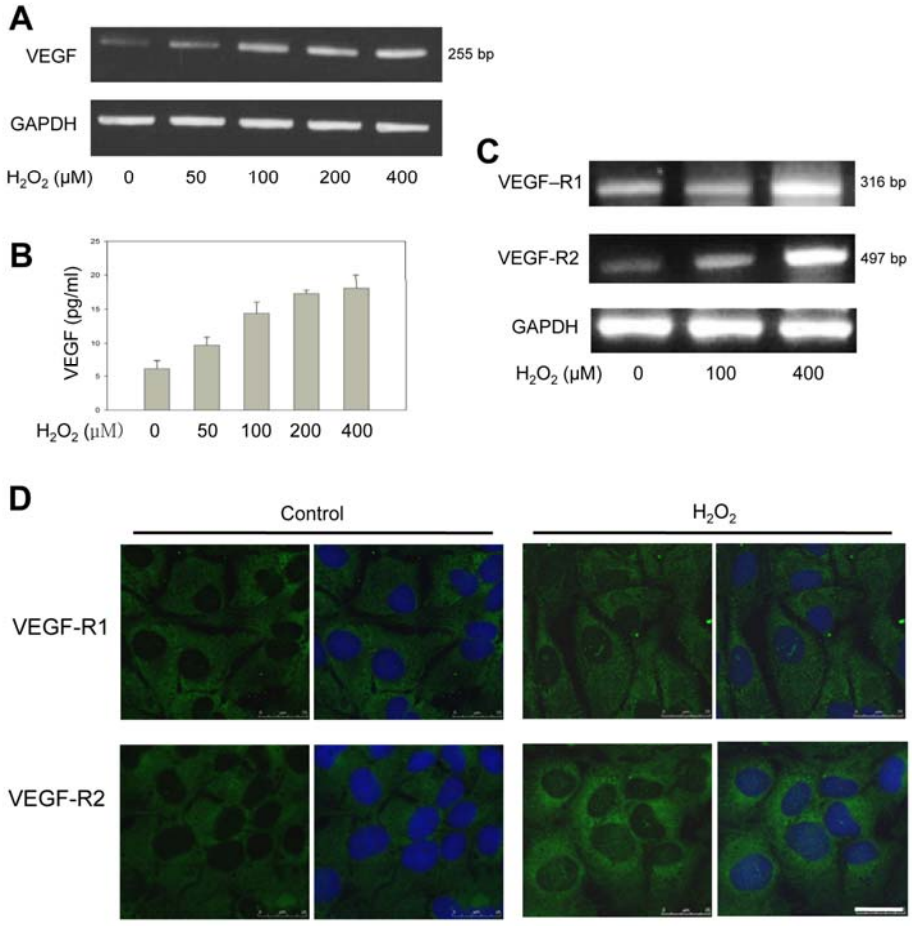

Figure 2. Expression of VEGF-A and VEGF receptors by H_2O_2 in ARPE-19 cells.

A. After 1 hour H_2O_2 treatment, VEGF-A mRNA expression in APRE-19 cells were determined with dose dependent manner. B. VEGF-A excretion into the medium was measured by ELISA. After 16 hours treatment of H_2O_2, supernatant were collected and analyzed by ELISA. Each data are represented as means ± standard deviations (SD) from three independent experiments. C. VEGF R1 and VEGF R2 mRNA expression was determined after H_2O_2 treatment. Each mRNA level was measured 1 hour after inoculation of various

concentration of H_2O_2. D. Expression pattern of VEGF R1 and VEGF R2 was investigated by immunocytochemical staining. Cells were exposed to 300 μM H_2O_2 for 6 hours, fixed with formaldehyde, and incubated with anti-VEGF R1 or anti-VEGF R2 antibody for 2 hours at room temperature, followed by 1 hour incubation with FITC-conjugated secondary antibody. Images were obtained with a confocal microscope (TSE SPE instrument; Leica microsystem, Germany). Green: VEGF R1 or VEGF R2; Blue: DAPI (bar=25 μm).

3. The VEGF-A/VEGF R2 axis, and not the VEGF-A/VEGF R1 axis, mediates the autocrine VEGF-A cell survival effect

Two high-affinity VEGF-A receptors, VEGF R1 and VEGF R2, are membrane-spanning receptor tyrosine kinases that bind VEGF-A, but their effects on VEGF-A signaling are very different. VEGF-A signaling through VEGF R2 produces several cellular responses, including a strong mitogenic signal and a survival signal both for endothelial cells and many other cell types.[4, 30] However, VEGF-A binding to VEGF R1 does not produce a strong mitogenic signal in endothelial cells. Here, we found that of the two high-affinity VEGF-A receptors, VEGF R1 and VEGF R2, only VEGF R2 mediated cell survival signals. H_2O_2-induced cell death was promoted by pretreatment with anti-VEGF R2 neutralizing antibody, but not with use of anti-VEGF R1 neutralizing antibody (Figure 3 A, B). Unlike the situation with VEGF-A, which is a ligand for both VEGF R1 and VEGF R2, PlGF binds only to VEGF R1 and not to

VEGF R2. In a previous result, H_2O_2-induced cell death was rescued by supplementation with recombinant human $VEGF_{165}$, but PlGF did not prevent cell death caused by anti-VEGF-A neutralizing antibody (Figure 3 D).

Phosphorylation levels of VEGF R2 were measured by immunoblotting for phosphotyrosine after immunoprecipitation of VEGF R2. When stimulated with H_2O_2, the phosphorylated VEGF R2/total VEGF R2 ratio was increased by approximately 190% but did not rise upon pretreatment with anti-VEGF-A antibody (Figure 4 B). Phosphorylation of VEGF R2 in RPE cells was induced by oxidative stress, however, pretreatment with anti-VEGF-A neutralizing antibody inhibited phosphorylation. These results are consistent with our earlier data indicating that RPE cells can survive oxidative stress with the assistance of autocrine VEGF-A signaling.

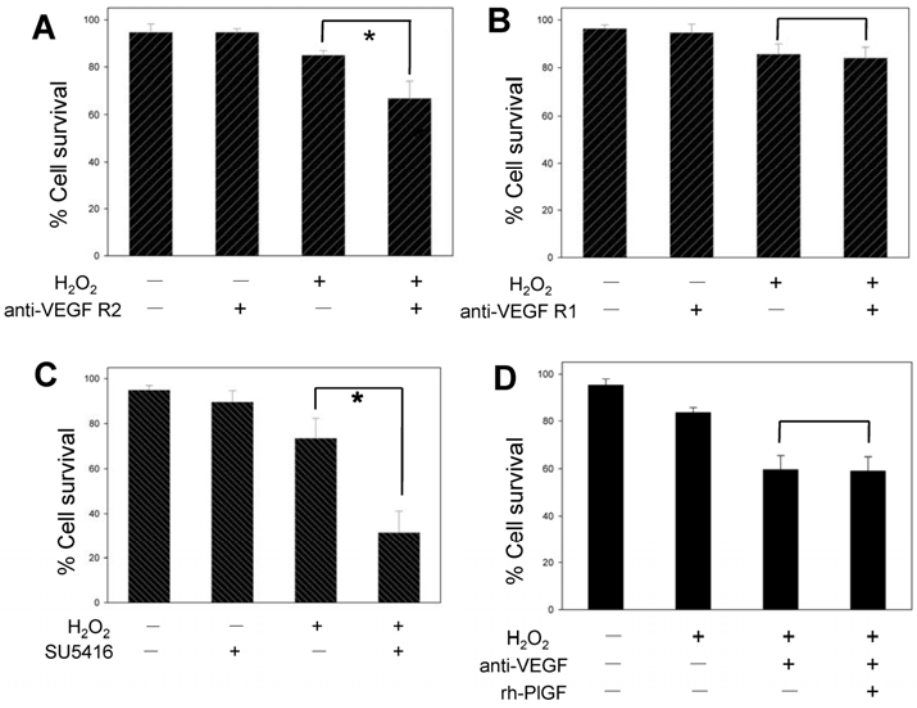

Figure 3. The VEGF/VEGF R2 axis, and not that of VEGF/VEGF R1, mediates the VEGF cell survival effect in ARPE-19 cells.

Each antibody was added 2 hours before treatment with 200 μM H_2O_2 for 16 hours. Cell death was analyzed by FACS using cells tagged with FITC-labeled Annexin V and propidium iodide. H_2O_2-induced cell death was aggravated by pretreatment with anti-VEGF R2 (A) but not by pretreatment with anti-VEGF R1 antibody (B). C. Cell survival analysis using FACS showed that ARPE-19 cell survival was reduced by pretreatment with an VEGF R2-specific PTK inhibitor (SU5416) in the presence of 300 μM H_2O_2 for 16 hours.

D. Above, H_2O_2 -induced cell death was promoted by pretreatment with anti-VEGF antibody but cells were rescued if rh-$VEGF_{165}$ was added. However, pretreatment with rh-PlGF, a substrate of VEGF R1 only (thus not of VEGF R2), could not rescue the cell death caused by pretreatment with anti-VEGF antibody.

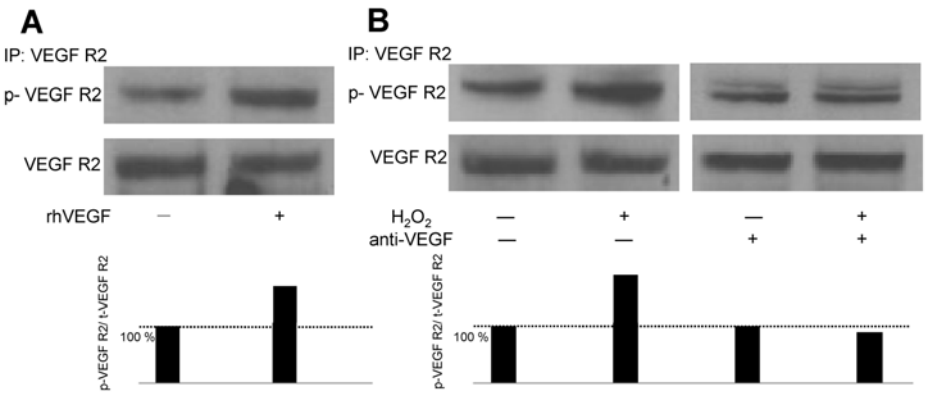

Figure 4. Determination of phosphorylated VEGF R2 in APRE-19 by H_2O_2 treatment.

A. For positive control, phosphorylation of VEGF R2 in ARPE-19 cells were determined by treating recombinant human VEGF-A_{165}. A nearly confluent monolayer of ARPE-19 cells were treated 20ng/ml of VEGF-A in serum free medium for 24 hours. Then cells were collected and lysed by protein lysis buffer. Immunoprecipation was performed 400μg of cell lysate with using 1μg of anti-VEGF R2 and 40μl of protein G sepharose. Immunoblot was performed with phosphor-VEGF R2 antibody.

B. Phosphor-VEGF R2 expression under oxidative stress in the absence and presence of anti-VEGF antibody was determined by immunoprecipitation for VEGF R2. Cells were treated 800 μM of H_2O_2 for 15 minutes. Then, cells were lysed by cell lysis buffer. Then, immunoprecipitation and immunblot were performed by same as above. Due to differences in loading of VEGF R2, the phosphor-VEGF R2/total VEGF R2 levels of this experiment are noted here: lane1,100%; lane 2, 190%; lane 3, 100%; lane 4, 90%.

4. The autocrine VEGF-A axis influences phosphorylation of the Akt signal protein

In RPE cells, it has been previously reported that the PI3k-Akt pathway stimulated by H_2O_2 is involved in protection from oxidant-induced cell death both under normal conditions and in disease states such as AMD.[12] The PI3K/Akt pathway has been proposed to be activated in an VEGF R2-dependent fashion in other cell types.[31, 32] Survival signaling from VEGF R2 in endothelial cells also has been reported to involve the PI3K/Akt pathway.[31] We thus explored whether blocking of the autocrine VEGF-A loop influenced Akt phosphorylation.

RPE cells were cultured with 300 μM H_2O_2 in the presence or absence of anti-VEGF-A neutralizing antibodies and tyrosine phosphorylation of Akt was measured in cell lysates. H_2O_2-induced phosphorylation of Akt was abrogated by pretreatment with a neutralizing antibody against VEGF-A or SU5416 (Figure 5 A, C). The data thus suggest that VEGF-A/VEGF R2/PI3K/Akt pathway activation may be involved in resistance to cell death caused by H_2O_2 stress.

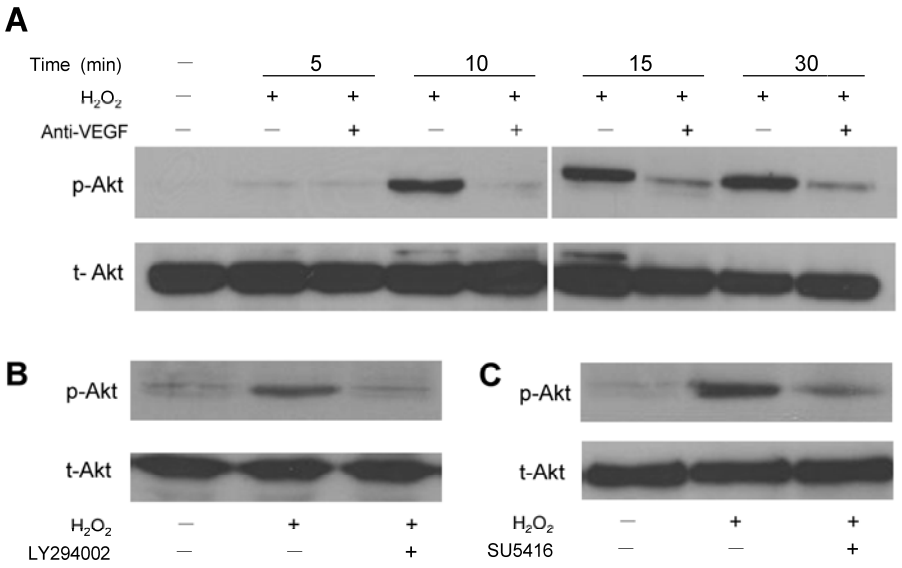

Figure 5. Akt phosphoryation by the autocrine VEGF-A and its receptor activation pathway.

A. An immunoblot probed with antibody to phosphor-Akt (p-Akt) (Ser473) and antibody to total-Akt (t-Akt), a control for gel loading. ARPE-19 cells were treated with 300 μM H_2O_2 for various times with or without pretreatment with anti-VEGF antibody. The immunoblot was probed with antibody to p-Akt (Ser473) and antibody to t-Akt 15 minutes after treatment with H_2O_2. B. Pretreatment with a PI3K-specific inhibitor (LY294002), acting upstream of Akt, blocked phosphorylation of Akt. C. Pretreatment with an VEGF R2-specific RTK inhibitor (SU5416) blocked phosphorylation of Akt.

5. Soluble VEGF R-1 regulates the autocrine VEGF-A signal

It has been reported that s VEGF R1 acts as an effective signaling modulator by regulating the availability of free VEGF-A in the microenvironment.[33, 34] The action of VEGF-A is dependent not only on the concentration of free VEGF-A and the expression level of VEGF R2 on cell surface but also on the concentration of the negative regulator (e.g. s VEGF R1). Under oxidative stress, transcription of both VEGF-A and s VEGF R1 was concomitantly induced (Figure 6 A). The transcription level of s VEGF R1, however, appeared to be regulated by the environmental free VEGF-A concentration. When the free available VEGF-A level was reduced, the transcription of s VEGF R1 decreased, but when VEGF-A was present at high concentrations, the s VEGF R1 level rose (Figure 6 B).

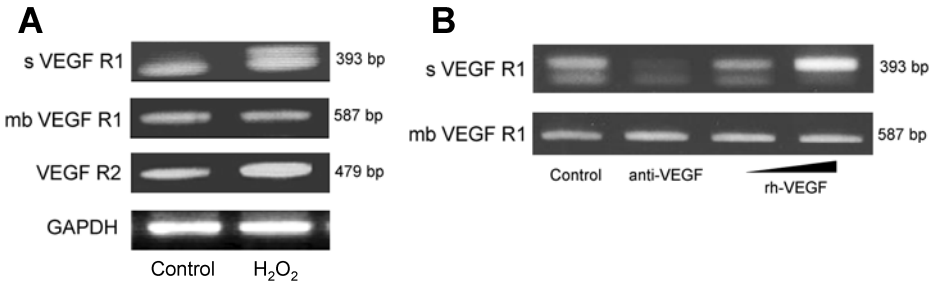

Figure 6. Expression of soluble VEGF R1 by autocrine VEGF signaling under conditions of H_2O_2 stress.

Soluble VEGF R1 (s VEGF R1) acts as an effective signaling modulator by regulating the availability of free VEGF in the microenvironment with VEGF R2 then functioning as the primary receptor for VEGF.

A. Gene expression of s VEGF R1, membrane bound VEGF R1 (mb VEGF R1), and VEGF R2, after 1 hour of H_2O_2 stress.

B. Gene expression of s VEGF R1 and mb VEGF R1 after treatment with anti-VEGF antibody or rh-VEGF.

6. Influence of Bevacizumab on survival of RPE cells under oxidative stress

Intravitreal injection of a humanized monoclonal antibody against VEGF-A (Bevacizumab, Genentech/Roche) currently finds wide clinical application. Addition of a high concentration (2.5 mg/mL) bevacizumab to 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) (Figure 7). However, under higher stress levels (200 or 300 μM H_2O_2), pretreatment with bevacizumab induced a significantly higher level of cell death (Figure 7).

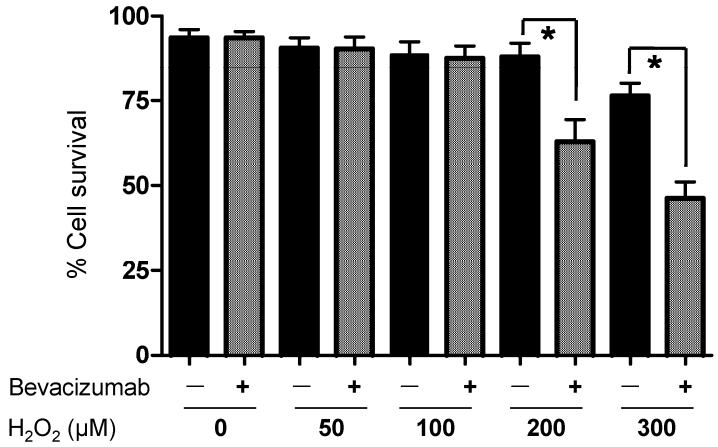

Figure 7. Effect of Bevacizumab on H_2O_2-induced ARPE-19 cell death. Cell survival analysis by FACS, using annexin V-FITC/DAPI-labeled cells, after pretreatment with bevacizumab (2.5 mg/mL) and H_2O_2 at various concentrations for 16 hours.

IV. DISCUSSION

The presence of functional VEGF-A receptors on RPE cells, transmitting signals similar to those mediated by receptors on endothelial cells, suggested that targeting of these receptor tyrosine kinases, either through the use of neutralizing antibody or kinase inhibitors, could have clinical potential, permitting modulation of RPE survival or proliferation through autocrine VEGF-A signaling.[4, 27] The main therapeutic mechanisms of anti-VEGF-A agents are based on anti-leakage effects and regression or maturation of CNV. Even with such an effect, progressive fibrosis and residual inflammatory processes are postulated to cause damage to RPE cells and photoreceptors.[6] RPE cell survival is crucial for maintaining the normal function of the overlying neurosensory retina and the underlying choriocapillaries. In the CNV regression area, RPE cells also proliferate and wrap around new vessels, thus forming a novel outer blood-retinal barrier (BRB).[8, 19]

Our results imply that neutralization of VEGF-A signaling with an anti-VEGF-A agent in AMD eyes may influence RPE cell survival, which is essential for visual recovery and reduction of AMD recurrence. It may therefore be important to modulate the extent of VEGF-A blockade, or to specifically and selectively inhibit only one or a few of the angiogenic actions of VEGF-A, when considering VEGF-A inhibition as a treatment strategy.

In RPE cells, Akt signaling was postulated to compensate for oxidative injury and to prevent apoptotic cell death.[12] When PI3K-Akt was blocked,

H_2O_2-induced RPE cell apoptosis and cell death were significantly enhanced.[12] Here, we found that autocrine VEGF-A signaling affected the Akt signaling pathway, which may be used by RPE cells to survive under conditions of oxidative stress.[12]

In pathological specimens of CNV, RPE cells showed excessive proliferation and resultant subretinal scarring.[8] It is not known whether this is attributable to loss of RPE cell function under chronic oxidative stress, or perturbation of RPE function by underlying AMD pathogenesis.[8] Our study was performed on low-passage, low-density cultures of ARPE-19 cells that showed relatively undifferentiated growth characteristics and were quite sensitive to oxidative stress.[35] When pathology (*e.g.,* AMD) is present, RPE cells adjacent to CNV experience transformation and proliferation. Thus, RPE cells under our experimental conditions may simulate those of an *in vivo* pathological lesion, compared to long-term culture of RPE cells. *In vivo*, RPE cells are always exposed to oxidative stress from lipid peroxides and anti-VEGF-A agents are currently clinically used to treat RPE pathology, but not when the RPE is normal. Another important indication for anti-VEGF-A agent treatment is diabetic retinopathy, where RPE cells are exposed to a pathological level of oxidative stress *in vivo*.

We found that RPE cells secreted not only VEGF-A but also s VEGF R1 and production of s VEGF R1 appeared to be regulated by the environmental level of VEGF-A. s VEGF R1 is a naturally occurring protein antagonist of

VEGF-A, formed by alternative splicing of the pre-mRNA for the full-length receptor.[33, 34] s VEGF R1 negatively modulates developmental blood vessel formation by inhibition of signaling through VEGF R2. We found that s VEGF R1 may possibly play a regulatory role in RPE cells. *In vivo*, fine-tuning of the effective VEGF-A level in the outer retina is very important, because aberrant angiogenesis in the retina may cause severe tissue damage. Thus, we hypothesize that the effective VEGF-A level in RPE cells is tightly regulated by synchronous production of s VEGF R1, secreted extracellular domain of VEGF R1.

Bevacizumab is a full-length, recombinant, humanized monoclonal antibody binding to all VEGF-A isoforms. Because of this general binding pattern for VEGF-A, bevacizumab is presumed to be as effective as ranibizumab in the treatment of intraocular neovascularization. Experimental investigations in rats, rabbits, and primates showed that intravitreal bevacizumab at different concentration did not cause any functional and morphological retinal toxicity.[36-38] In vitro cellular assays examining exposure to bevacizumab have shown little toxic effects on ganglion cells, neuroretinal cells, RPE cells, choroidal endothelial cells, and corneal epithelial cells.[39-43] However, recent rabbit eye study showed that increasing the dosage with intravitreal bevacizumab can cause nuclear DNA fragmentation in the outer retinal layers shown by the TUNEL method.[44] Also, in a mouse model, systemic neutralization of VEGF led to significant cell death in the inner and outer

nuclear cell layer and loss of visual function.[45] As shown in our study, high doses of bevacizumab significantly induced RPE cell death under conditions of higher oxidative stress, which may be attributable to blocking of the VEGF-A autocrine survival signal (Figure 7). However, we used a greater dose of bevacizumab than is employed clinically and RPE cell death was induced only at higher levels of oxidative stress. Further clinical evaluation of the long-term safety of bevacizumab is required.

The present study provides evidence that VEGF-A assists in RPE cell survival when cells are exposed to oxidative stress; the autocrine VEGF-A/VEGF R2/PI3K/Akt pathway is involved. Our results imply that neutralization of VEGF-A signaling, with an anti-VEGF-A agent, in AMD eyes, may influence RPE cell survival. A high level of VEGF-A secreted from RPE cells under oxidative stress conditions may participate in the pathogenesis of exudative AMD (by stimulating CNV), however, VEGF-A may have a beneficial effect in assisting RPE cell resistance against oxidative stress. Bevacizumab, now extensively used in the ophthalmic field, may also affect RPE cell survival under conditions of high oxidative stress. Thus, the extent or specificity of VEGF-A blockade, and the level of oxidative stress, might affect treatment outcomes (survival of RPE cells, restoration of outer BRB, or geographic atrophy) when anti-VEGF-A treatment is employed in neovascular AMD patients.

V. CONCLUSION

Autocrine VEGF-A enhanced RPE cell survival under oxidative stress; the autocrine VEGF-A/VEGF R2/PI3K/Akt pathway is involved. A high level of VEGF-A secreted from RPE cells under oxidative stress conditions may participate in the pathogenesis of exudative AMD (by stimulating CNV), however, VEGF-A may have a beneficial effect in assisting RPE cell resistance against oxidative stress. Neutralization of VEGF-A signaling, as in age-related macular degeneration eyes, may influence RPE cell survival.

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< ABSTRACT(IN KOREAN)>

산화스트레스 하에서 망막색소상피의 오토크라인으로 분비된 혈관내피세포성장인자의 VEGF R-2/PI3K/Akt pathway를 통한 생존 효과

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변석호

혈관내피세포성장인자 (Vascular endothelial growth factor; VEGF)는 산화스트레스 하에서 망막색소상피에서 강하게 분비되며, VEGF는 여러가지 종류의 세포들에 있어서 생존인자로 작용하는 사실이 알려져 있다. 본 연구에서는 망막색소상피에서 분비되는 VEGF가 오토크라인 생존 시그널을 활성화하여 산화스트레스 하에서 생존을 위한 적응 과정임을 확인하고자 하였다.

ARPE-19 세포주를 이용하여 과산화수소를 이용하여 산화스트레스에 의한 세포사를 annexin V-fluorescein isothiocyanate를 이용한 flow cytometric analysis를 통하여 정량하였다. VEGF에 대한 중화항체를 이용하거나, VEGF 수용체 타이로신 인산화 억제제를 이용하거나, VEGF 수용체를 중화하는 항체들을 이용하여 전처치 후 산화스트레스를 가하여 생존률을 측정하였다. 또한, 준 정량적인 RT-PCR이나

Western blot을 이용하여 VEGF, VEGF R1, VEGF R2의 발현이 산화스트레스 하에서 증가함을 확인하였다. 망막색소상피에서 VEGF R2의 인산화를 측정하기 위하여 면역침강후 Western blot을 시행하였다.

과산화수소에 의한 세포사는 VEGF에 대한 중화항체나 VEGF R2에 대한 중화항체를 전처치 하였을 때 의미있게 증가되는 소견을 보였다. 산화스트레스에 의한 VEGF R2의 인산화는 VEGF에 대한 중화항체를 전처치 하였을 때에는 감소되었다. Akt의 인산화 역시 전 처치시에 감소하였다.

산화스트레스 하에서 오토크라인하게 망막색소상피에서 분비되는 VEGF는 VEGF R2/PI3K/Akt pathway를 통하여 세포의 생존에 관여하는 것으로 보인다. 현재에도 노인성 황반변성에서 널리 사용되는 VEGF를 중화하는 치료는 망막색소상피의 생존에 영향을 줄 가능성이 있다.

핵심되는 말 : 망막색소상피; 산화스트레스; 노인성 황반변성; 혈관내피세포성장인자; 세포사; VEGF R2; Flk-1; Akt;

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